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VEGFR-3 deficiency in astrocytes exacerbates Japanese encephalitis virus-induced neuroinflammation in mouse brains

Xi-Lin Wang[1,#], Qi Zhao[1,#], Hong-Yang Liu[1], Yong-Sheng Dai[1], Ming-Shun Han[2], Xiao-Jing Li[3], Feng Li[4], Jing Ye[5], Sheng-Bo Cao[5,*], Lin-Lin Qi[1,*], Bin Wei[1,*]

[1] *School of Medicine*, *School of Life Sciences*, *Shanghai University*, *Shanghai* 200444, *China*

[2] *State Key Laboratory of Cell Biology*, *Shanghai Institute of Biochemistry and Cell Biology*, *Center for Excellence in Molecular Cell Science*, *Chinese Academy of Sciences*, *University of Chinese Academy of Sciences*, *Shanghai* 200031, *China*

[3] *Institute for Translational Brain Research*, *State Key Laboratory of Medical Neurobiology*, *MOE Frontiers Center for Brain Science*, *Fudan University*, *Shanghai* 200433, *China*

[4] *State Key Laboratory of Virology*, *Wuhan Institute of Virology*, *Chinese Academy of Sciences*, *University of Chinese Academy of Sciences*, *Wuhan*, *Hubei* 430071, *China*

[5] *State Key Laboratory of Agricultural Microbiology*, *Huazhong Agricultural University*, *Wuhan*, *Hubei* 430070, *China*

ABSTRACT

Japanese encephalitis virus (JEV), a highly neurotropic flavivirus, preferentially targets neurons, where it replicates efficiently and induces extensive neuroinflammation and neural injury within the central nervous system (CNS), resulting in severe neurological deficits and high mortality. Vascular endothelial growth factor receptor 3 (VEGFR-3), a receptor tyrosine kinase encoded by *FLT4*, plays an essential role in vascular system regulation, especially in the development and maintenance of the lymphatic system. While previous studies have shown that macrophage-expressed VEGFR-3 suppresses bacterial sepsis and neuroinflammatory responses, its function in astrocytes during viral encephalitis remains unclear. To elucidate the astrocytic role of VEGFR-3 in JEV pathogenesis, mice carrying a conditional deletion of the VEGFR-3 ligand-binding domain specifically in astrocytes were generated. Astrocyte-specific VEGFR-3 deficiency led to increased brain viral load, amplified transcription of proinflammatory genes, and reduced survival following JEV challenge. JEV-infected brains with astrocytic VEGFR-3 deficiency exhibited heightened expression of multiple inflammatory cytokines and chemokines. Complementary *in vitro* experiments further confirmed that chemical inhibition of VEGFR-3 intracellular kinase activity enhanced inflammatory cytokine gene expression in JEV-infected astrocytes. Collectively, these findings identify astrocytic VEGFR-3 as a critical negative regulator of virus-triggered neuroinflammation, implicating it as a protective modulator of host responses during JEV infection.

Keywords: VEGFR-3; JEV; Neuroinflammatory response; Astrocytes

INTRODUCTION

Neurotropic viruses encompass a broad spectrum of pathogens capable of infecting the central nervous system (CNS) and eliciting a range of neurological disturbances (Yang et al., 2023). Among these, Japanese encephalitis virus (JEV), a classic mosquito-borne neurotropic flavivirus (Liu et al., 2025), invades the brain and provokes intense activation of neurons and glial cells, resulting in a severe inflammatory response that damages specific brain regions and leads to CNS dysfunction. Neuroinflammation represents a central pathological feature in encephalitic progression, serving both protective and deleterious roles depending on its magnitude and duration (Chang et al., 2024).

As one of the most abundant and functionally versatile glial cell types in the CNS, astrocytes play critical roles in essential homeostatic functions, including blood-brain barrier (BBB)

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#Authors contributed equally to this work

*Corresponding authors, E-mail: sbcao@mail.hzau.edu.cn; qilinlin@shu.edu.cn; weibinwhy@shu.edu.cn

maintenance (Abbott et al., 2006), synaptic function, metabolic support, and immune modulation (Wang et al., 2025). Their ability to sense and respond to various external cues, such as cytokines (e.g., IL-1β and TNF-α), chemokines, and pathogen-associated molecular patterns (PAMPs), enables rapid conversion into reactive states, which subsequently secrete inflammatory mediators to modulate inflammatory responses. However, persistent activation of astrocytes in later stages of viral infection can lead to excessive inflammation, contributing to secondary neurodegeneration and neural tissue damage (Zheng et al., 2023). Therefore, elucidating the effector molecules in astrocytes that can mitigate neurotropic viral infections is crucial for controlling neurological damage and associated inflammatory responses.

Vascular endothelial growth factor receptor 3 (VEGFR-3), also known as *FLT4*, is a tyrosine kinase receptor primarily involved in lymphangiogenesis and the regulation of vascular endothelial cells. Once thought to be restricted to lymphatic vessels (Adams & Alitalo, 2007), VEGFR-3 is now recognized to be expressed in diverse cell types, including macrophages, where it negatively regulates inflammatory responses and limits tissue injury. Our prior work demonstrated that macrophage VEGFR-3 inhibits Toll-like receptor 4 (TLR4)-NF-κB-mediated inflammation, thereby attenuating sepsis associated with bacterial infections (Zhang et al., 2014). In the context of neurotropic viral challenge, vascular endothelial growth factor C (VEGF-C)/VEGFR-3 signaling promotes crosstalk between neurons and macrophages, suppressing inflammation and protecting neural integrity (Qi et al., 2023). JEV-induced VEGF-C has also been shown to expand functional meningeal lymphatic vessels, thereby ameliorating encephalitis triggered by neurotropic viral infections (Li et al., 2022).

Emerging evidence also implicates astrocytic VEGFR-3 in the regulation of neuronal excitability, with activation attenuating seizure-induced hyperexcitability via mTOR-dependent signaling pathways (Brewster, 2021). However, its contribution to astrocyte-driven responses during neurotropic viral infection remains poorly understood. The present study investigated the functional role of VEGFR-3 in astrocytes during JEV infection using a conditional deletion model targeting the ligand-binding domain of the receptor. Results demonstrated that VEGFR-3 in astrocytes serves as a crucial inhibitory regulator of inflammation, restraining excessive cytokine production and reducing viral pathogenesis. These findings not only deepen understanding of VEGFR-3 involvement in neuroinflammation but also identify novel targets for therapeutic intervention in neuroinflammation and neurological disorders.

MATERIALS AND METHODS

Animals

To generate *Vegfr-3*^{fl} mice with targeted deletion of the ligand-binding domain (LBD), exons 4–6 of the *Vegfr-3* gene were flanked with loxP sites. *Vegfr-3*$^{fl/fl}$ mice were then crossed with *Gfap-cre*$^{+/+}$ mice to generate *Vegfr-3*$^{fl/fl}$;*Gfap-cre*+/− mice, in which *Vegfr-3* was specifically knocked out in astrocytes. *Vegfr-3*$^{fl/fl}$;*Gfap-cre*−/− mice were used as controls.

All mice used in this study were aged 6–12 weeks and included both sexes. The mice were housed in individually ventilated cages (3–5 per cage) under specific pathogen-free conditions at Shanghai University. Environmental parameters were maintained at 20°C, 55% humidity, and a 12 h light-dark cycle (lights on from 0700h to 1900h), with food and water provided *ad libitum*. Mice were anesthetized via intraperitoneal injection before any experimental procedures. The study strictly adhered to institutional guidelines and complied with the Shanghai Municipal Regulations on the Management of Laboratory Animals under the approval of the Ethics Committee of Shanghai University (approval No. ECSHU 2023-050).

Cell culture

U87 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM; Sigma, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C with 5% CO_2 in a humidified atmosphere. For stimulation experiments, U87 cells were pretreated with SAR131675 (s2842; Selleck, USA) for 2 h prior to infection with JEV at a multiplicity of infection (MOI) of 5 for 24 h. Inflammatory gene expression was subsequently assessed.

Antibodies and chemical reagents

Antibodies and compounds used in this study included: JEV-E (1:1 000, GTX125867; Genetex, USA) and SAR131675 (s2842; Selleck, USA).

Viruses

The JEV P3 strain was supplied by Professor Shengbo Cao from Huazhong Agricultural University. This strain was propagated intracerebrally in suckling mice via a 10 μL inoculum containing 1×10^2 $TCID_{50}$ of the virus. The virus stocks were titrated via a conventional plaque assay and stored in aliquots at –80°C until subsequent experimental use.

Quantitative real-time polymerase chain reaction

Total RNA was extracted from cells or tissues with TRIzol reagent (Vazyme, China), followed by the generation of cDNA via reverse transcriptase M-MLV transcriptase (Takara, Japan) and random hexamers (Sangon, China). Relative qPCR was performed on a Stratagene MX3000P Real-time PCR System (Agilent Technologies, USA) with Cham QTM qRT-PCR SYBR Green Master Mix (Vazyme, China).

Animal experiments

Mice were anesthetized via intraperitoneal injection of a combined anesthetic, achieving sedation within approximately 1–3 min, and subsequently secured in a mouse restrainer. A 100 μL dilution containing 5×10^6 plaque-forming units (PFU) of JEV was administered via tail vein injection. Body weight and activity were monitored daily at consistent time points. Experimental procedures were initiated upon the onset of neurological symptoms.

Disease severity was assessed using a standardized clinical scoring system. A score of 0 indicated normal movement, absence of piloerection, and no signs of body rigidity or hindlimb paralysis. A score of 1 reflected normal movement without paralysis, but with piloerection, reduced activity, mild hindlimb extension, and a hunched posture. A score of 2 represented similar symptoms but with more pronounced piloerection and marked movement slowness. A score of 3 signified restricted mobility, piloerection, mild body stiffness, occasional jerks, and slight hindlimb extension without paralysis. A score of 4 denoted restricted movement, pronounced piloerection, significant body stiffness, hindlimb paralysis, and intermittent tremors. A score of 5 represented

severe movement restriction, piloerection, body stiffness, hindlimb paralysis, and continuous tremors, culminating in death.

Immunostaining

Brains from JEV-infected mice were fixed in 4% paraformaldehyde (PFA) (Sigma, USA) at 4°C for 24 h, then cryoprotected in 30% sucrose in phosphate-buffered saline (PBS) (Sangon, China) until fully immersed. Tissues were embedded in OCT (Sakura Finetek, USA) compound, snap frozen on dry ice, and stored at –20°C until use. Cryosections were prepared using a cryostat, rinsed in PBS, and incubated in a blocking/permeabilization buffer containing PBS, 0.1% Triton X-100, and 2% normal donkey serum. Sections were then incubated with primary antibodies and counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, Germany). Images were captured using a Zeiss Z1 fluorescence microscope (Germany).

Histological analysis

Following saline perfusion, brains were fixed in 4% PFA for 24–48 h, dehydrated through an alcohol gradient, cleared with xylene, and embedded in paraffin. Serial sections were prepared with a microtome and stained with hematoxylin and eosin (H&E). Stained sections were examined using an Olympus BX53 fluorescence microscope (Japan). Perivascular cuffing—defined as dense perivascular aggregates of inflammatory cells such as lymphocytes, monocytes, or macrophages—was evaluated based on characteristic “cuff-like” structures surrounding small vessels (Liu et al., 2022; Xing et al., 2022). These perivascular structures were distinguished by the contrast between vessel walls and adjacent tissue, with inflammatory cells exhibiting basophilic (dark-stained) nuclei and eosinophilic (lightly stained) cytoplasm (Moe et al., 2024). Quantification was performed across the left cerebral hemisphere in coronal sections, with each data point representing a single mouse.

Enzyme-linked immunosorbent assay (ELISA)

Cytokine concentrations, including TNF-α, IL-6, IFN-γ, and CCL2, were measured in serum and brain tissue homogenates using a Cytometric Bead Array (CBA) Kit (552364; BD, USA) according to the manufacturer’s instructions. IFN-β levels were determined using a commercial ELISA kit (K6965; pBl Assay Science, USA).

Statistical analysis

To ensure the reliability of the results, each experiment was repeated at least twice. Data are presented as mean±standard deviation (SD) or standard error of the mean (SEM). Student’s *t*-test was used for comparisons between two groups, whereas one-way or two-way analysis of variance (ANOVA) was used for multiple comparisons. Statistical calculations were performed using GraphPad Prism v.9.0.0, with $P<0.05$ considered statistically significant.

RESULTS

VEGFR-3 deficiency in astrocytes exacerbates JEV infection and increases mortality in mice

To investigate the role of astrocytic VEGFR-3 during viral infection, *Gfap-cre* mice were crossed with *Vegfr-3*$^{flox/flox}$ mice to generate *Vegfr-3*$^{fl/fl}$;*Gfap-cre* mice, in which the LBD of VEGFR-3 was selectively knocked out in astrocytes. Following peripheral inoculation with JEV, *Vegfr-3*$^{fl/fl}$;*Gfap-cre* mice exhibited significantly reduced survival compared to *Vegfr-3*$^{fl/fl}$ controls (Figure 1A). Quantitative analysis of *JEV-C* mRNA revealed a markedly higher viral load in the brains of *Vegfr-3*$^{fl/fl}$;*Gfap-cre* mice compared to *Vegfr-3*$^{fl/fl}$ mice (Figure 1B). JEV-E immunofluorescence staining further confirmed enhanced viral accumulation in the brains of *Vegfr-3*$^{fl/fl}$;*Gfap-cre* mice relative to controls (Figure 1C). In addition, H&E staining of mouse brain tissue indicated more pronounced neuroinflammatory changes in *Vegfr-3*$^{fl/fl}$;*Gfap-cre* mice, including extensive immune cell infiltration around blood vessels (black arrowheads) and beneath the meninges (yellow arrowheads) (Figure 1D). Collectively, these findings demonstrate that loss of VEGFR-3 in astrocytes exacerbates JEV-induced neuropathology by amplifying viral burden and leukocyte infiltration, ultimately compromising host survival.

Astrocytic VEGFR-3 deficiency in JEV-infected mice induces an inflammation-biased transcriptional program

To elucidate the molecular mechanisms underlying the increased mortality and viral burden associated with astrocytic VEGFR-3 deficiency during JEV infection, transcriptomic profiling was conducted on brain tissues from *Vegfr-3*$^{fl/fl}$ and *Vegfr-3*$^{fl/fl}$;*Gfap-cre* mice. RNA sequencing (RNA-seq) identified 1 273 differentially expressed genes (DEGs), including 571 up-regulated and 702 down-regulated genes ($|\log_2 FC|\geq 1$, Q-value≤0.05) (Figure 2A). Among the most prominently up-regulated transcripts were proinflammatory mediators such as *Ccl8*, *Ifng*, and *Il10*, while *Il12b* expression was down-regulated. KEGG pathway analysis of DEGs revealed significant enrichment in multiple signaling cascades, including inflammation-related pathways such as MAPK signaling, cytokine-cytokine receptor interaction, and TNF signaling (Figure 2B). Gene Ontology (GO) molecular function analysis further highlighted significant enrichment in cytokine and chemokine activity, as well as protein binding (Figure 2C), while cellular component analysis revealed significant changes in membrane-associated components and receptor complexes (Figure 2D). Further pathway analysis revealed that VEGFR-3 deletion in astrocytes triggered broad transcriptomic remodeling beyond canonical inflammatory signaling (Supplementary Figure S1). KEGG enrichment included not only inflammatory signaling cascades but also significant perturbations in the cGMP-PKG, PI3K-Akt, and JAK-STAT signaling pathways (Figure 2B; Supplementary Figure S1A). In parallel, disease-related pathway analysis suggested potential links to multiple pathological processes (Supplementary Figure S1B). Hierarchical clustering analysis of DEGs demonstrated distinct expression patterns between groups (Figure 2E; Supplementary Figure S1C, D). Heatmap visualization further illustrated coordinated dysregulation of gene sets involved in diverse biological processes, including inflammatory responses (e.g., *Ifng*, *Il10*). Astrocyte-specific deletion of VEGFR-3, along with alterations in neural-specific gene expression and enrichment of synaptic pathways, revealed that these inflammatory responses occurred within the CNS. These findings suggest that astrocytic VEGFR-3 signaling plays a crucial role in regulating CNS inflammatory responses during viral infection.

Astrocytic VEGFR-3 deficiency during JEV infection leads to localized neuroinflammation

To determine the contribution of astrocytic VEGFR-3 to central and systemic inflammatory dynamics during viral challenge, expression of key immune mediators was evaluated at the

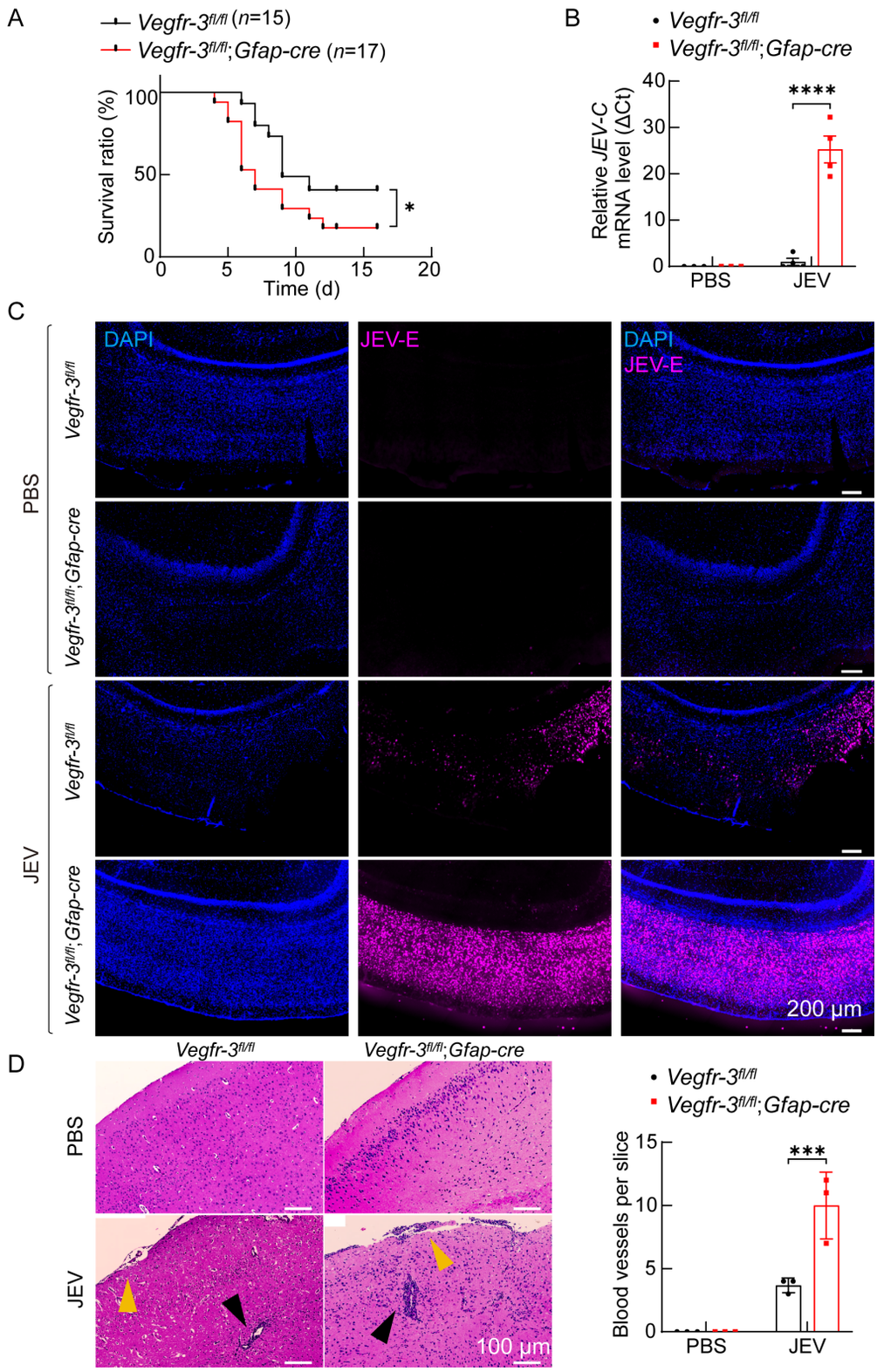

Figure 1 VEGFR-3 deficiency in astrocytes exacerbates JEV infection in mice, leading to a reduction in survival and an increase in viral load in the brain

A: Survival curves of *Vegfr-3*$^{fl/fl}$ (*n*=15) and *Vegfr-3*$^{fl/fl}$;*Gfap-cre* (*n*=17) mice with JEV infection. Statistical significance was determined using log-rank test, *: *P*<0.05. B: Relative mRNA expression of *JEV-C* in *Vegfr-3*$^{fl/fl}$ and *Vegfr-3*$^{fl/fl}$;*Gfap-cre* mouse brains at 5 days post-infection. Data are presented as mean±SEM, ****: *P*<0.0001. C: Immunofluorescence staining for JEV-E (magenta) and DAPI (blue) in brain sections from *Vegfr-3*$^{fl/fl}$ and *Vegfr-3*$^{fl/fl}$;*Gfap-cre* mice following PBS or JEV treatment at the peak of infection severity in most mice (approximately 5 days post-infection). Scale bar: 200 μm. D: Representative H&E staining of the cerebral cortex from *Vegfr-3*$^{fl/fl}$ and *Vegfr-3*$^{fl/fl}$;*Gfap-cre* mice under PBS treatment or JEV infection at approximately 5 days post-infection. Black arrowheads indicate typical perivascular cuffs; yellow arrowheads indicate belt of infiltrating immune cells under the cerebral cortex. Scale bar: 100 μm. Graph on the right quantifies blood vessels per tissue slice. Data are presented as mean±SD, ***: *P*<0.001.

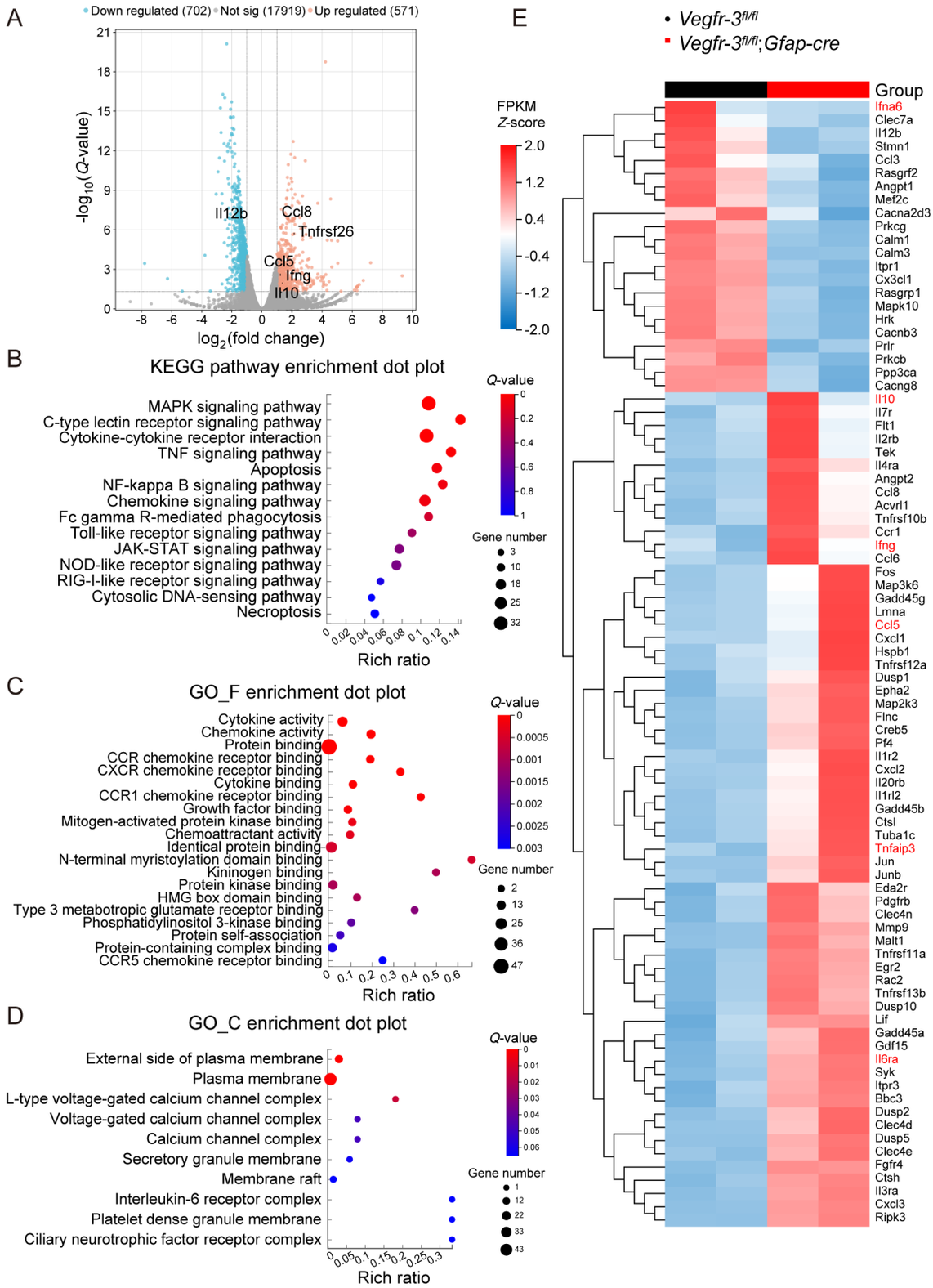

Figure 2 VEGFR-3 deficiency in astrocytes up-regulates transcription of inflammatory genes in JEV-infected mice

A: Volcano plot showing differential gene expression between *Vegfr-3*$^{fl/fl}$ and *Vegfr-3*$^{fl/fl}$;*Gfap-cre* mice ($|\log_2 FC| \geq 1$, Q-value≤0.05). Red dots represent up-regulated genes (n=571), blue dots represent down-regulated genes (n=702), and gray dots indicate non-significantly changed genes (n=17 919). *Vegfr-3*$^{fl/fl}$ and *Vegfr-3*$^{fl/fl}$;*Gfap-cre* mouse brains were collected at 6 days post-infection. ns: Not significant. B: KEGG pathway enrichment analysis of differentially expressed genes (DEGs). Dot size represents gene number and color indicates Q-value. C: GO molecular function (GO_F) enrichment analysis. Dot size represents gene number and color indicates Q-value. D: GO cellular component (GO_C) enrichment analysis. Dot size represents gene number and color indicates Q-value. E: Heatmap showing hierarchical clustering of selected DEGs. Expression values are shown as Z-score normalized FPKM across samples from both groups. Each group contained two samples, with each sample representing a single mouse. Each column in the heatmap represents an individual mouse.

mRNA and protein levels in *Vegfr-3*$^{fl/fl}$ and *Vegfr-3*$^{fl/fl}$;*Gfap-cre* mice following JEV infection. qPCR analysis of brain tissue revealed marked up-regulation of proinflammatory and antiviral genes, including *Ifng*, *Ccl2*, *Il6*, *Tnfa*, *Ifnb*, *Il10*, *Ccl5*, and *Ifna*, in JEV-infected *Vegfr-3*$^{fl/fl}$;*Gfap-cre* mice compared with controls (Figure 3A–H). Protein quantification using CBA assays confirmed elevated IFN-γ and CCL2 protein levels in brain homogenates of mutant mice following JEV infection (Figure 3I, J), while IL-6, TNF-α, and IFN-β levels exhibited upward trends that did not reach statistical significance (Figure 3K–M). These results suggest that VEGFR-3 signaling in astrocytes constrains neuroinflammatory responses to JEV infection.

Since we have observed significant changes in the mouse brain, we are also interested in understanding how peripheral inflammation is affected by the absence of VEGFR-3 in astrocytes. Similarly, serum profiling via CBA assay (Figure 4A–D) revealed no significant differences in key inflammatory mediators, including IFN-γ, CCL2, IL-6, and TNF-α, between genotypes. Consistent with a CNS-restricted inflammatory phenotype, cytokine concentrations in peripheral blood and major organs, including liver, lung, and spleen, remained comparable between *Vegfr-3*$^{fl/fl}$ and *Vegfr-3*$^{fl/fl}$;*Gfap-cre* mice following JEV infection (Supplementary Figures S2–S5). qPCR analysis of these peripheral tissues revealed no significant differences in the expression of key inflammatory genes (*Ifng*, *Il1b*, *Il6*, *Tnfa*, *Ifna*) or in viral RNA levels (*JEV-C*), except for *Il1a*, which was significantly reduced in *Vegfr-3*$^{fl/fl}$;*Gfap-cre* mice. These findings indicate that astrocytic VEGFR-3 deletion exacerbates the inflammatory response within the CNS without substantially altering peripheral immune responses during JEV infection.

VEGFR-3 inhibition increases astrocytic inflammation during JEV infection

To examine whether VEGFR-3 signaling constrains astrocytic inflammation during viral challenge, U87-MG human astrocytoma cells were treated with SAR131675, a selective inhibitor of VEGFR-3 enzymatic activity (Alam et al., 2012), prior to JEV infection. qPCR analysis revealed that SAR131675 pretreatment significantly elevated the expression of proinflammatory cytokines *IL-6*, *TNFA*, and *IFNB* following viral exposure compared to vehicle-treated controls (Figure 5A–C). Although *IFNG* expression was up-regulated by JEV infection, its expression remained unaffected by VEGFR-3 inhibition (Figure 5D). These findings indicate that chemical inhibition of VEGFR-3 signaling enhances the inflammatory response of astrocyte-like cells to JEV infection, reinforcing the role of VEGFR-3 as a negative regulator of virus-evoked cytokine responses in astrocytes.

DISCUSSION

Astrocyte-specific deletion of the extracellular LBD of VEGFR-3 markedly exacerbated JEV-induced neuropathology, resulting in greater viral burden and intensified neuroinflammation. VEGFR-3 is widely recognized as a key receptor mediating VEGF-C and VEGF-D signaling in neurons, where activation of downstream ERK and Akt pathways promotes neurogenesis and cell survival, underscoring its therapeutic potential for neurological disorders. Beyond neurons, VEGFR-3 is essential for the development of meningeal lymphatic vessels and for oligodendrocyte survival and myelination; however, its function in astrocytic responses to neurotropic viruses remains unclear.

This study investigated how astrocyte-specific VEGFR-3 deletion modulates JEV infection and associated molecular mechanisms (Aksan & Mauceri, 2025). Our previous findings demonstrated that VEGFR-3 negatively regulates the inflammatory response of macrophages during bacterial infection (Zhang et al., 2014). In these cells, VEGFR-3 inhibits TLR4-driven inflammatory signaling, modulates metabolism, enhances bacterial clearance, and suppresses caspase-1-mediated inflammasome activation and pyroptosis (Ma et al., 2022; Zhang et al., 2014). During viral encephalitis, neuron-secreted VEGF-C engages VEGFR-3 on macrophages, disrupting inflammatory feedback with neurons and dampening CNS inflammation (Qi et al., 2023). In contrast, astrocyte-derived VEGF-D binding to VEGFR-3 disrupts IL-3/IL-3Rα signaling between astrocytes and microglia, triggering mutual pro-inflammatory activation and exacerbating neuroinflammation in ischemic stroke models (Wang et al., 2025). VEGFR-3 activation in astrocytes also reprograms microglial lipid metabolism, leading to lipid droplet accumulation and pro-inflammatory polarization. Together, these findings illustrate the multifaceted, context-dependent function of VEGFR-3 across diverse CNS cell populations.

Viral infection further enhanced VEGF-C expression in the brain, which, in turn, attenuated macrophage inflammation via VEGFR-3 and stimulated the expansion of meningeal lymphatic vessels (MLVs), thereby facilitating viral clearance and amplifying immune priming in draining lymph nodes (Li et al., 2022; Qi et al., 2023). VEGFR-3 has also been implicated in neural progenitor cells, neurons, and oligodendrocytes during early cortical development (Choi et al., 2010; Le Bras et al., 2006). Multipotent neural stem cells (NSCs) express VEGFR-3, which regulates autocrine signaling and cell fate specification (Calvo et al., 2011; Han et al., 2015). In astrocytes, VEGFR-3 responds to VEGF-C via paracrine and autocrine pathways, modulating reactivity and the pathogenesis of ischemic injury (Shin et al., 2008). The current results identified both VEGFR-3 expression level and kinase activity in astrocytes as critical determinants of the neuroinflammatory response to JEV infection. In mice lacking the VEGFR-3 LBD (*Vegfr3*$^{\Delta LBD/\Delta LBD}$) or treated with VEGFR-3 kinase inhibitors, JEV challenge exacerbated encephalitic severity, TNF-α production, and neuronal apoptosis, emphasizing that VEGFR-3 controls brain inflammation through coordinated regulation of its extracellular and intracellular domains. Similar mechanisms have been described in peripheral bacterial infections, where both the LBD and kinase activity of VEGFR-3 jointly inhibit inflammatory signaling (Zhang et al., 2014). In summary, both the tyrosine kinase activity and the extracellular LBD of VEGFR-3 play a regulatory role in downstream signal transduction. While our study investigated the regulation of inflammation by VEGFR-3 in astrocytes, but whether it regulates neuronal cell death during viral infection in the CNS remains to be further investigated.

Astrocytes, among the most abundant glial cells in the CNS, play essential roles in maintaining neural homeostasis, modulating synaptic activity, and shaping immune responses within the brain parenchyma (Edison, 2024; Linnerbauer et al., 2020). Upon viral challenge, these cells initiate innate immune responses and secrete proinflammatory cytokines such as IL-1α and TNF, thereby contributing to the orchestration of neuroinflammatory signaling (Jorgačevski & Potokar, 2023).

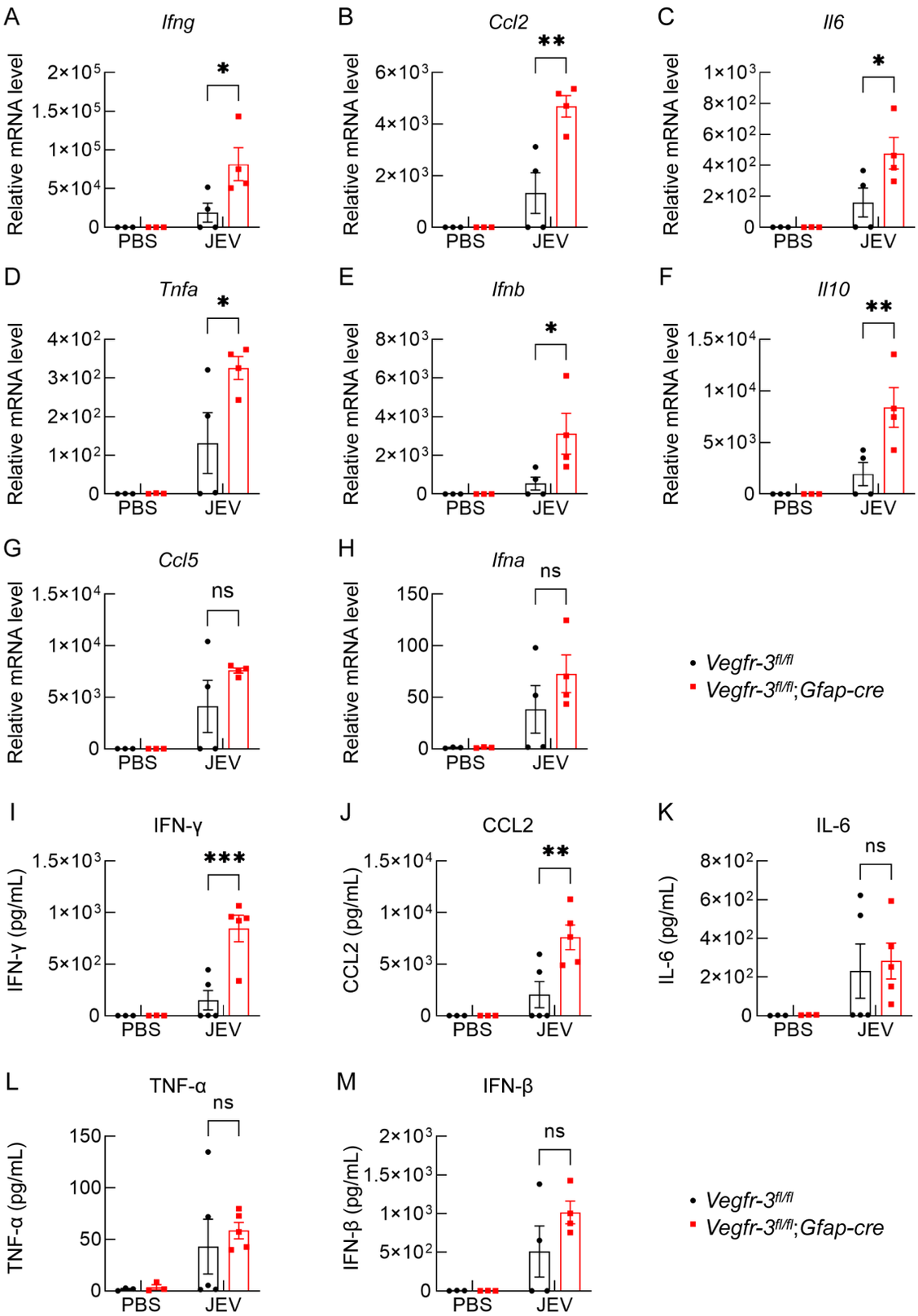

Figure 3 Elevated expression levels of inflammatory cytokines and chemokines in JEV-infected brains of mice with astrocyte-specific VEGFR-3 deficiency

A–H: Relative mRNA expression levels of inflammatory cytokines and chemokines in the brains of *Vegfr-3*$^{fl/fl}$ and *Vegfr-3*$^{fl/fl}$;*Gfap-cre* mice treated with JEV or PBS at the peak of infection severity (approximately 5 days post-infection). Analyzed genes included: *Ifng* (A), *Ccl2* (B), *Il6* (C), *Tnfa* (D), *Ifnb* (E), *Il10* (F), *Ccl5* (G), and *Ifna* (H). Data are presented as mean±SEM, ns: Not significant; *: P<0.05. I–M: Protein levels of inflammatory mediators in brain homogenates measured by cytometric bead array (CBA) for IFN-γ (I), CCL2 (J), IL-6 (K), TNF-α (L), and by ELISA for IFN-β (M). Data are presented as mean±SEM, ns: Not significant; **: P<0.01; ***: P<0.001.

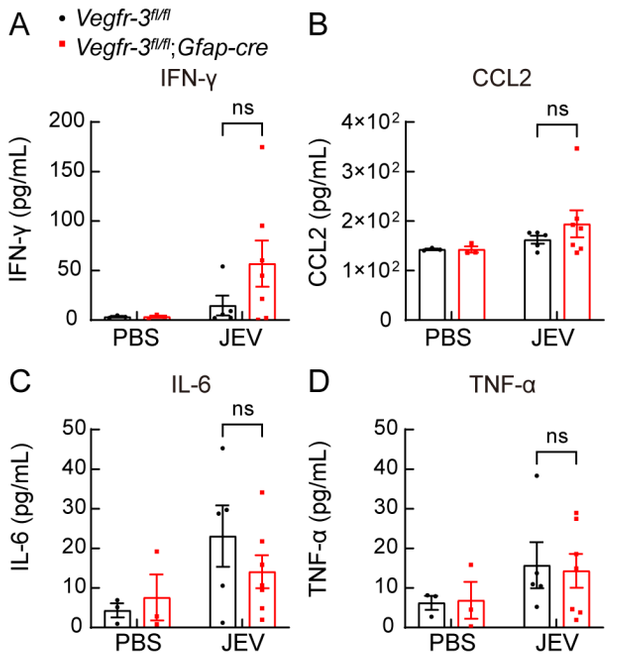

Figure 4 VEGFR-3 deficiency in astrocytes does not alter peripheral blood cytokine levels during JEV infection

A–D: Cytokine and chemokine levels in serum from *Vegfr-3*$^{fl/fl}$ and *Vegfr-3*$^{fl/fl}$;*Gfap-cre* mice treated with JEV or PBS, measured by cytometric bead array (CBA): IFN-γ (A), CCL2 (B), IL-6 (C), and TNF-α (D). Data are presented as mean±SEM, ns: Not significant.

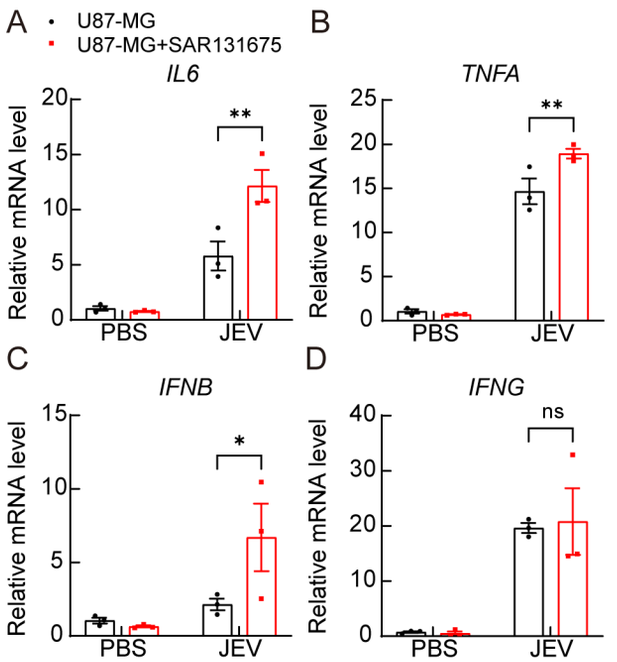

Figure 5 Inhibition of VEGFR-3 kinase activity enhances inflammatory cytokine gene expression in JEV-infected astrocytes

A–D: Relative mRNA expression levels of *IL-6* (A), *TNFA* (B), *IFNB* (C), and *IFNG* (D) in U87-MG cells pretreated with VEGFR-3 inhibitor SAR131675 or vehicle control, followed by JEV or PBS exposure for 24 h. Data are presented as mean±SEM, ns: Not significant; *: P<0.05; **: P<0.01.

Transcriptomic profiling of JEV-infected brains revealed that astrocyte-specific VEGFR-3 deficiency altered multiple inflammation-associated pathways, including MAPK signaling, cytokine-cytokine receptor interaction, and TNF signaling. These findings were derived from RNA-seq of brain tissues from *Vegfr-3*$^{fl/fl}$ and *Vegfr-3*$^{fl/fl}$;*Gfap-cre* mice following viral infection, revealing significant enrichment of inflammation-related pathways among the DEGs. Hyperactivation of MAPK signaling is known to drive excessive cytokine production and amplify neuroinflammatory damage in cerebral ischemia/reperfusion models (Xu et al., 2021). Cytokine networks within the CNS mediate inflammatory cascades, where microglia-derived IL-1α, TNF, and C1q induce astrocyte transformation into neurotoxic A1 subtypes (Sun et al., 2025; Zhang et al., 2025). TNF signaling via TNFR1/2 activation promotes reactive astrocytosis, with hippocampal TNFR1 up-regulation correlating with A1 astrocyte activation and neuronal apoptosis in depression models (Gao et al., 2024; Kim et al., 2022). However, whether VEGFR-3 directly regulates neuroinflammation through these pathways in astrocytes requires further investigation. Astrocytes exhibit dynamic phenotypic plasticity under inflammatory stress, initially adopting a neuroprotective role before transitioning toward neurotoxic states as pathological stimuli persist (Ding et al., 2021). However, the temporal trajectory of these transitions during viral infection, their contribution to neuronal dysfunction, and the potential involvement of VEGFR-3 in governing astrocytic phenotype shifts remain unresolved.

Following viral infection, astrocytes secrete a broad array of cytokines that not only regulate local immune dynamics but also influence peripheral immune cell infiltration. Previous studies have shown that in the context of enterovirus 71 (EV71) infection, astrocytes activate the p38MAPK signaling pathway via complement C5a-C5aR signaling, thereby driving the secretion of proinflammatory mediators such as IL-6 and TNF-α and contributing to neuronal injury (Giovannoni & Quintana, 2020; Luo et al., 2019). Astrocytes also exhibit the capacity to detect and respond to inflammatory signals from peripheral immune cells. For example, TH1-derived IFN-γ up-regulates IFNGR1 and MHC II expression in astrocytes, enabling them to function as nonprofessional antigen-presenting cells and further amplify CNS immune responses (Jorgačevski & Potokar, 2023; Wong et al., 1984). In the current study, astrocyte-specific VEGFR-3 deficiency resulted in elevated brain levels of IFN-γ and CCL2, accompanied by increased immune cell infiltration. Whether VEGFR-3 signaling intrinsically restricts cytokine production in astrocytes or modulates microglial activation through the regulation of additional secreted factors warrants further mechanistic investigation.

DATA AVAILABILITY

All sequencing datasets were deposited into the Science Data Bank (DOI: 10.57760/sciencedb.25874), Genome Sequence Archive (GSA, accession No. CRA026218), and National Center for Biotechnology Information (NCBI, BioProjectID PRJNA1269274) databases.

SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS CONTRIBUTIONS

L.L.Q., X.L.W., and Q.Z.: writing-original draft; L.L.Q., X.L.W., Q.Z., and B.W.: writing-review & editing; L.L.Q., X.L.W., Q.Z., H.Y.L., Y.S.D., and

M.S.H.: investigation and analysis; L.L.Q., X.J.L., and B.W.: Funding acquisition; L.L.Q., B.W., J.Y., S.B.C., F.L., and X.J.L.: Supervision. All authors read and approved the final version of the manuscript.

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