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ARTICLE

Anti-TNF-α restricts dengue virus-induced neuropathy

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Abstract

Proinflammatory TNF-α facilitates dengue virus (DENV) infection in endovascular dysfunction and neurotoxicity. The introduction of TNF-α blocking therapy with Abs is performed to test its therapeutic effect in this study. In DENV-infected mice, TNF-α production in the brain accompanied the progression of neurotoxicity and encephalitis. DENV infection caused the loss of hippocampal neurons with TNF-α expression around damaged regions, and immunostaining showed the induction of apoptosis in hippocampal neurons. TNF-α was expressed in active microglia and astrocytes in DENV-infected mice. TNF-α facilitated DENV-induced neurotoxicity in vitro in murine Neuro-2a cells. Using a currently established encephalitic mouse model in which DENV infection causes progressive hunchback posture, limbic seizures, limbic weakness, paralysis, and lethality 7 days postinfection, we showed that TNF-α transgenic mice represented the progressive disease development and administration of neutralizing TNF-α Ab reduced dengue encephalitis and mortality. These results demonstrate an immunopathogenesis of TNF-α for mediating DENV-induced encephalitis-associated neurotoxicity and that targeting TNF-α can be used as a strategy against dengue encephalitis.

KEYWORDS

dengue virus, encephalitis, infection, mice, neurotoxicity, TNF-α

1 | INTRODUCTION

Infection of dengue virus (DENV) commonly causes patients to suffer from high fever, severe headache, pain behind the eyes, muscle and joint pain, and rash. However, severe dengue cases may present plasma leakage, hemorrhage, neurologic dysfunction, and organ failure and may be lethal to both children and adults.[1] Currently, the neuropathogenesis of DENV infection has been widely reported; however, the pathogenic mechanisms of DENV-induced neuropathy remain unclear.[2,3] Generally, neurologic manifestations of dengue cases include encephalitis, myositis, myelitis, Guillain-Barré syndrome, and mononeuropathies. Although several mouse models of DENV infection have been developed,[4,5] there is still a lack of integration ideal for investigating pathogenic mechanisms of viral and host involvement.[6] It is challenging for physicians to treat patients with effective drugs against DENV infection and dengue disease progression because of its complicated pathogenesis.

DENV comprises a positive sense single-stranded RNA genome that encodes 3 structural proteins: the capsid, premembrane, and envelope (E) proteins, and 7 nonstructural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5.[7] All viral proteins contribute to viral infection, replication, and pathogenesis by mediating virus–host interaction, interfering antiviral interferon responses, and causing aberrant immune activation. Host risk factors, such as genetics, Ab-dependent enhancement, T cell responses, complement activation, autoimmune responses, cytokine storms, and underlying diseases, are speculated to be pathogenic in severe dengue.[6,8] Following acute viremia, several proinflammatory and vasoactive cytokines are correlated with disease severity in dengue patients at the critical phase of plasma leakage.[9] Taken together, the viral and host factors are concurrently involved in dengue pathogenesis and there are no preventive and effective therapeutic agents against DENV infection and dengue disease progression.

Abbreviations: DENV, dengue virus; BHK, Baby hamster kidney; CHX, cycloheximide; ACD, actinomycin D; WT, wild-type; Nrf-2, nuclear factor (erythroid-derived 2)-like 2.

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TNF-α is the hub regulator of the inflammatory responses in many viral infections. Despite its proinflammatory role in antiviral activity, aberrant production of TNF-α acts as a pathogenic cytokine in severe inflammatory cytokine storms. The introduction of immune blockade by targeting TNF-α with Abs and/or antagonists has been a new era in the treatment of inflammatory human disease with high medical demand.[10] An increase in TNF-α production, which has a well-established role in promoting vascular leakage, shows a positive correlation with the severity of illness in patients with hemorrhage.[11] Targeting TNF-α production pharmacologically and genetically diminishes hemorrhage development during DENV infection.[12,13] We currently demonstrate an increase in TNF-α in DENV-infected monocytes/macrophages in the brains of immunocompetent mice with viral encephalitis.[14,15] All of these findings suggest a pathogenic role for TNF-α in dengue-associated hemorrhagic disorders; however, the potential role of TNF-α production in CNS impairment still needs to be investigated. By using the established model of DENV-induced acute encephalitis, this study is aimed at validating the possible pathogenic effects of TNF-α by in vivo administration of neutralizing TNF-α Abs.

2 | MATERIALS AND METHODS

2.1 | Cells, virus strains, and reagents

Baby hamster kidney (BHK)-21 cells (CCL10; ATCC) and murine Neuro-2a cells (CCL131; ATCC), cultured in DMEM (Invitrogen Life Technologies, Rockville, MD), and *Aedes albopictus* C6/36 cells (CRL1660; ATCC), grown on plastic in RPMI (Invitrogen Life Technologies), were carried out for DENV infection accordingly.[16] DENV2 PL046, a Taiwanese human isolate obtained from the Centers for Disease Control in Taiwan, was propagated in C6/36 cells and viral titers were quantified by plaque assay using the BHK-21 cells. The following reagents and Abs were used in these studies: recombinant murine and human TNF-α (mTNF-α and hTNF-α; PeproTech, Rocky Hill, NJ); DAPI, cycloheximide (CHX), actinomycin D (ACD), and mouse mAb specific for β-actin (Sigma–Aldrich, St. Louis, MO); Abs against TNF-α (clone MP6-XT22; BioLegend, San Diego, CA); Abs against TNF-α and GFAP (Abcam, Cambridge, MA); Abs against NeuN and Iba1 (GeneTex, San Antonio, TX); Abs against active caspase-3 (Cell Signaling Technology, Beverly, MA); and Alexa Fluor 488- and Alexa Fluor 594-conjugated goat anti-mouse and goat anti-rabbit (Invitrogen, y Carlsbad, CA).

2.2 | Animals

Protocols according to guidelines established by the Ministry of Science and Technology, Taiwan were approved by the Laboratory Animal Care and Use Committee of National Cheng Kung University (Approval number IACUC #104062) and the Institutional Animal Care and User Committee of National Defense Medical Center (IACUC number: 16–261). C57BL/6NTac hTNFα transgenic mice, purchased from Taconic (Germantown, NY), and age-matched wild-type (WT) mice were provided from Yi-Fan Chen Laboratory. Mice were obtained from hTNFα TG mouse mating with WT mouse and genotyped using regular PCR (Forward primer, 5′-ACTGAAAGCATGATCCGGGACG-3′; Reverse primer, 5′-ATCTCTCAGCTCCACGCCATTG-3′).

2.3 | DENV infection in vivo and in vitro

Seven-day-old ICR suckling mice, hTNFα TG mice, and age-matched WT mice were inoculated intracerebrally with 2.5×10^5 PFU and intraperitoneally with 7.5×10^5 PFU of DENV2 (PL046), which was combined with or without anti-mTNF-α (250 μg/kg) treatment. Disease scoring was carried out according to our previous studies.[17] Cells were resuspended at a concentration of 7×10^4 cells/ml in the appropriate medium with DENV (MOI = 1) and incubated for 90 min at 37°C with or without drug cotreatment. The presence of viral supernatants was evaluated using plaque assays. Protocols for plaque assay were performed according to our previous studies.[16,17]

2.4 | Nissl stain

Mouse brains were prepared in tissue blocks and sliced. The tissue slices were fixed in 10% neutral-buffered formalin and embedded in paraffin wax. A Nissl staining kit (MDS Analytical Technologies, Sunnyvale, CA) was used to analyze Nissl bodies in the cytoplasm of neurons after fixing and freezing in paraformaldehyde, as described previously.[17]

2.5 | Immunofluorescence

Immunofluorescence staining was carried out according to the protocols (https://www.thermofisher.com/tw/zt/home/references/protocols/cell-and-tissue-analysis/protocols/ihc-staining-formalin-fixed-paraffin-embedded-tissues.html). The tissue slices were stained with Abs against active caspase-3, NeuN, GFAP, and activated microglial marker Iba-1. DAPI was used for nuclear staining. After being washed with PBS twice, tissue sections were visualized under a fluorescent microscope (EVOS FL imaging system; Thermo Fisher Scientific, Inc., Waltham, MA) or a laser-scanning confocal microscope (TCS SP5; Leica Mikrosysteme Vertrieb GmbH, Wetzlar, Germany).

2.6 | ELISA

The concentration of TNF-α in the brain extracts was determined using ELISA kits (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

2.7 | Cytotoxicity and caspase-3 activity assay

Cell death was assessed using Cytotoxicity Detection kit assays (Roche Diagnostics, Lewes, UK) according to the manufacturer's instructions. A caspase-3 assay kit (Calbiochem, La Jolla, CA) was performed to detect cell apoptosis according to the manufacturer's instructions. Following detection by using a Spectra MAX 340PC microplate reader (Molecular Devices, Sunnyvale, CA), OD measurements were performed.

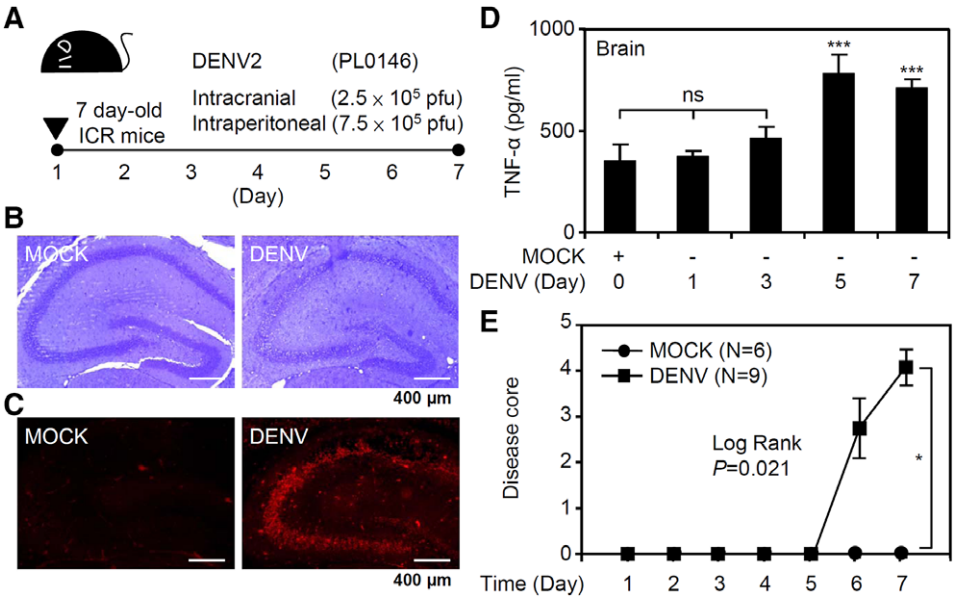

FIGURE 1 **DENV infection induces the production of TNF-α in brain accompanied by the induction of encephalitis-like symptoms.** (A) Seven-day-old ICR suckling mice were inoculated with DENV2 PL046 by concurrent intracranial (2.5×10^5 PFU) and intraperitoneal (7.5×10^5 PFU) injections. (B) Representative Nissl staining and (C) immunofluorescence staining of active caspase-3 (*red*) showed neuropathy in DENV2 PL046-infected brains of ICR mice day 7 postinfection. (D) Tissue supernatant of mouse brains infected with DENV2 PL046 were collected ($N = 5$). ELISA analysis was further carried out to detect TNF-α production. $^{***}P < 0.001$, compared with MOCK. ns, not significant. (E) Time course changes in the encephalitic score are shown. $^{*}P < 0.05$, compared with MOCK. The quantitative data are depicted as the mean ± SD of all tests

2.8 | Statistical analysis

Data obtained from 3 independent experiments, presented as the mean ± SD, were analyzed by an unpaired Student's *t*-test or 1-way ANOVA with Tukey's multiple-comparison test. Statistical significance was set at $P < 0.05$.

3 | RESULTS

3.1 | TNF-α is produced in DENV-infected brains of ICR mice with neuropathy

By using an in vivo model of DENV infection described previously,[17] Seven-day-old WT ICR suckling mice were infected with DENV2 PL046 by concurrent intracranial (2.5×10^5 PFU) and intraperitoneal (7.5×10^5 PFU) injections (Fig. 1A). At 7 days postinfection, Nissl staining showed a decreased amount of neuronal cell staining in DENV-infected brains, particularly in the hippocampal region (Fig. 1B). Following immunostaining of active caspase-3, an indicator of cell apoptosis, the results showed that DENV infection caused neuronal cell apoptosis with caspase-3 activation (Fig. 1C). To analyze the production of TNF-α, brain extracts were collected and an ELISA was performed accordingly.[14] The level of TNF-α was significantly ($P < 0.001$) greater at 7 days postinfection (Fig. 1D). We monitored a time-dependent change in clinical scores, which were graded according to the severity of illness as follows: 0 for healthy; 1 for minor illness, including weight loss, reduced mobility, and a hunchback body orientation; 2 for limbic seizures; 3 for moving with difficulty and anterior limb or posterior limb weakness; 4 for paralysis; and 5 for death. The results showed a significant increase in illness severity ($P < 0.05$) in mice with DENV infection (Fig. 1E). These results indicate that DENV infection causes TNF-α production accompanied by neuropathy, including the induction of neuronal cell death followed by the progression of encephalitis-like symptoms.

3.2 | Expression of TNF-α is highly associated with neuronal cell death in DENV-infected brains of ICR mice

Our previous study showed that DENV infection in neuronal cells was accompanied by the induction of neurotoxicity, including neuronal cell loss and apoptosis.[17] To investigate the correlation between TNF-α production and neuropathy, expression of TNF-α was monitored accordingly. Immunofluorescence staining of TNF-α and NeuN, a general marker of neuronal cells, revealed that TNF-α was produced in non-neuronal cells around the hippocampal neurons (Fig. 2A). Further immunofluorescence staining of TNF-α and active caspase-3, a marker of cell apoptosis, revealed that TNF-α was produced around the apoptotic regions in DENV-infected brains (Fig. 2B). These data indicate that, in DENV-infected mice, TNF-α production is accompanied by the induction of neurotoxicity, suggesting an immunopathogenic role of CNS inflammation.

3.3 | TNF-α is expressed mainly in active microglia and astrocytes of DENV-infected brains of ICR mice

Through immunostaining and ELISA, our work confirmed that expression of TNF-α was induced in the brains of DENV-infected ICR mice.

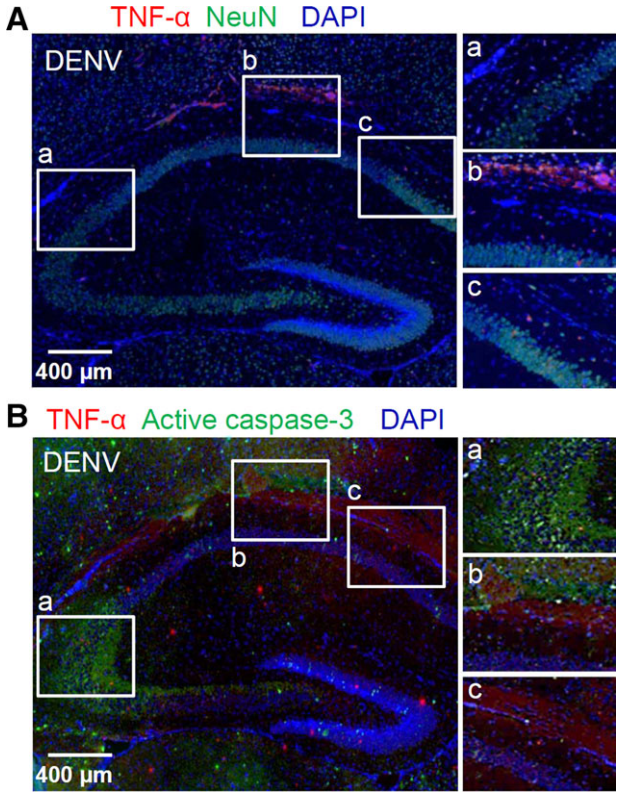

FIGURE 2 DENV infection induces TNF-α production accompanied by neuronal cell death in vivo. Representative immunofluorescence staining of (A) TNF-α (*red*) plus NeuN (*green*), a marker for neuronal cells, and (B) TNF-α plus active caspase-3 (*green*) in DENV2 PL046-infected brains of ICR mice day 7 postinfection. DAPI is a nuclear stain (*blue*). Selective enlarged graphs are shown as a, b, and c

We previously found that DENV infection causes TNF-α production in mononuclear phagocytes.[14,15] To verify the TNF-α-expressing cells in DENV-infected brains of ICR mice, several cell markers were utilized. Immunofluorescence staining (Fig. 3) showed that TNF-α was not expressed in NeuN-positive cells. However, TNF-α was expressed in GFAP-positive astrocytes and Iba1-positive microglia in vivo. The results indicate that, in DENV-infected mice, TNF-α is expressed in active microglia and astrocytes around the apoptotic regions.

3.4 | TNF-α facilitates DENV-induced neurotoxicity in vitro in murine Neuro-2a cells

Our previous work showed that DENV infection directly causes neurotoxicity in vitro in neuronal cells.[16] Regarding the proapoptotic role of TNF-α, we next assessed the neurotoxic effects of TNF-α under DENV infection in murine neuronal cell line Neuro-2a. By detecting LDH release, a general cytotoxic event occurring under cellular injury, we demonstrated that DENV infection significantly ($P < 0.05$) induced neurotoxicity, whereas exogenous administration of mTNF-α significantly ($P < 0.05$) facilitated DENV-induced neurotoxicity (Fig. 4A). To confirm this finding, activation of caspase-3 was determined by detecting its activity. The results showed that additional treatment of mTNF-α significantly ($P < 0.05$) increased the effect of cell apoptosis

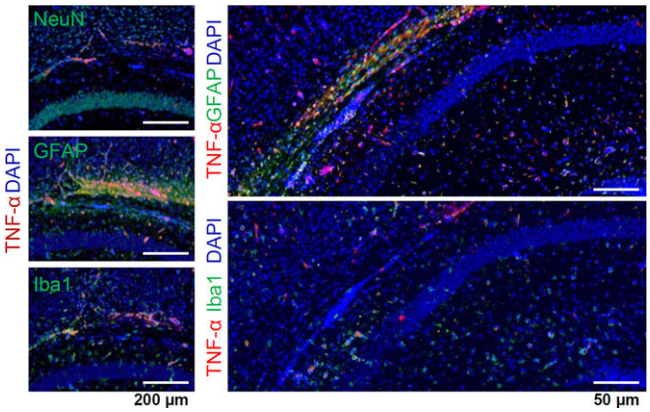

FIGURE 3 TNF-α is mainly produced in astrocytes and microglia of DENV-infected mice. Representative immunofluorescence staining of TNF-α (*red*), NeuN (a marker for neuronal cells, *green*), GFAP (a marker for astrocytes, *green*), and Iba1 (a marker for microglia, *green*) in DENV2 PL046-infected brains of ICR mice day 7 postinfection. DAPI is a nuclear stain (*blue*). Selective enlarged graphs of GFAP and Iba1 are shown

in DENV-infected cells (Fig. 4B). These data demonstrate a combined induction of neurotoxicity caused by TNF-α and DENV infection.

3.5 | Targeting TNF-α retards DENV-induced encephalitis-like symptoms

TNF-α knockout mice have been used for verifying the pathogenic role of TNF-α in DENV-induced hemorrhagic responses.[13] In this study, an overexpression approach by using hTNF-α transgenic mice, obtained from TACONIC (B6.Cg(SJL)-Tg(TNF) N21),[18] was performed. Following the infectious model of our previous work,[17] WT and transgenic mice were infected with DENV (Fig. 5A). Compared with WT, RT-PCR confirmed the specific expression of hTNF-α in transgenic mice (Fig. 5B). Following DENV infection, an early progression of encephalitic symptoms (Fig. 5C) and mortality (Fig. 5D) could be observed in transgenic mice. To verify the cytotoxic effects of hTNF-α on DENV-infected murine neuronal cells, LDH release assay (Fig. 5E) and caspase-3 activity measurement (Fig. 5F) all showed that exogenous administration of hTNF-α significantly ($P < 0.05$) facilitated DENV-induced neurotoxicity and caspase-3 activation in murine Neuro-2a cells. These results imply that TNF-α facilitates DENV-induced encephalitis-like symptoms and neurotoxicity in mice.

To further evaluate the therapeutic effects of targeting TNF-α, a therapeutic protocol using neutralizing Abs against mTNF-α was carried out in WT ICR suckling mice (Fig. 6A). In this study, commercial anti-mTNF-α neutralizing Abs were used to test for proof of concept in cellular protection from infection and inflammation in vivo. Neutralizing mTNF-α Abs (250 μg/kg) were coadministered at Day 0 postinfection. Following Ab treatment, neutralization of TNF-α slightly retarded the progression of encephalitic symptoms (Fig. 6B) and significantly ($P < 0.05$) reduced mortality (Fig. 6C). Further Nissl staining and active caspase-3 immunostaining showed that blocking DENV-induced TNF-α also reduced DENV-induced neurotoxicity (Fig. 6D) and

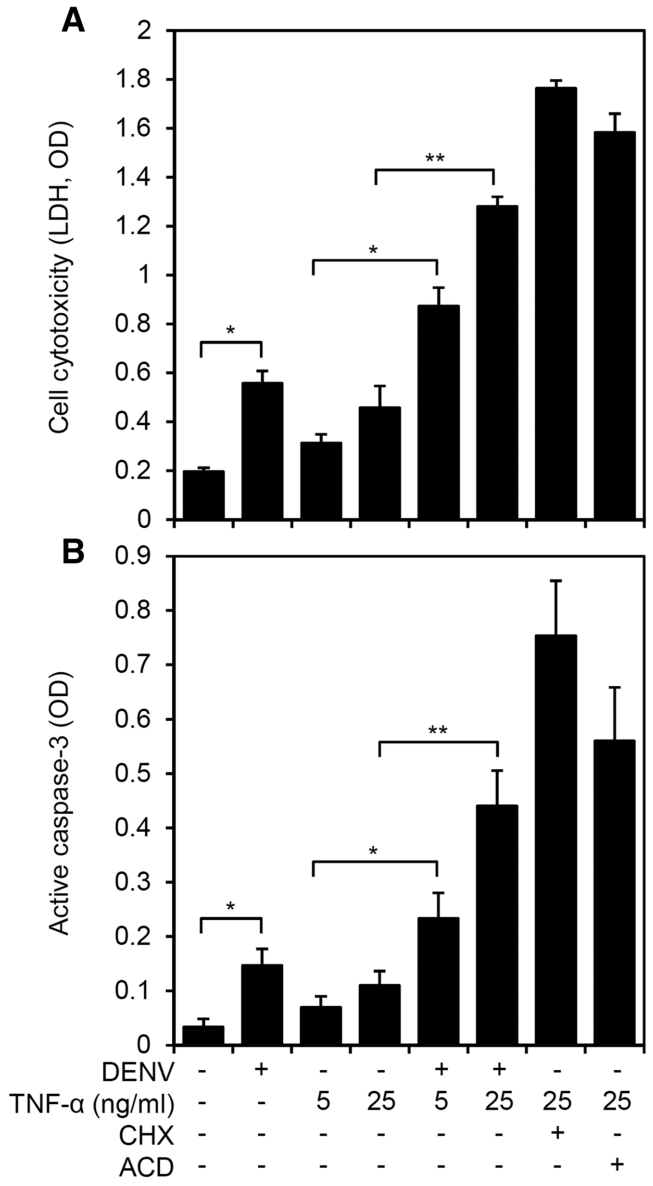

FIGURE 4 Exogenous administration of TNF-α facilitates DENV-induced neurotoxicity in vitro. Following preinfection with DENV (at a multiplicity of infection of 10) for 2 h, mTNF-α (5 or 25 ng/ml) was added for an additional 48 h. (A) Lactate dehydrogenase (LDH) release and (B) caspase-3 activity assays showed cell cytotoxicity and apoptosis. Cycloheximide (CHX; 10 μM) and actinomycin D (ACD, 100 nM) were used as positive controls. All quantitative data are shown as the mean $\pm$ SD of 3 independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$

caspase-3 activation (Fig. 6E). These results imply that targeting TNF-α could be a therapeutic strategy against DENV-induced neuropathy.

4 | DISCUSSION

Severe dengue is well defined as a potentially deadly complication in patients with plasma leaking, fluid accumulation, respiratory distress, severe bleeding, or organ impairment.[1] Although vascular leakage is major problem of more severe dengue patients, a relative rare situation with CNS impairment in dengue patients is also diagnosed with sever dengue. Neurologic manifestations have been reported in severe dengue cases with unknown pathogenic mechanisms while CNS inflammation is generally involved.[2,3] Because of its aberrant expression with high correlation to disease severity and its pathogenic role of causing inflammatory and cytotoxic responses, TNF-α overproduction may be harmful for dengue pathogenesis. For vascular leakage, an increased level of circulating serum TNF-α has been proposed as a risk factor[19]; however, its pathogenic role in dengue CNS impairment remains undefined. By applying an in vivo model of DENV infection in immunocompetent ICR suckling mice through concurrent intraperitoneal and intracranial routes, as previously described,[17] we found that DENV induced TNF-α production in the brain around the injured neuronal cells with caspase-3 activation. Further experiments demonstrated that either astrocytes or microglial cells produced TNF-α. Exogenous administration of TNF-α and forced expression of TNF-α facilitated DENV-induced neurotoxicity. Pharmacologically targeting TNF-α effectively retarded DENV-induced progression of encephalitis-like symptoms in mice. These results validate the pathogenic role of TNF-α not only systemically but also in organ-specific CNS impairment.

We demonstrated that DENV infection causes CNS inflammation, neurotoxicity, and blood–brain barrier destruction which may explain why encephalitic DENV-infected mice display progressive hunchback posture, limbic seizures, limbic weakness, paralysis, and lethality as described by Tsai et al.[17] TNF-α is hypothesized to be a crucial inflammatory factor involving CNS impairment due to its proinflammatory and proapoptotic role in neuropathy.[20] According to our findings, DENV infection induces TNF-α production in brain and TNF-α is highly correlated with encephalitic progression and the induction of neuronal cell apoptosis. This study, along with our previous works,[16,17] show that DENV caused neurotoxicity in vivo and in vitro as shown by histopathologic changes and apoptotic staining. We further demonstrated an enhanced effect of TNF-α on DENV-induced neurotoxicity in vitro. These results suggest the involvement of aberrant TNF-α production caused by DENV infection for dengue CNS impairment.

Patient with DENV infection have elevated levels of serum TNF-α, which can stimulate endothelial cell apoptosis.[11,21] Exogenous administration of TNF-α is able to cause endothelial cell apoptosis, whereas TNF-α knockout mice are resistant to DENV-induced hemorrhagic responses.[13] In severe dengue with dengue hemorrhagic fever, vascular leak occurs at the critical phase. It is anticipated that endothelial dysfunction followed by vascular leakage is a result of inflammatory responses, particularly TNF-α overproduction. Interestingly, a single-nucleotide polymorphism of TNF-α at the TNF-308A allele was identified with a clinical association of hemorrhagic manifestations in severe dengue patients.[22] To test this association, hTNF-α transgenic mice were validated for the enhanced effects of TNF-α overexpression on DENV infection and dengue encephalitic progression. All of the studies indicate the pathogenic role of TNF-α in facilitating not only dengue disease's vascular leakage but also its neurotoxicity. Importantly, treatment with anti-TNF-α neutralizing Ab not only reduced mortality but also prevented vascular leakage following DENV infection.[12,23] In this

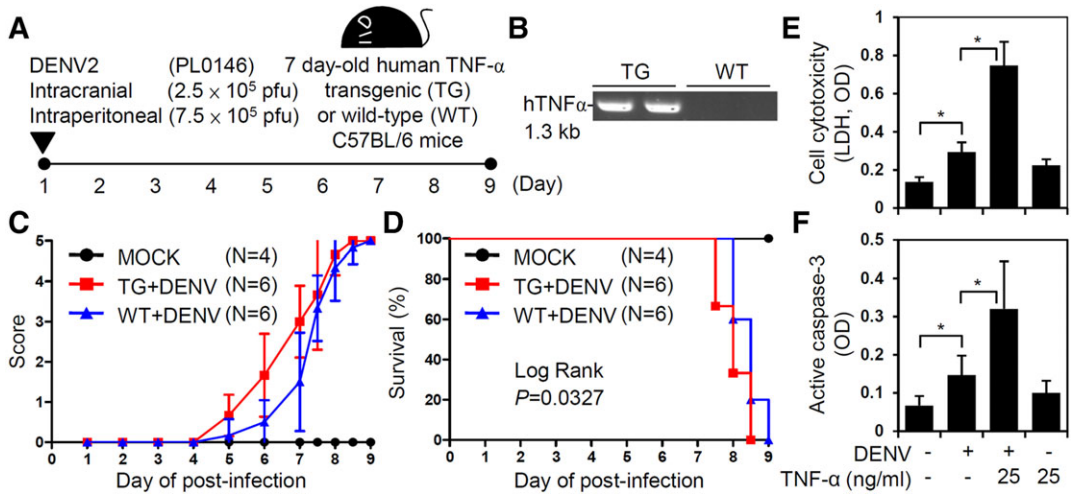

FIGURE 5 **Human TNF-α transgenic mice are susceptible to dengue encephalitis.** (A) Seven-day-old human TNF-α (hTNF-α) transgenic C57BL/6 mice and wild-type (WT) mice were inoculated with DENV2 PL046 by concurrent intracranial (2.5×10^5 PFU) and intraperitoneal (7.5×10^5 PFU) injections. (B) Representative RT-PCR analysis of hTNF-α mRNA expression. Time-kinetic changes in (C) encephalitic scores and (D) survival rates were measured ($N = 4$ or 6). The log-rank test is valid and the p value is shown. Following preinfection with DENV (at a multiplicity of infection of 10) for 2 h, hTNF-α (25 ng/ml) was added for an additional 48 h. (E) Lactate dehydrogenase (LDH) release and (F) caspase-3 activity assays showed cell cytotoxicity and apoptosis. All quantitative data are shown as the mean ± SD of 3 independent experiments. $^*P < 0.05$

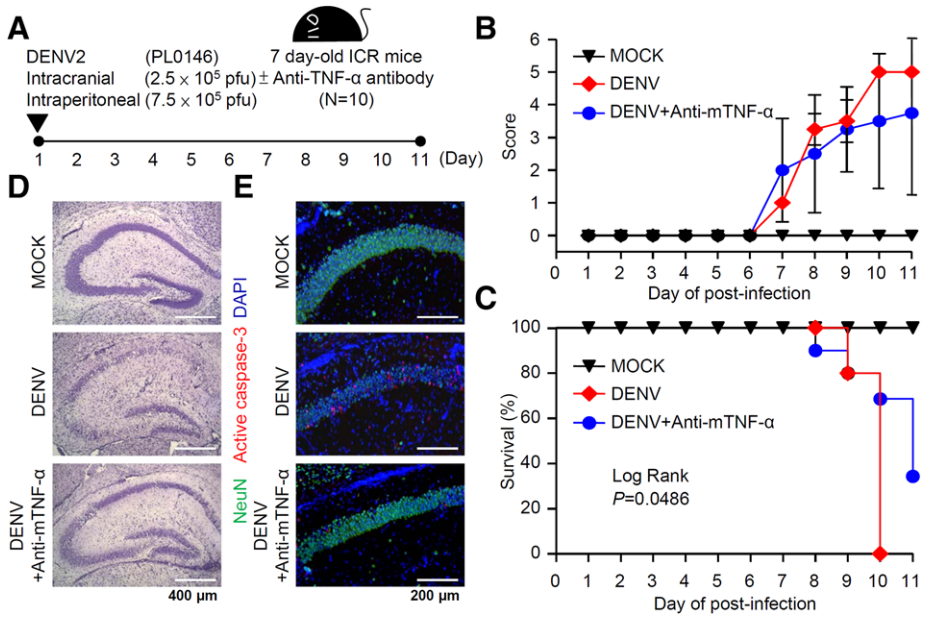

FIGURE 6 **Treatment of neutralizing Ab against TNF-α partly retard dengue encephalitis and neurotoxicity.** (A) Seven-day-old wild-type (WT) ICR mice were inoculated with DENV2 by concurrent intracranial (2.5×10^5 PFU) and intraperitoneal (7.5×10^5 PFU) injections with or without mTNF-α neutralizing Abs (mTNF-α; 250 μg/kg) cotreatment. Time-kinetic changes in (B) encephalitic scores and (C) survival rates were measured ($N = 10$). The log-rank test is valid and the P value is shown. Representative (D) Nissl staining and (E) immunofluorescence staining of active caspase-3 (*red*) plus NeuN (*green*). DAPI is a nuclear stain (*blue*)

study, we further validated the therapeutic effects against dengue CNS impairment by targeting TNF-α in the brain.

For DENV-induced TNF-α production, our previous studies[15] showed the potential regulation of TNF-α production by DENV-activated NF-κB independent of TLR 3 signaling. Additionally, forced expression of the NS2B3 viral protein enhances DENV-induced TNF-α production probably by activating NF-κB,[15] whereas NS2B3 is shown as an activator of NF-κB for inflammatory activation and hemorrhagic responses.[24] We also demonstrated that DENV infection causes activation of nuclear factor (erythroid-derived 2)-like 2 (Nrf2), an essential regulator of antioxidant response, to increase C-type lectin domain family 5, member A (CLEC5A) expression for TNF-α production.[14] CLEC5A, also known as myeloid DAP12-associating lectin, is critical for DENV inflammatory signaling receptor and is responsible for cytokine and chemokine production following an interaction with DENV E protein.[25,26] Through an interaction with TLR2 and TLR6, DENV NS1

protein also triggers immunopathogenic effects by up-regulating TNF-α production.[27] According to these findings, it is speculated that host factors, such as NF-κB, Nrf2, and CLEC5A signaling, and viral factors, such as E, NS2B3, and NS1, are involved in DENV-induced encephalitic CNS inflammation, as well as neurotoxicity.

Dengue encephalitis is a growing infectious disease that needs further effective preventive interventions, such as antiviral agents, vaccines, vector control, and anti-inflammation. Together, our current work that DENV infection causes TNF-α production in the brains of mice with dengue encephalitis[14] and that immune blockade by using neutralizing Abs and antagonists against proinflammatory and proapoptotic TNF-α, may confer immediate protection against dengue diseases, such as systemic hemorrhagic responses as well as dengue encephalitis. This study demonstrates an immunopathogenesis of TNF-α for mediating DENV-induced encephalitis-associated neurotoxicity and targeting TNF-α as a strategy against dengue encephalitis. While this study has demonstrated the therapeutic effects of targeting TNF-α, evidence of patients' study showing TNF-α overproduction in CNS specimens and more models of DENV infection and more modes of immune blockade, including administrative routes, time points and dosage used for treatment, should be further validated.

AUTHORSHIP

M.K.J., J.C.K., T.T.T., M.R.H., and T.J.S. performed the experiments and interpreted data. W.C.H. and Y.F.C. provided reagents. M.K.J. and C.F.L. designed the research, made the figures, and wrote the manuscript. M.K.J., P.C.T., Y.T.W., and C.F.L. analyzed the results.

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DISCLOSURES

The authors declare no conflicts of interest.

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