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The Critical Role of Interleukin-6 in Protection against Neurotropic Flavivirus Infection

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by

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ABSTRACT

West Nile virus (WNV) and Japanese encephalitis virus (JEV) are emerging mosquito-borne flaviviruses that cause encephalitis globally. Interleukin-6 (IL-6) upregulation is associated with viral infections in mice and humans, implying that it may influence the outcome of the disease. In this study, we investigated the role of IL-6 during neurotropic flavivirus infections. We found that IL-6 neutralization in human neuronal cells and its deletion in primary mouse cells led to increased flavivirus titers, suggesting that IL-6 inhibits flavivirus replication in vitro. In vivo experiments using IL-6 knockout mice (IL-$6^{-/-}$) showed higher mortality and viral titers in the periphery and brain, revealing IL-6's protective role against WNV and JEV infections. We also examined how IL-6 modulates immune responses. We observed reduced cytokine levels in the infected human neuronal cells with neutralized IL-6 and in the serum of IL-$6^{-/-}$ mice despite high viral load. We further detected downregulated type I interferon response in the IL-$6^{-/-}$ mice.

Our subsequent research involves comprehensive transcriptome analysis of WNV-infected murine brains to elucidate the function of IL-6. We used RNA-seq to compare IL-$6^{-/-}$ mice with wild-type (WT) mice, revealing key genes with differential expressions in IL-$6^{-/-}$ mice, primarily related to inflammation, cell death, and interferon pathways. IL-6 deficiency in mice weakened interferon-stimulated gene (ISG) activation, potentially compromising the antiviral response. These mice exhibited upregulated proinflammatory cytokine and chemokine genes but downregulated immunoregulatory genes. Ingenuity Pathway Analysis (IPA) highlighted increased immune cell recruitment and inflammatory pathways in IL-$6^{-/-}$ mice, along with decreased expressions of immune checkpoint regulators and NF-κB and MAPK pathway suppressors. An examination of differentially expressed pattern recognition receptors and neuroinflammation-associated genes in IL-$6^{-/-}$ mice brains corroborated these findings by revealing an upregulation

of antigen presentation, immune cell activation, leukocyte recruitment, and NF-κB pathway, but downregulation of genes crucial for stimulating interferon and antiviral responses. Overall, this study demonstrates that the absence of IL-6 increases the severity of WNV or JEV infection both in vitro and in vivo and sheds light on global immune pathway modulation by IL-6 during a neurotropic flavivirus infection.

INDEX WORDS: West Nile virus, Japanese encephalitis virus, Flavivirus, Encephalitis, Interleukin-6 (IL-6), Host-pathogen interaction, Neuronal cells, Mouse models, RNA-seq, Neuroinflammation

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West Nile Virus	WNV
National Institute of Allergy and Infectious Diseases	NIAID
Japanese Encephalitis Virus	JEV
West Nile Neuroinvasive Disease	WNND
Acute flaccid paralysis	AFP
Acute encephalitis syndrome	AES
Polymerase chain reaction	PCR
Central nervous system	CNS
Blood-brain barrier	BBB
Matrix metalloproteases	MMPs
Tight junction	TJ
High-mobility group box 1	HMGB1
Tumor necrosis factor-α	TNF-α
Toll-like receptor	TLR
Terminal deoxynucleotidyl transferase dUTP nick end labeling	TUNEL
c-Jun N-terminal kinase	JNK
Interleukin	IL
Chemokines	CC, CXC
Mouse embryonic fibroblast	MEF
IL-6 binding receptor protein	IL-6R
Soluble IL-6R	sIL-6R
Suppressor of cytokine signaling	SOCS

Lymphocyte choriomeningitis virus	LCMV
IL-6 knockout	IL-6$^{-/-}$
Type I interferon	IFN- I
Quantitative real-time reverse transcriptase-polymerase chain reaction	qRT-PCR
Wild type	WT
Bone marrow-derived macrophage	BMDM
Multiplicity of infection	MOI
Enzyme-Linked Immune Sorbent Assay	ELISA
Animal biosafety level-3	ABSL-3
Institutional Animal Care and Use Committee	IACUC
Dulbecco's Modified Eagle *Medium*	DMEM
Tris-Acetate-EDTA	TAE
Plaque forming unit	PFU
Two-way analysis of variance	ANOVA
Standard deviation	SD
Granulocyte-macrophage colony-stimulating factor	GM-CSF
IFN-α/β receptor knockout	IFN-αβR$^{-/-}$
Interferon alpha and beta receptor subunit 1	IFNAR1
Tick-borne encephalitis virus	TBEV
Langat virus	LGTV
Signal transducer and activator of transcription	STAT
Janus kinase	JAK
Interferon-stimulated genes	ISGs

Ingenuity Pathway Analysis	IPA
Nuclear factor kappa B	NF-κB
Fragments per kilobase of transcript per million mapped reads	FPKM
False discovery rate	FDR
Differentially expressed genes	DEGs
Dendritic cells	DC
Natural killer cells	NK
T helper cells	Th
Cytotoxic T lymphocytes	CTL
Interferon regulatory factor	IRF
Interferon-induced protein with tetratricopeptide repeats	IFIT
Interferon induced protein with helicase C domain	IFIH
Interleukin-1 receptor antagonist protein	IL-1RN
Tumor necrosis factor superfamily member	TNFSF
Triggering receptor expressed on myeloid cells	TREM
Macrophage migration inhibitory factor	MIF
Cluster of differentiation	CD
Mitochondrial antiviral signaling	MAVS
Mitogen-activated protein kinase	MAPK
Inhibitor of nuclear factor-κB (IκB) kinase	IKK
Pattern recognition receptors	PRR
Pathogen-associated molecular pattern molecules	PAMP
Oligoadenylate synthase proteins	Oas

Nucleotide-binding oligomerization domain, Leucine rich Repeat and Pyrin domain containing	NLRP
Neuronal apoptosis inhibitor proteins	Naip
C-type lectin	CLEC
Human leukocyte antigen	HLA
Ras-related C3 botulinum toxin substrate	RAC
Neutrophil chemotactic factor	NCF
Intercellular adhesion molecule	ICAM
Reactive oxygen species	ROS
Baculoviral IAP repeat-containing gene	BIRC
Cytochrome b-245, beta chain	CYBB
Post-transcriptional gene silencing	PTGS
Dengue virus	DENV
Clustered Regularly Interspaced Short Palindromic Repeats	CRISPR
Type 1 IFN receptor knockout	A129
Type 1/2 IFN receptor knockout	AG129
Lipid nanoparticles	LNP
Severe Acute Respiratory Syndrome Coronavirus-2	SARS-CoV-2
Human angiotensin-converting enzyme 2 under the cytokeratin 18 promoter	K18-hACE2
Z-DNA binding protein 1	ZBP1
Mixed lineage kinase domain like pseudokinase	MLKL
Receptor-Interacting Protein Kinase 3	RIPK3
Receptor-binding domain	RBD

Variants of concern	VoC
Mouse-adapted SARS-CoV-2	MA10

1 INTRODUCTION

1.1 Origin, Transmission, and Outbreaks

West Nile virus (WNV) and Japanese encephalitis virus (JEV) are mosquito-borne flaviviruses with global significance **[1]**. WNV, originating in Africa and first identified in Uganda's West Nile district in 1937, is maintained through bird reservoir hosts and mosquito vectors **[2-4]**. It is transmitted to humans and other animals through mosquito bites and spread globally because of increased travel, trade, and the movement of infected birds **[5, 6]**. Its emergence in North America in the late 1990s led to recurrent epidemics in the United States and sporadic outbreaks in Europe, Israel, and endemic regions in Africa **[7-10]**. In contrast, JEV originated in Southeast Asia, notably in Japan in the early 1930s **[11]**. It cycles primarily between mosquitoes, birds, and pigs, with humans as incidental hosts **[12]**. JEV continues to cause outbreaks in Asia, with countries like India, Southeast Asian nations, and China, often coinciding with monsoon seasons **[13-16]**. Both viruses underscore the importance of surveillance and mosquito control as public health measures to mitigate their impact on human populations.

1.2 Cases and Deaths

Since its arrival in New York in 1999 **[7]**, WNV, an NIAID Category B Priority Pathogen, has become the main cause of arboviral encephalitis in the United States, resulting in over 7 million infections, 28,000 neuroinvasive disease cases, 20,000 hospitalizations, and 2,600 deaths between 1999-2022 **[17]**. Similarly, JEV is Asia’s primary cause of virus-induced encephalitis, accounting for around 68,000 cases per year and an estimated 20,000 deaths, with long-term neurologic sequelae among half of the survivors **[18]**.

1.3 Symptoms and Clinical Manifestations

WNV and JEV infections present a spectrum of clinical manifestations. WNV infection can range from mild flu-like symptoms such as fever, headache, body ache, fatigue, skin rash, and swollen lymph nodes to moderate cases with high fever, neck stiffness, muscle weakness, tremor, and gastrointestinal symptoms. Severe WNV cases, known as West Nile Neuroinvasive Disease (WNND), involve encephalitis, meningitis, acute flaccid paralysis (AFP), and myelitis **[19, 20]**. Approximately 1 in 150 WNV-infected individuals develop WNND, especially older adults and the immunocompromised at higher risk **[21]**. JEV can also cause mild or asymptomatic febrile illness but might progress to severe neurological diseases, including Japanese encephalitis (JE) and acute encephalitis syndrome (AES), often affecting children more profoundly **[22, 23]**. Early medical intervention is crucial for both WNV and JEV; however, specific antiviral therapy is currently unavailable for these infections.

1.4 Treatment and Prevention

Management of WNV and JEV-induced diseases revolves around supportive care to alleviate symptoms and complications caused by these viruses **[24]**. Prevention involves mosquito control, public awareness campaigns promoting mosquito repellent and protective clothing use, and vaccination efforts where applicable, such as equine vaccination for WNV and human vaccination for JEV in risk areas **[25-28]**. Future research may yield new treatment options, but supportive care remains the primary approach to managing these viral infections.

1.5 JEV Vaccines

Available vaccines against JEV come in two forms: inactivated (killed) and live attenuated vaccines, administered as injections or a single dose, respectively **[29, 30]**. They might be accessible in monovalent (JE only) or combination formulations. Common brands include

IXIARO and JE-Vax **[31]**. These vaccines are recommended for individuals in or traveling to JE-endemic regions, particularly those with increased mosquito exposure, such as travelers, outdoor workers, and military personnel **[31]**. In endemic areas, vaccination campaigns may target specific age groups, with children as a primary focus **[32]**. Consultation with healthcare professionals is essential to determine vaccination timing, and booster doses may be advised for long-term protection **[33]**. Adverse effects of vaccination are generally mild, including discomfort at the injection site, headache, and fatigue, with rare severe reactions **[34]**. In the United States, IXIARO is the licensed JEV vaccine for individuals aged two months and older, administered as a two-dose series with the option for booster doses, depending on the travel context **[35]**.

1.6 Virus Structure

WNV and JEV possess single-stranded, positive-sense RNA genomes containing 5' and 3' UTRs that serve as templates for replication and protein synthesis **[36, 37]**. The capsid protein envelops the viral RNA, forming the nucleocapsid, which protects the genome **[38]**. The envelope protein on the virus's surface facilitates host cell entry, attachment, and fusion **[39]**. Also, both viruses' envelope proteins have glycosylation sites, influencing protein folding and interactions with host cells **[40]**. The membrane protein lines the inner envelope surface, aiding virion stability **[41]**. Moreover, both viruses encode non-structural proteins essential for replication and host response modulation, with NS3 and NS5 playing pivotal roles as NS3 exhibits protease and helicase activities, and NS5 functions as the viral RNA-dependent RNA polymerase responsible for replicating the viral genome **[42-44]**. Virions overall exhibit icosahedral symmetry, yielding spherical shapes **[45]**.

1.7 Characteristic Features of Neurological Infections Caused by WNV and JEV

Characteristics such as neuroinvasion (the ability to invade the brain), neurotropism (the affinity for infecting neural cells), and neurovirulence (the capacity to induce neurological symptoms) have been well-documented in human WNV and JEV cases. Neuroinvasion by WNV was evident through virus isolation, histochemistry, and PCR investigations in the infected brain tissue, prominent in regions such as the brainstem, basal ganglia, thalami, and spinal cord anterior horn cells **[46-48]**. Neurotropism was demonstrated by necrosis and neuronal loss upon WNV infection, particularly in areas where WNV antigens were detected **[48-50]**. These antigens were focal and sparse, except in severely immunosuppressed patients, where they were more widespread throughout central nervous system (CNS) **[48, 51]**. Lastly, its neurovirulence was apparent through the development of conditions like encephalitis, cranial nerve dysfunction, and motor neuron damage **[52-54]**. Prolonged clinical courses in some WNV cases were associated with demyelination, gliosis, and occasional perivascular infiltrates **[51]**. Moreover, murine models showcased WNV's affinity for infecting neurons in the CNS, as evidenced by neuronal degeneration, cytopathic changes, and cell mortality during infection **[55-57]**. According to in vitro studies, while neurons and astrocytes were found to support productive WNV infection, the virus's ability to replicate in microglial cells was limited **[58]**. Similarly, JEV targeted specific brain regions, including the cerebral cortex, olfactory area, basal ganglia, hippocampus, and brainstem, leading to neuronal damage **[59-61]**. Pathological changes in JEV-infected humans encompassed inflammation around blood vessels, glial cell proliferation, neuronal necrosis, edema, bleeding, and leukocyte infiltration, often leading to severe neurological consequences **[62, 63]**. Interestingly, in vitro and animal model studies revealed that JEV preferred to infect neural precursor cells and glial cells over neurons **[64]**.

1.8 Pathogenesis

Once a WNV or JEV-infected mosquito bites an individual, flavivirus gains entry into host cells by binding its envelope protein (E) with specific receptors located on the host cell surface. These receptors include glycosaminoglycans, tight junction proteins, integrins, laminin, C-type lectin, and phosphatidylserine receptors **[65]**. The virus primarily infects resident dermal cells (dendritic cells, fibroblasts, endothelial cells, and pericytes), which triggers local immune responses. It can then spread via the bloodstream, causing flu-like symptoms or asymptomatic viremia **[66]**. Later, the virus disseminates through the lymphatic system to multiple organs (e.g., heart, liver, spleen), potentially causing secondary viremia **[67]**. In severe cases, these viruses cross the blood-brain barrier (BBB), entering the CNS (**Figure 1**) and leading to conditions like encephalitis, meningitis, seizures, and paralysis **[68-70]**. The BBB, which controls the transport of immune cells and molecules between the bloodstream and brain, is breached through multiple mechanisms, including disruption of the BBB by its direct permeabilization or through the release of inflammatory mediators, the movement of virus-infected leukocytes between endothelial cell junctions, passive transport of virus across endothelial cells, and transport via the peripheral nervous system **[71]**. WNV and JEV directly damage the BBB's integrity by inducing the production of matrix metalloproteases (MMPs), which cleave tight junction (TJ) proteins and collagen IV **[72]**. Additionally, JEV induces mast cell secretion of chymase, a protease that cleaves BBB proteins **[73]**. Also, WNV induces an inflammatory response involving immune cells like macrophages and T cells within the CNS **[74]**. Another investigation demonstrated that JEV replicated in macrophage/monocytes in the periphery and migrated to the CNS, contributing to the inflammatory response **[75]**. Furthermore, ICAM-1 and VCAM induced by WNV infection and high-mobility group box 1 (HMGB1), secreted by JEV-infected endothelial cells, acted as a

chemoattractant and adhesion factor, stimulating infected immune cell migrations into the CNS **[76, 77]**. Transcytosis, an alternate pathway for WNV and JEV to enter the CNS, was utilized in a study where WNV and JEV-like particles localized in vesicles, crossing endothelial cells and pericytes **[78, 79]**. Moreover, WNV infection spread through motor nerve cells, causing severe neuromuscular pathology **[80]**. The ability for WNV's retrograde transport is especially relevant for neuroinvasion, as it allows the virus direct access to the CNS from the periphery without damaging the BBB **[81]**.

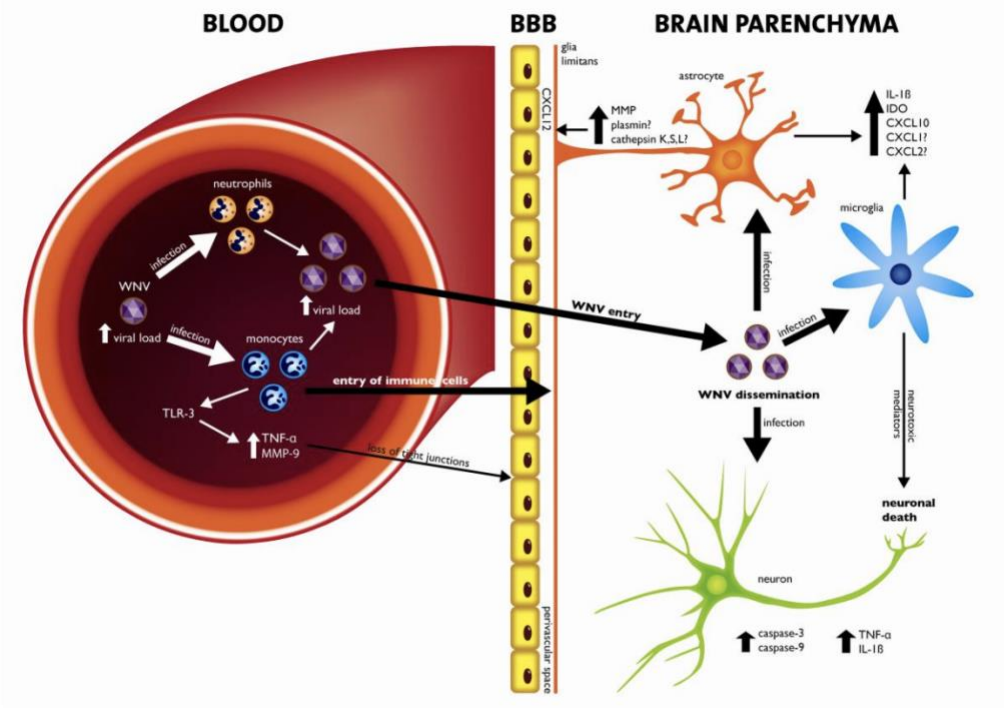

Figure 1: Regulation of BBB permeability, WNV entry into the brain, and the factors contributing to WNV-induced neuroinvasive disease. After WNV inoculation in the skin, the virus infects and reproduces in monocytes and neutrophils. TLR3 recognition of WNV replication in monocytes triggers the production of TNF-α and MMP-9 in a dose-dependent manner. This production leads to the disruption of tight junctions, enabling WNV and immune cells to enter the brain. The expression of CXCL12 is crucial for retaining immune cells in the CNS's perivascular spaces. Activated/infected monocytes and glial cells produce MMPs, cathepsins, and plasmin, potentially aiding cell migration from perivascular spaces into the brain parenchyma. Additionally, infected glial cells and neurons release neurotoxic substances, culminating in neuronal death. A significant portion of neuronal death is mediated through the caspase-9 and caspase-3 pathway, which is dependent on WNV's capsid. TNF-α: Tumor necrosis factor-α; MMP: Matrix

metalloproteinases; TLR: Toll-like receptor; CXCL: chemokine; IL: Interleukin; glial limitans: a network of foot processes of astrocytes **[70]**.

Once WNV and JEV reach the CNS, direct infection of neurons can lead to neuronal damage and cell death, causing severe neurological symptoms **[69]**. WNV induced apoptosis in neuronal cells by upregulating caspase 3, as indicated by Annexin V staining, DNA fragmentation, and TUNEL assay **[56, 82]**. JEV encoded viroporins that increased membrane permeability, potentially contributing to apoptosis **[83]**. A separate study showed that JEV infection led to TNFR-1 overexpression, activating extrinsic apoptosis pathways, and upregulated JNK and p53, associated with intrinsic apoptosis pathways **[84]**. Interestingly, inflammatory factors produced by JEV-infected microglia, even after virus inactivation, contributed to neuronal death, highlighting the role of bystander neuron damage in neuropathogenesis **[85]**.

1.9 Interleukin-6

Cytokine storm, an excessive expression of cytokines and chemokines, has been documented during flavivirus infections **[86, 87]**. This phenomenon can lead to septic shock, tissue damage, and multiple organ failure in various pathological conditions **[88]**. Cytokine storm also has protective functions in terms of virus clearance and memory response **[89]**.

Interleukin-6 (IL-6), a key cytokine, is well-known for its regulatory role in various aspects of the host immune response, including inflammation, autoimmunity, the acute phase response, and tissue repair **[90-94]**. Under normal conditions, circulating IL-6 levels remain low, typically 1 to 5 pg/ml. However, during a cytokine storm, these levels can rise more than 1,000-fold **[95]**. Evidence has also indicated an upregulation of IL-6 in response to viral and bacterial infections in mice and humans **[96, 97]**, suggesting its potential role in modulating disease severity caused by pathogens.

Consistent with others' research, our previous work demonstrated a substantial increase in IL-6 expression in the brains of mice and primary mouse cells, including mouse embryonic fibroblasts (MEFs), macrophages, dendritic cells, astrocytes, and neurons, following WNV infection **[87, 98]**. However, our understanding of the specific role that IL-6 plays during the progression of neurotropic flavivirus infections remains incomplete. Further investigation is required to elucidate its precise contributions to these infections.

1.10 Mode of Action of IL-6

IL-6 is secreted by various cell types, including fibroblasts, keratinocytes, mesangial cells, vascular endothelial cells, mast cells, macrophages, dendritic cells, and T and B cells **[99]**. Upon secretion, IL-6 initiates two types of signaling pathways: the classic signaling pathway, where IL-6 binds to the IL-6 binding receptor protein (IL-6R) and activates the universally expressed receptor gp130 **[100]**, and the trans-signaling pathway, where IL-6 binds to soluble IL-6R (sIL-6R) in the extracellular fluid, forming the IL-6/sIL-6R complex, which then couples to gp130 on the plasma membrane **[101]**. sIL-6R is produced once membrane-bound IL-6R get cleaved by the metalloprotease ADAM17 **[102] (Figure 2)**. The protective and regenerative functions of IL-6 rely on classic signaling through membrane-bound IL-6R, while the pro-inflammatory effects are primarily mediated through trans-signaling **[103, 104]**.

IL-6 or IL-6R cannot independently bind to gp130; the complex of IL-6 and IL-6R binds to gp130 and induces its dimerization **[105]**. IL-6R expression is limited to a subset of cells like hepatocytes and certain leukocytes; therefore, cells lacking IL-6R cannot be stimulated by IL-6 in the classic pathway **[106]**. On the contrary, in the trans-signaling pathway, IL-6 can stimulate any cell in the presence of sIL-6R since all cells express gp130 **[107]**. Most IL-6R-expressing cells express far more gp130 than IL-6R molecules. Therefore, stimulation of such cells with IL-6 alone

will only lead to the engagement of a few gp130 molecules, whereas stimulation with the complex of IL-6 and sIL-6R will stimulate all cellular gp130 proteins **[103]**.

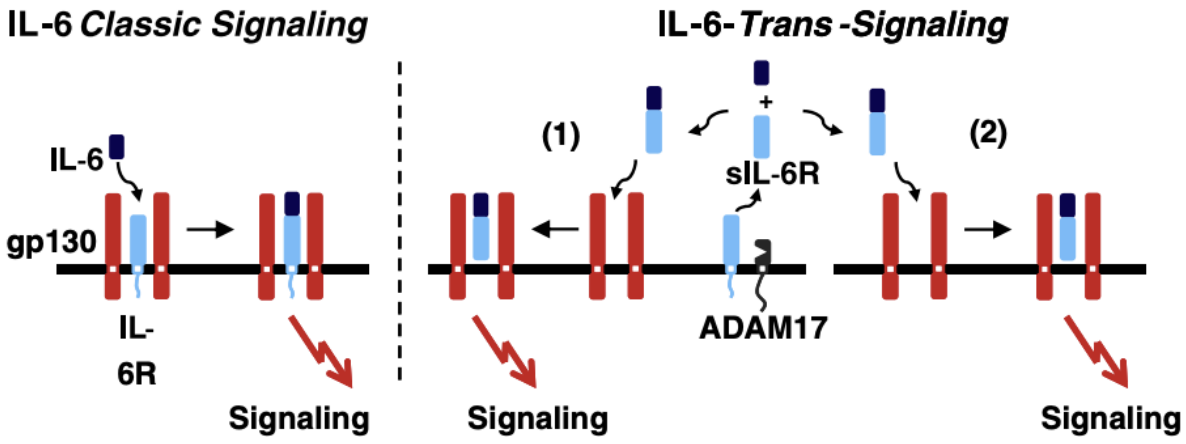

Figure 2: Classic and trans-signaling of IL-6. Cells expressing both gp130 and the IL-6R are responsive to IL-6 (classic signaling). However, cells that express only gp130 are stimulated by the IL-6/sIL-6R complex (trans-signaling). The sIL-6R is proteolytically cleaved of the membrane-bound precursor by the metalloprotease ADAM17 and binds IL-6 with comparable affinity. The IL-6/sIL-6R complex mediates gp130 activation in a (1) autocrine or (2) paracrine fashion **[108]**.

Both signaling pathways activate intracellular signal transduction through JAK, STAT3, and other pathways **[109]**. STAT3 activation triggers the production of suppressor of cytokine signaling 1 (SOCS1) and SOCS3, which bind to tyrosine-phosphorylated JAK and gp130, respectively, establishing a negative feedback loop to regulate IL-6 signaling **[110, 111]**. However, during inflammatory conditions, IL-6 production is induced by myeloid cells upon Toll-like receptor stimulation, often accompanied by cytokines like IL-1β and TNF-α, resulting in a significant amplification of IL-6 production **[103]**.

1.11 Evidence of Protective Roles of IL-6

Numerous studies have highlighted the protective role of IL-6 in the context of viral infections. The significance of IL-6 in the survival of mice infected with influenza virus and herpes simplex virus was identified using IL-6-deficient mice **[112-114]**. Additionally, mice experiments

involving the blockade of IL-6 or its receptor, IL-6R, using monoclonal antibodies during lymphocyte choriomeningitis virus (LCMV) infection, showed a detrimental effect on virus clearance **[115]**. Similarly, in vitro studies indicated that neutralizing endogenous IL-6 results in an increased burden of hepatitis B virus **[116]**. Further evidence came from studies involving mice with disrupted IL-6 genes, which exhibited impaired immune responses following infection with the Vaccinia virus **[117]**. Moreover, when wild-type mice were infected with a genetically engineered rabies virus carrying the IL-6 gene, they demonstrated heightened resistance to viral infection compared to those infected with the parental virus **[118]**. These findings collectively underscore the crucial and diverse roles of IL-6 in various viral infections.

1.12 Significance of Our Findings

Our research encompasses a multifaceted approach, combining cell-based and animal model-based studies to elucidate the role of IL-6 in neurotropic infections caused by flaviviruses. Notably, WNV and JEV prefer to infect myeloid cells (e.g., lymphoid dendritic cells and macrophages) in peripheral tissues, as well as neuronal cells within the CNS in vivo **[119, 120]**. However, the specific functions of IL-6 in these cells during flavivirus infections remain largely unexplored. Therefore, first, our research employs an in vitro approach to dissect the effect of IL-6 on peripheral and brain cells following flavivirus infection.

We aim to neutralize IL-6 activity in human neuronal cells after flavivirus infection to assess the impact of IL-6 on virus replication and the expression of key cytokines within these cells. Interestingly, WNV and JEV infections share common pathogenesis in mice concerning their primary target cells **[121, 122]**. Therefore, our next plan involves isolating and infecting some of these target cells, including those of fibroblast origin, myeloid lineage, and brain cells, from both

wild-type and IL-6 knockout (IL-$6^{-/-}$) mice, with WNV or JEV, allowing us to explore if IL-6 exerts similar effects on virus yields in primary mouse cells from peripheral tissues and the brain.

Mouse is an excellent model for inducing severe neurological symptoms that mimic human flavivirus diseases, hence facilitating our understanding of the molecular mechanisms underlying WNV or JEV-induced pathogenesis **[123]**. Previous studies using IL-$6^{-/-}$ mice have underscored the significance of IL-6 in the survival of mice infected with influenza virus, herpes simplex virus, and vaccinia virus **[112-114, 117]**. Thus, we will employ an IL-$6^{-/-}$ mouse model to characterize the role of IL-6 in flavivirus infections by conducting a longitudinal study to evaluate mouse survival, viral load dynamics, and immune responses in both the periphery and the brain following infection with WNV or JEV.

To gain deeper insights into the specific consequences of IL-6 deficiency during the progression of neurotropic flavivirus infection in mice, we will conduct a comprehensive RNA-seq analysis of WNV-infected brains of WT and IL-$6^{-/-}$ mice. RNA-seq offers a highly precise mean of measuring global shifts in transcriptional activity, enabling the identification of potential transcriptomic signatures associated with severe infection outcomes. Furthermore, RNA-seq allows us to delve into the intricate dynamics of gene expression patterns throughout the course of infection **[124, 125]**. In this study, our objective is to uncover both early and late transcriptional events that shape the host response to neurotropic WNV infection, focusing on individual genes and relevant pathways. This approach will provide valuable insights into the temporal modulation of immune pathways and cellular processes mediated by IL-6. Overall, our results will deliver the first substantial evidence regarding the specific roles of IL-6 in the pathogenesis of WNV and JEV neurotropic infections.

2 DISSERTATION SCOPE

2.1 Background and Rationale

Flaviviruses, such as West Nile virus (WNV) and Japanese encephalitis virus (JEV), are emerging arboviruses that cause encephalitis epidemics worldwide and continue to spread globally **[126, 127]**. After its introduction to New York in 1999 **[7]**, WNV, categorized as a Priority Pathogen by NIAID, has emerged as the primary cause of arboviral encephalitis in the United States, leading to thousands of fatalities **[128]**. Japanese encephalitis virus (JEV), on the other hand, is Asia's most significant cause of mosquito-borne encephalitis, leading to a considerable loss of lives **[129]**. Besides encephalitis, both viruses cause meningitis, paralysis, and long-term neurological sequelae in humans **[1, 130]**. Despite serious public health concerns, no specific drug or therapy is available for treating these neurological diseases. Therefore, it is a pressing need to dissect the interaction between key host factors and flavivirus to develop therapeutics. Decades of research suggest that IL-6 is a critical cytokine that controls different aspects of the immune response, including inflammation, autoimmunity, and acute phase response **[90, 91, 93, 131, 132]**. Upregulation of IL-6 is seen during viral infections among mice and humans **[96, 97]**, implying its role in viral disease outcomes. Recently, we and others have demonstrated that WNV infection induces a dramatic upregulation of IL-6 in mouse brains and primary mouse cells, including mouse embryonic fibroblasts (MEFs), macrophages, dendritic cells, astrocytes, and neurons **[87, 133, 134]**. However, *there is a* ***significant gap*** *in our understanding of IL-6's function in neurotropic flavivirus infection.*

Our primary goal is to address this gap by defining the critical role of IL-6 in the neurotropic infection caused by WNV or JEV, therefore providing valuable insight into the development of the much-needed therapeutic interventions that will reduce the severity of diseases

caused by these flaviviruses with pandemic potential. We hypothesize that IL-6 significantly impacts flavivirus replication, host immune response, and disease severity during the progression of neurotropic infections.

2.2 Specific Aims

Specific Aim 1: ***To investigate the cell-specific function of IL-6 during flavivirus infection:*** WNV and JEV prefer to infect myeloid cells like lymphoid dendritic cells and macrophages in peripheral tissues and neuronal cells within the CNS in vivo **[119, 120]**. However, the specific function of IL-6 in these cells during flavivirus infection remains unclear. Therefore, we will employ an in vitro methodology to elucidate the role of IL-6 in both peripheral and brain cells following infection with flaviviruses.

Sub-aim 1.1: ***To evaluate the consequence of IL-6 blockade in human neuronal cells:*** IL-6 exhibits significant activities within the CNS **[135, 136]**. However, the role of IL-6 in neuronal cells during flavivirus infection has not been previously studied. Therefore, we will conduct experiments using human neuroblastoma cells, SK-N-SH, infected with WNV or JEV and treated with anti-IL-6 antibodies to investigate the impact of IL-6 on flavivirus replication. Additionally, we will assess the expression of various cytokines to examine how IL-6 affects the immune response in these infected human cells.

Sub-aim 1.2: ***To analyze the influence of the absence of IL-6 in primary murine cells:*** WNV and JEV infections in mice share common pathogenesis in terms of their primary target cells, including those of fibroblast origin, myeloid lineage, and brain cells **[121, 122]**. These specific cell types will be isolated from wild-type and IL-6 knockout ($IL\text{-}6^{-/-}$) mice and infected with either WNV or JEV. The virus titers will then be assessed at different time intervals.

Specific Aim 2: ***To determine the role of IL-6 in flavivirus-mediated disease outcome in mice:*** Mice provide an appropriate model for investigating the molecular mechanisms of WNV and JEV-induced pathogenesis **[123]**. Therefore, employing an IL-6 knockout (IL-$6^{-/-}$) mouse model allows a comprehensive analysis of IL-6's function in flavivirus infections. Furthermore, by infecting these knockout mice with similar encephalitic viruses, we can discern whether IL-6-mediated effects are unique to WNV infection **[87]** or have broader implications for the pathogenesis of other encephalitic viruses.

Sub-aim 2.1: ***To assess the function of IL-6 in the survival of mice after flavivirus infection:*** Previous research with IL-$6^{-/-}$ mice has highlighted the significance of IL-6 in the survival of mice infected with influenza virus and herpes simplex virus **[112-114]**. In this study, we will assess the impact of IL-6 on disease outcomes of wild-type and IL-$6^{-/-}$ mice following WNV or JEV infection by examining parameters such as morbidity, mortality, and viral loads at different time intervals.

Sub-aim 2.2: ***To inspect early and late immune responses in flavivirus-infected mice lacking the IL-6 gene:*** An earlier investigation demonstrated that the disruption of the IL-6 gene in mice significantly affected their immune response after vaccinia virus infection **[117]**. Here, to ascertain the influence of IL-6 on the immune response of our flavivirus-infected mice, we will conduct quantitative RT-PCR and immunoassays to measure the expressions of type I interferons (IFN-I) and protein levels of various cytokines and chemokines in the periphery and brain at different stages of infection.

Sub-aim 2.3: ***To examine the global immune response in the brains of flavivirus-infected mice in the absence of IL-6 gene:*** A better understanding of the global gene expression changes in IL-$6^{-/-}$ mice underlying the multi-step progression of pathogenicity during flavivirus infection

could help develop potential therapeutic strategies for treating similar flaviviruses. In this sub-aim, we will use RNA-seq technology to analyze differential gene expressions between WNV-infected wild-type and IL-$6^{-/-}$ mice brains. Our focus will be on genes associated with interferon, cytokine, and chemokine signaling, pattern recognition, and neuroinflammation pathways, thereby elucidating the key cellular factors connected to IL-6 gene expression. Additionally, we will conduct a comparative analysis of the most prominently activated canonical pathways, functional networks, and upstream regulators among the infected mouse strains.

3 THE CRITICAL ROLE OF INTERLEUKIN-6 IN PROTECTION AGAINST NEUROTROPIC FLAVIVIRUS INFECTION

West Nile virus (WNV) and Japanese encephalitis virus (JEV) are emerging mosquito-borne flaviviruses causing encephalitis globally. No specific drug or therapy exists to treat flavivirus-induced neurological diseases. The lack of specific therapeutics underscores an urgent need to determine the function of important host factors involved in flavivirus replication and disease progression. Interleukin-6 (IL-6) upregulation has been observed during viral infections in both mice and humans, implying that it may influence the disease outcome significantly. Herein, we investigated the function of IL-6 in the pathogenesis of neurotropic flavivirus infections. First, we examined the role of IL-6 in flavivirus-infected human neuroblastoma cells, SK-N-SH, and found that IL-6 neutralization increased the WNV or JEV replication and inhibited the expression of key cytokines. We further evaluated the role of IL-6 by infecting primary mouse cells derived from IL-6 knockout (IL-$6^{-/-}$) mice and wild-type (WT) mice with WNV or JEV. The results exhibited increased virus yields in the cells lacking the IL-6 gene. Next, our in vivo approach revealed that IL-$6^{-/-}$ mice had significantly higher morbidity and mortality after subcutaneous infection with the pathogenic WNV NY99 or JEV Nakayama strain compared to WT mice. The non-pathogenic WNV Eg101 strain did not cause mortality in WT mice but resulted in 60% mortality in IL-$6^{-/-}$ mice, indicating that IL-6 is required for the survival of mice after the peripheral inoculation of WNV or JEV. We also observed significantly higher viremia and brain viral load in IL-$6^{-/-}$ mice than in WT mice. Subsequently, we explored innate immune responses in WT and IL-$6^{-/-}$ mice after WNV NY99 infection. Our data demonstrated that the IL-$6^{-/-}$ mice had reduced levels of key cytokines in the serum during early infection but elevated levels of proinflammatory cytokines in the brain later, along with suppressed anti-inflammatory cytokines.

In addition, mRNA expression of IFN-α and IFN-β was significantly lower in the infected IL-6$^{-/-}$ mice. In conclusion, these data suggest that the lack of IL-6 exacerbates WNV or JEV infection in vitro and in vivo by causing an increase in virus replication and dysregulating host immune response.

3.1 Introduction

West Nile virus (WNV) and Japanese encephalitis virus (JEV) are emerging mosquito-borne flaviviruses that have caused global epidemics of viral encephalitis **[137]**. Since its arrival in New York in 1999 **[7]**, WNV, an NIAID Category B Priority Pathogen, has become the main cause of arboviral encephalitis in the United States, resulting in over 7 million infections, 28,000 neuroinvasive disease cases, 20,000 hospitalizations, and 2,600 deaths between 1999 and 2022 **[17, 138]**. Similarly, JEV is the leading cause of virus-induced encephalitis in Asia, responsible for approximately 68,000 cases annually and an estimated 20,000 fatalities, with nearly half of the survivors experiencing long-term neurologic sequelae **[18]**. Aside from encephalitis, both viruses can cause severe neurological diseases such as meningitis, paralysis, myelitis, and death **[11, 55]**. These viruses predominantly affect gray matter and prefer targeting specific regions of the nervous system, including the basal ganglia, cerebellum, and spinal cord **[139]**. Neurons are their prime target while replicating in the brain **[63]**. The subsequent pathology is marked by neuronal loss, glial cell activation, blood-brain barrier disruption, and leukocyte infiltration to the parenchyma and perivascular space **[55, 71, 72, 140-145]**. Both viruses continue to spread and are causing human diseases in new parts of the world **[146]**. However, no effective therapies are available for treating individuals infected with WNV or JEV.

Interleukin-6 (IL-6) is a key cytokine that regulates various aspects of the host immune response. It has a broad range of effects on inflammation, autoimmunity, and acute phase response

[90-93]. Moreover, IL-6 is upregulated during viral and bacterial infections in mice and humans **[96, 97]**, implying that it may also play an important role in disease pathogenesis. Consistent with others' studies, we have previously demonstrated that WNV infection induces a dramatic upregulation of IL-6 in mouse brains and primary mouse cells, including mouse embryonic fibroblasts (MEFs), macrophages, dendritic cells, astrocytes, and neurons **[87, 98]**. Interestingly, several investigations have exhibited the protective role of IL-6 during viral infections. For example, the neutralization of endogenous IL-6 increased the virus burden following hepatitis B infection in vitro **[116]**. Furthermore, IL-6-deficient mice were used to reveal the importance of IL-6 for the survival of mice infected with the influenza virus and herpes simplex virus **[112-114]**. Another study showed that blocking IL-6 or IL-6R (IL-6 receptor) by monoclonal antibodies during lymphocyte choriomeningitis virus (LCMV) infection restricted virus clearance in mice **[115]**. Similarly, disruption of the IL-6 gene in mice impaired the immune response after infection with the vaccinia virus **[117]**. However, our knowledge of the function of IL-6 during the progression of a neurotropic flavivirus infection is lacking.

In the present study, we show the critical role of IL-6 in the neurotropic infections induced by WNV or JEV in human neuronal cells, primary murine cells, and mice. Our data revealed that human neuronal cells, primary murine neuronal cultures, mouse embryonic fibroblasts (MEFs), and bone marrow-derived macrophages (BMDMs) lacking IL-6 produced higher virus titers compared to the corresponding cells derived from wild-type (WT) mice upon infection with WNV or JEV. The IL-$6^{-/-}$ mice exhibited increased morbidity and mortality than WT mice when infected with either lethal or non-lethal strains of WNV or JEV. Higher viral loads were observed in the serum and brains of IL-$6^{-/-}$ mice at different time points of the infection. Additionally, reduced antiviral interferon response and cytokine production were detected in the absence of IL-6 gene.

Collectively, these results provide the first evidence of the important role of IL-6 in limiting the severity of WNV and JEV infections.

3.2 Materials and Methods

3.2.1 Flavivirus Infection and IL-6 Neutralization in Human Neuroblastoma Cells

A transformed human neuroblastoma cell line, SK-N-SH, was purchased from the American Tissue Culture Collection and propagated as described previously **[147]**. Cells below passage 10 were used for all experiments. SK-N-SH cells seeded in 12-well plates (1.2×10^5 cells/well) were infected with WNV NY99 or JEV Nakayama strain at the multiplicity of infection (MOI) of 0.1 as described previously **[147]**. Briefly, the cells were incubated with virus for 1 hour at 37°C. After incubation, the cells were replenished with fresh media or media containing neutralizing monoclonal antibodies against IL-6 (Catalog # MAB2061, R&D Systems). The concentrations of anti-IL-6 antibodies employed in this study were 5, 10, and 20 μg/mL. Supernatant and cell lysates were collected at 12, 24, and 48 hours **[148]**. The viral load in the supernatant was measured by plaque formation assay using Vero cells as described previously **[149]**.

3.2.2 Enzyme-Linked Immune Sorbent Assay (ELISA)

IL-6 neutralization was confirmed by measuring the protein levels of IL-6 in the WNV-infected SK-N-SH cell culture supernatants using the Quantakine Human IL-6 ELISA kit (R&D Systems) **[147]**. The tests were conducted following the guidelines provided by the manufacturer, and the plates were analyzed using a Victor 3 microtiter reader as described previously **[147]**.

3.2.3 Cell Viability Assay

SK-N-SH cells were seeded in 96-well plates (2×10^4 cells/well) and treated with 5, 10, and 20 μg/mL anti-IL-6 antibodies for 48 hours. Then, cell viability was assessed using CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) as described previously **[150]**.

3.2.4 Quantitative Real-Time Reverse Transcriptase-PCR Analysis

Total RNA was isolated from WNV-infected SK-N-SH cell lysates using a Qiagen RNeasy Mini kit (Qiagen, Germantown, MD, USA) and utilized to generate cDNAs using an iScript™ cDNA synthesis kit (Bio-Rad, Catalog #1708891). Quantitative RT-PCR (qRT-PCR) was conducted using the produced cDNAs utilizing SsoAdvanced™ Universal SYBR® Green Supermix (Biorad, Catalog # 1725271) to determine the expression levels of key cytokines **[151]**. The fold change in WNV-infected cells compared to mock-infected cells was calculated after normalizing to the housekeeping GAPDH gene **[133]**. The primer sequences used for qRT-PCR are listed in Table 1.

Table 1: Primer sequences used for qRT-PCR.

Gene (Accession No.)	Primer Sequence (5′–3′)
IL-6 (NM_000600)	
Forward	CCAGGAGCCCAGCTATGAAC
Reverse	CCCAGGGAGAAGGCAACTG
TNF-α (NM_013693)	
Forward	CCAGTCTGTATCCTTCTAA
Reverse	TCTTGTGTTTCTGAGTAGT
IL-1β (NM_000576)	
Forward	AGCACCTTCTTTCCCTTCATC
Reverse	GGACCAGACATCACCAAGC

3.2.5 Animals

C57BL/6J (WT) mice and IL-6$^{-/-}$ mice (002650) were acquired from the Jackson Laboratory (Bar Harbor, ME, USA). These mice were bred and genotyped in the animal facility located at Georgia State University. All WNV and JEV infection experiments were conducted in an animal biosafety level-3 (ABSL-3) laboratory following the designated protocol (A21067). The protocol was validated by National Institutes of Health and Georgia State University IACUC (Institutional Animal Care and Use Committee).

3.2.6 WNV and JEV Infection of Primary Mouse Cells

One-day-old pups from established colonies of WT and IL-6$^{-/-}$ mice were used to isolate MEFs and mouse cortical neurons as previously described **[152, 153]**. MEFs were cultured in DMEM media containing 10% FBS and 1% penicillin/streptomycin antibiotic. Neurons were

seeded on poly-D-lysine-coated plates and maintained in serum-free neurobasal A media supplemented with B27 (Gibco) for one week before infection. For isolating BMDM, eight-week-old WT and IL-6$^{-/-}$ mice were euthanized, and bone marrow cells were generated from the hind limbs as previously described **[154]**. The cultures were maintained in DMEM media supplemented with 10% FBS and 40 ng/mL macrophage colony-stimulating factor for one week before flavivirus infection.

MEFs, BMDMs, and neuronal cultures were infected with JEV Nakayama, WNV NY99, or WNV Eg101 at the MOI of 0.1 for one hour or inoculated with PBS (mock). Supernatants and cell lysates were collected at 12, 24, 48, and 72 hours after infection. Virus titers were then measured in the culture supernatants by plaque assay as previously described **[155]**.

3.2.7 IL-6 Deletion Efficiency in Primary Mouse Cells

Total RNA was extracted from MEF and BMDM cell pellets using a Qiagen RNeasy Mini kit (Qiagen) to generate cDNAs utilizing an iScript^{TM} cDNA synthesis kit (Bio-Rad). qRT-PCR was performed using the cDNAs to quantify the expression levels of IL-6 as previously described **[151]**. The primer sequences employed for amplifying IL-6 during qRT-PCR are listed in Table 1. The fold change in IL-6 expression in IL-6$^{-/-}$ cells, relative to WT cells, was calculated after normalizing to the housekeeping GAPDH gene **[133]**.

For the gel electrophoresis, a 2% agarose gel was prepared using 1X TAE (Tris-Acetate-EDTA) buffer. Once the gel solidified, a mixture of 2 µl of 6X SYBR Green loading dye and 10 µl of the qRT-PCR product were loaded into each lane. Additionally, a 10 µl sample of a 100-bp ladder was loaded into one lane as a size reference. The gel was initially electrophorized at 100 V for 15 minutes and then at 110 V for 45 minutes. The gel was visualized using BioDoc-It™ 220 imaging system (UVP, Upland, CA, USA).

3.2.8 Animal Infection Experiments and Plaque Assay

Eight-week-old WT and IL-6$^{-/-}$ mice were transferred to the ABSL-3 facility and acclimated to the local surroundings before initiating experiments. For survival studies, WT and IL-6$^{-/-}$ mice were inoculated with PBS (mock) or 100 PFU of WNV NY99 strain or 1,000 PFU of WNV Eg101 strain or 1,000 PFU of JEV Nakayama strain via the subcutaneous route. On days 2, 3, 4, and 6 after inoculation, 100 μL of blood was drawn from the tail veins to determine viremia and serum protein levels of cytokines and chemokines. These animals were monitored daily for clinical signs until day 21. A clinical scoring sheet was utilized to evaluate their appearance (coat condition, posture, grooming), signs of neurological disease (lethargy, ataxia, paralysis), and behavior (active, subdued when stimulated, unresponsiveness). Mice displaying severe symptoms, such as tremor and paralysis, were euthanized by CO_2 inhalation followed by cervical dislocation. In separate experiments, WT and IL-6$^{-/-}$ mice were subcutaneously inoculated with PBS, WNV NY99 (100 PFU), WNV Eg101 (1,000 PFU), or JEV Nakayama (1,000 PFU). On days 3, 6, and 8 after infection, the mice were anesthetized with isoflurane, and blood was collected through heart puncture. The mice were then perfused with PBS, and their brains were harvested and flash-frozen in 2-methyl butane, followed by tissue homogenization. Serum and brain homogenates were analyzed by performing plaque assays as previously described **[155]** to quantify WNV and JEV titers. Viral loads were expressed as PFU per milliliter of serum and PFU per gram of brain tissue.

3.2.9 Multiplex Immunoassay

The protein concentrations of inflammatory cytokines and chemokines in the mouse serum and brain homogenates were measured using a multiplex immunoassay kit (MILLIPLEX MAP Mouse Cytokine/Chemokine Kit, Millipore) **[156]**.

3.2.10 Type I Interferon Expression in Mouse Brains

Total RNA isolation from the brain homogenates was performed using a Qiagen RNeasy Mini kit (Qiagen), adhering to the manufacturer's instructions. cDNA was synthesized using an iScript™ cDNA synthesis kit (Biorad) and was employed for qRT-PCR to determine the expression levels of IFN-α and IFN-β **[151]**. The fold change in their expressions within the infected tissues was calculated against mock-infected tissues after normalization to the GAPDH gene **[133]**. The primer sequences utilized for qRT-PCR are listed in Table 2.

Table 2: Primer sequences utilized for qRT-PCR.

Gene (Accession No.)	Primer Sequence (5′–3′)
IFN-α (NM_010502)	
Forward	CTCTGTGCTTTCCTGATG
Reverse	CTGAGGTTATGAGTCTGAG
IFN-β (NM_010510)	
Forward	GCCTTTGCCATCCAAGAGATGC
Reverse	ACACTGTCTGCTGGTGGAGTTC

3.2.11 Statistical Analysis

GraphPad Prism 7.0 was used to perform a two-way analysis of variance (ANOVA) to measure *p* values for WNV and JEV titers in cell cultures, IL-6 protein levels in cell culture supernatants, the fold changes of cytokine expressions, IL-6 gene deletion efficiency test in primary mouse cells, type I IFN expression in mouse brain, and the quantification of the proteins in mouse serum. The Mann–Whitney test was used to calculate the *p* values for the viability test of antibody-treated SK-N-SH cells, virus titers in mouse serum and brain, and the protein levels

of various cytokines and chemokines in brains. Survival curves were compared using a Kaplan–Meier log-rank test. Unpaired Student's *t*-test was applied to measure *p* for the clinical scores. *p* values < 0.05 were used to indicate a statistically significant difference.

3.3 Results

3.3.1 Neutralization of IL-6 in Flavivirus-Infected Human Neuroblastoma Cells Causes an Increase in Viral Load and a Decrease in Proinflammatory Cytokine Expression Levels

To determine the role of IL-6 in human neuronal cells during flavivirus infection, we infected human neuroblastoma cells, SK-N-SH, with WNV NY99 or JEV Nakayama strain at the MOI of 0.1 and treated these infected cells with various concentrations of anti-IL-6 antibody. To verify the neutralization of IL-6 in the antibody-treated samples, we performed an ELISA to measure the IL-6 protein levels in culture supernatants obtained from WNV infection. We observed very low levels of IL-6 in mock-infected cells. There was a significant upregulation of IL-6 in the infected cells without antibody treatment at all time timepoints. As expected, treatment with anti-IL-6 antibody decreased the IL-6 concentration significantly in the infected cells. Our results showed that the antibody effectively blocked IL-6 in a time- and dose-dependent manner, evidenced by the lowest concentration of IL-6 (2.8 pg/mL) measured after 48 hours of treatment with 20 µg/mL antibody **(Figure 3A)**.

To examine the effect of IL-6 neutralization on the susceptibility of SK-N-SH cells to WNV or JEV infection, we analyzed virus titers in the culture supernatants of infected and antibody-treated cells collected at 12, 24, and 48 hours after infection. Plaque assay data demonstrated that all samples had gradually increased virus yields from 12 to 48 hours. When comparing the virus titers between infected samples with and without antibody treatment,

flavivirus loads were higher in the antibody-treated samples at each time point. However, the increase in viral loads reached statistical significance in a dose-dependent manner only at 48 hours **(Figure 3B, 3G)**. These data suggest that blocking IL-6 in SK-N-SH cells enhances WNV and JEV replication.

We also assessed the viability of antibody-treated SK-N-SH cells to determine the possible cytotoxicity effect of 5, 10, and 20 μg/mL anti-IL-6 antibody on the cells after 48 hours of treatment. A cell proliferation assay revealed that the antibody doses employed in this experiment did not cause significant cell death **(Figure 3C)**.

We next investigated the effect of IL-6 neutralization on the expression of key proinflammatory cytokines, TNF-α and IL-1β, in mock- and WNV-infected SK-N-SH cells by performing a qRT-PCR using RNA extracted from the cell lysates. TNF-α and IL-1β are known to initiate innate immune response, and mediate the activation, recruitment, and adherence of circulating macrophages and neutrophils to the infection site **[157]**. We observed a significant upregulation of IL-6, TNF-α, and IL-1β in the infected cells compared to mock-infected cells at 24 and 48 hours. However, despite higher viral load, exposure to 10 and 20 μg/mL concentrations of anti-IL-6 antibody led to a significant downregulation of these cytokines in the treated cells after 24 and 48 hours **(Figure 3D, 3E, 3F)**. The data from this experiment indicate that IL-6 neutralization inhibited the expression of critical cytokines, TNF-α and IL-1β, in the WNV-infected cells.

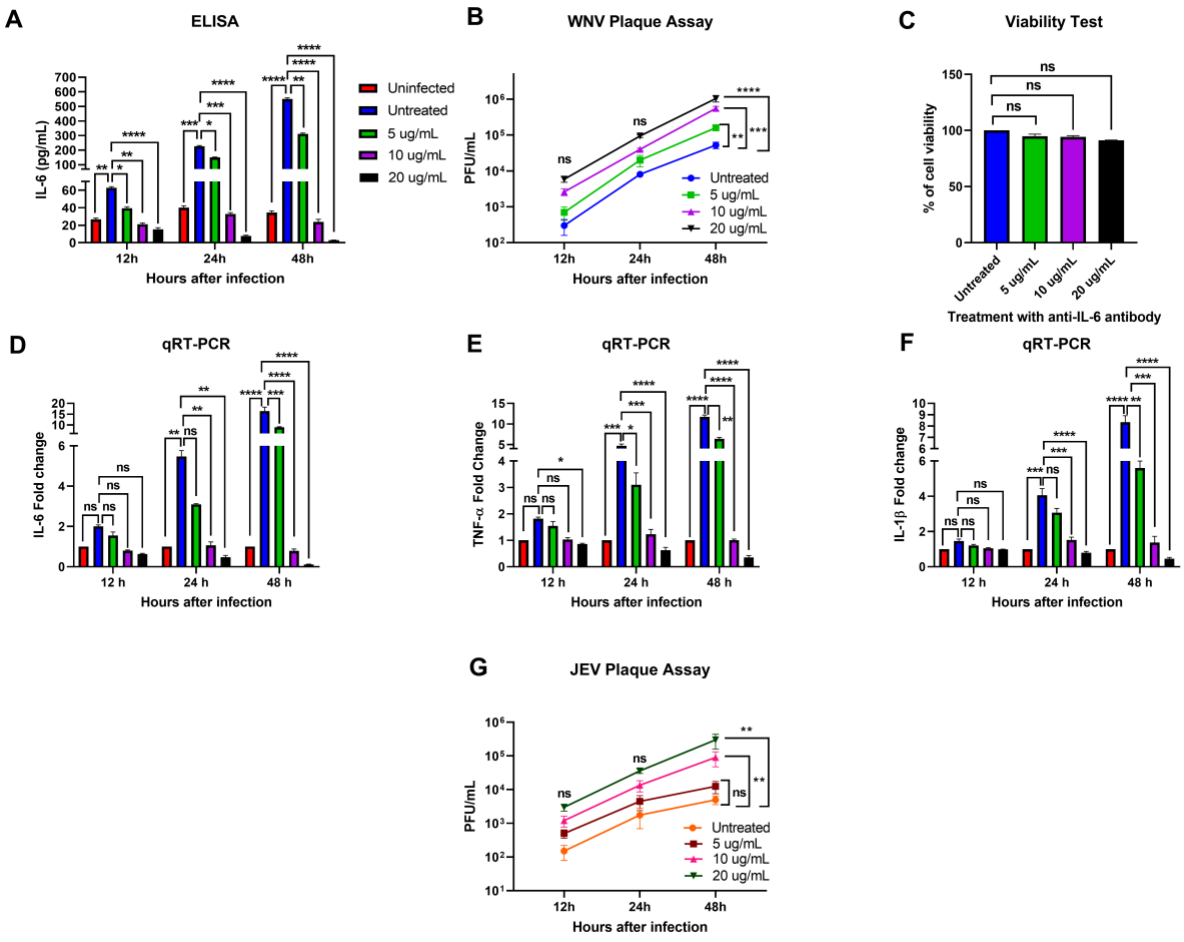

Figure 3: Effect of IL-6 neutralization in flavivirus-infected human neuronal cells. The human neuroblastoma cell line, SK-N-SH, was infected with WNV NY99 or JEV Nakayama strain (as described in Materials and Methods), followed by treatment with 5, 10, and 20 µg/mL of monoclonal antibody against IL-6. The supernatant and cell lysates were collected at different time points. **(A)** IL-6 protein levels were measured in the cell culture supernatant by ELISA. The data expressed are the mean concentration (pg/mL) ± SD of IL-6 in the supernatant, representing two separate experiments. **(B, G)** Viral loads in the cell culture supernatants were quantified by plaque assay. Results from two independent studies in duplicate are presented as PFU/mL ± SD. **(C)** Cell viability of SK-N-SH cells was assessed by conducting a cell proliferation assay at 48 hours after antibody treatment. Data are presented as mean ± SD for two independent experiments performed in triplicate. **(D, E, F)** cDNA templates from mock, WNV-infected, and anti-IL-6 antibody-treated WNV-infected SK-N-SH cells were used to evaluate the fold-change of IL-6, TNF-α, and IL-1β at different time points using qRT-PCR. Changes in the levels of these cytokines were normalized to the housekeeping gene, GAPDH, and the fold change in infected cells as compared to mock-infected controls was calculated. Data represent mean ± SD of three independent experiments conducted in duplicate. Statistical significance is presented as $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$.

3.3.2 IL-6 Restricts WNV and JEV Replication in Primary Mouse Cells

To further define the role of IL-6 during flavivirus infection, we planned an in vitro experiment using primary murine cells. We performed a comprehensive analysis of virus growth in cortical neurons, MEFs, and BMDMs isolated from WT and IL-6$^{-/-}$ mice. The absence of the IL-6 gene in the IL-6$^{-/-}$ cells was first verified by qRT-PCR and gel electrophoresis. No expression of IL-6 was detected in the cells derived from the IL-6$^{-/-}$ mice **(Figure 4A, 4B)**. These primary cells were then infected with WNV NY99, WNV Eg101, or JEV Nakayama strain at the MOI of 0.1, and the supernatants were harvested at various time points after infection (12, 24, 48, and 72 hours). Virus titers were then quantified in the cell supernatants using plaque assays. We observed that neuronal cultures derived from IL-6$^{-/-}$ mice exhibited significantly higher virus titers upon WNV NY99 or WNV Eg101 infection than those from WT mice **(Figure 4C, 4D)**. Furthermore, MEFs and BMDMs isolated from IL-6$^{-/-}$ mice produced significantly increased virus yields after infection with JEV compared to cells derived from WT mice **(Figure 4E, 4F)**. These experiments show that the knockout of the IL-6 gene increases the replication of lethal or non-lethal strains of WNV or JEV in primary mouse cells derived from both peripheral tissues and the CNS, indicating the protective role of IL-6 in both areas.

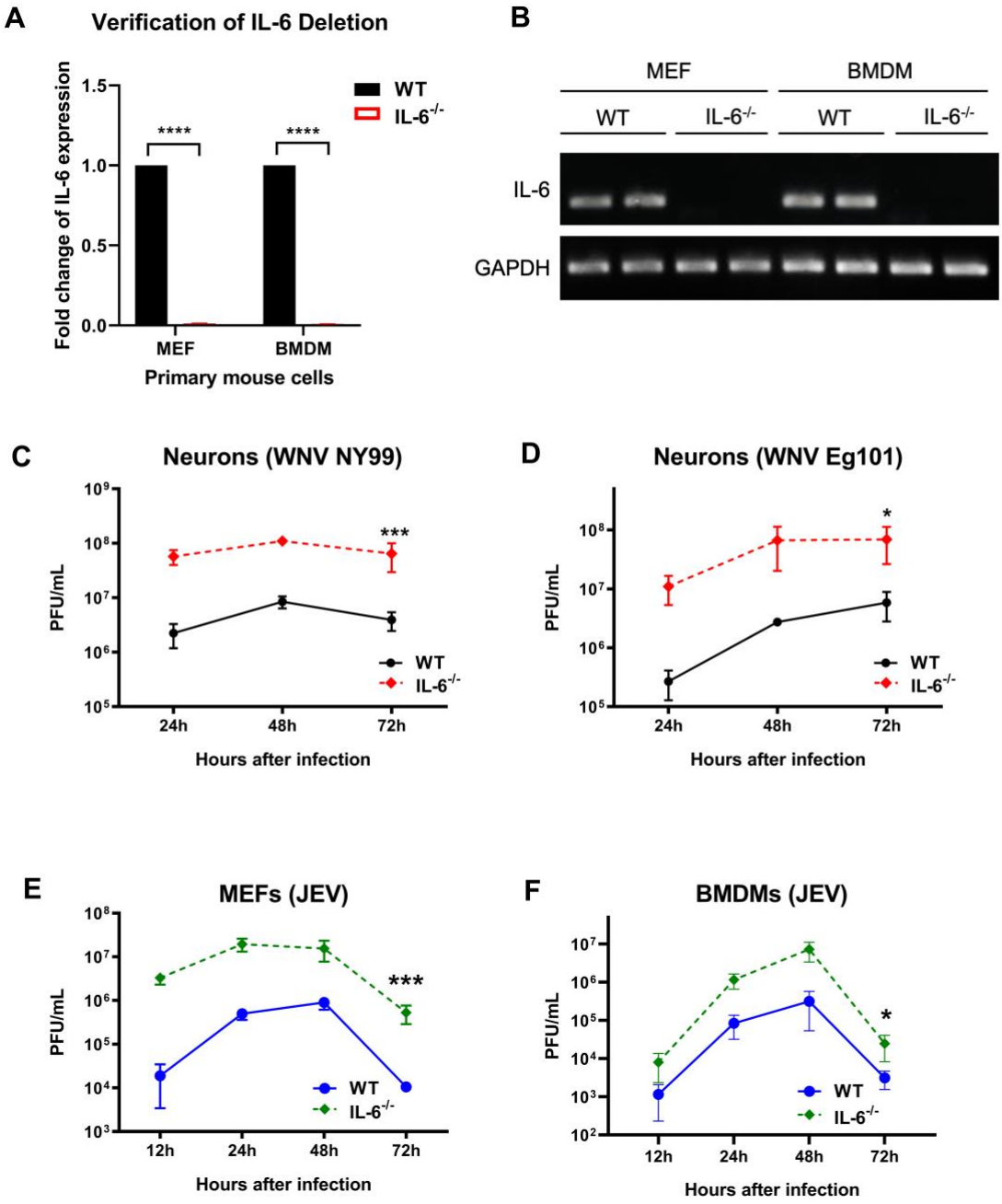

Figure 4: Flavivirus titers in the infected primary mouse cells isolated from WT and IL-6$^{-/-}$ mice. (A, B) qRT-PCR and gel electrophoresis were performed to confirm the deletion of IL-6 gene in primary cells derived from IL-6$^{-/-}$ mice. **(C)** WNV NY99, **(D)** WNV Eg101, or **(E, F)** JEV Nakayama were used to infect neuronal cultures, MEFs, and BMDMs (as described in Materials and Methods), and viral titers in the cell culture supernatants were determined at various time points by plaque assay. Results from at least three separate studies carried out in duplicate are presented as PFU/mL ± SD. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$.

3.3.3 IL-6 Limits WNV and JEV Pathogenesis in Mice Following Peripheral Infection

Next, to determine the role of IL-6 in WNV pathogenesis in vivo, we evaluated the morbidity and mortality of C57BL/6 (wild type) and IL-6$^{-/-}$ mice after WNV infection. Eight-week-old mice were subcutaneously inoculated with the lethal WNV strain NY99 (100 PFU) or the non-lethal WNV strain Eg101 (1,000 PFU) and monitored daily till day 21. Our records of clinical scores showed that all IL-6$^{-/-}$ mice manifested severe neurological symptoms after inoculation with WNV NY99 or WNV Eg101 **(Figure 5A, 5C)**. These symptoms included ruffled fur, hunched posture, ataxic gait, tremor, paralysis, and death. However, most WT mice infected with WNV NY99 had moderate clinical symptoms, while the WT mice infected with WNV Eg101 did not display any significant clinical symptoms **(Figure 5A, 5C)**.

The survival curve demonstrated that 100 PFU of WNV NY99 led to 40% mortality in WT mice, whereas it caused 100% mortality in IL-6$^{-/-}$ mice **(Figure 5B)**. The median survival time was also shorter for infected IL-6$^{-/-}$ mice than WT mice. As expected, there were no fatalities among WT mice infected with 1,000 PFU of WNV Eg101. However, infection of IL-6$^{-/-}$ mice with WNV Eg101 resulted in 60% mortality **(Figure 5D)**. These findings suggest that IL-6 is necessary for the survival of mice following WNV infection.

Subsequently, we inoculated eight-week-old WT and IL-6$^{-/-}$ mice subcutaneously with the JEV Nakayama strain (1,000 PFU) to assess the role of IL-6 in JEV pathogenesis in vivo. The clinical score data revealed that all IL-6$^{-/-}$ mice and some WT mice developed severe neurological symptoms after infection **(Figure 5E)**. Based on the survival curve, the infectious dose of 1,000 PFU resulted in 66% mortality in WT mice, whereas it caused 100% mortality in IL-6$^{-/-}$ mice **(Figure 5F)**. Like WNV infection, the median survival time was shorter for JEV-infected IL-6$^{-/-}$

mice than WT mice. The high morbidity and mortality observed in JEV-infected IL-6$^{-/-}$ mice suggest that IL-6 has a protective role in mice after peripheral JEV infection.

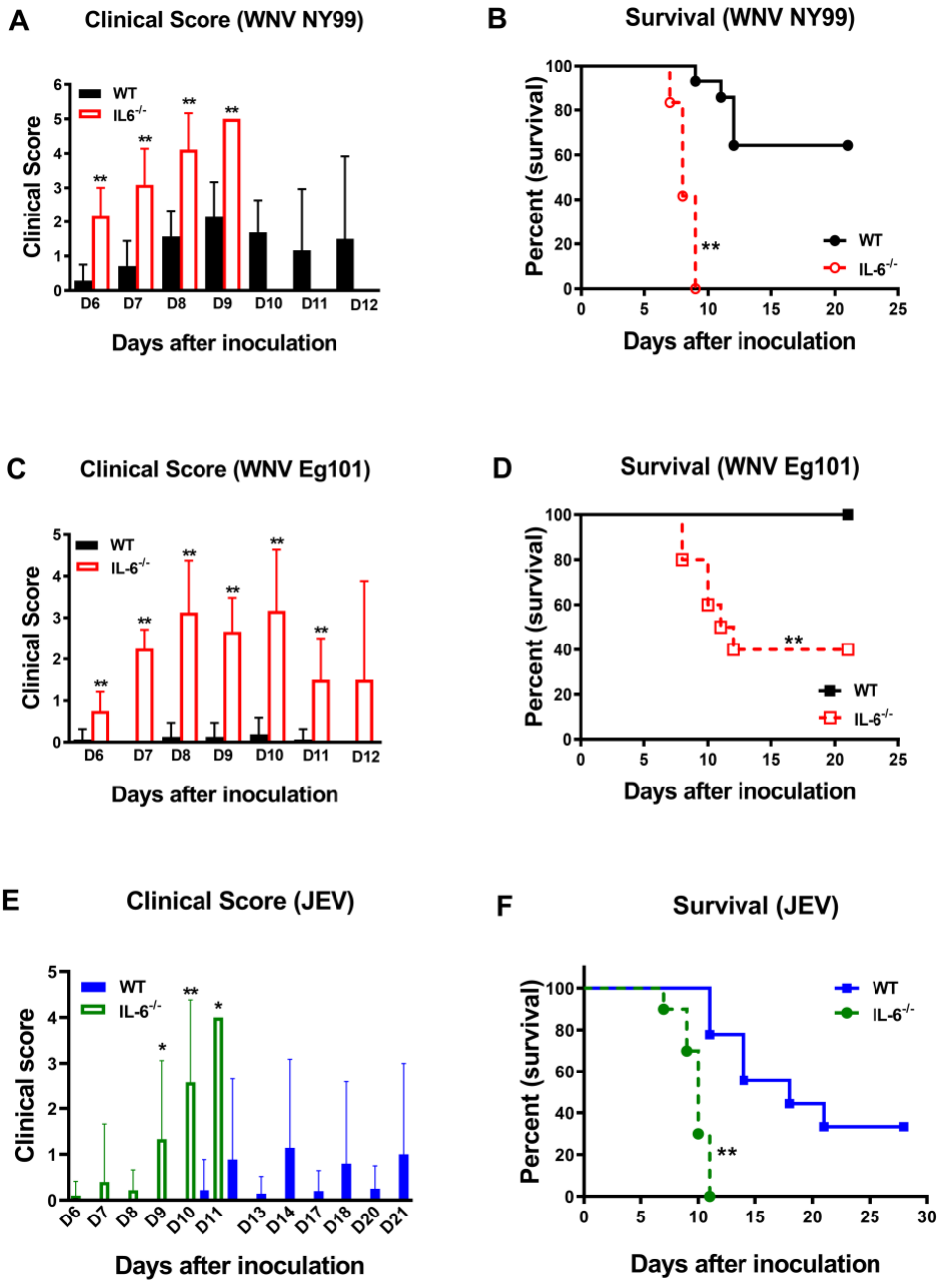

Figure 5: Clinical symptoms and survival of WT and IL-6$^{-/-}$ mice following WNV or JEV infection. WT and IL-6$^{-/-}$ mice were inoculated via footpads with **(A, B)** WNV NY99 (100 PFU), **(C, D)** WNV Eg101 (1,000 PFU), or **(E, F)** JEV Nakayama (1,000 PFU) strain. **(A, C, E)** Animals were checked daily for clinical signs. The clinical scores are as follows: 1 = ruffled fur/hunched posture; 2 = lethargy; 3 = abnormal walking; 4 = tremor/paralysis/moribund

(euthanized); and 5 = dead. Error bars indicate SD. **(B, D, F)** The difference between WT and IL-$6^{-/-}$ mice survival was determined. n = 12-20 mice per group, *$p < 0.05$, **$p < 0.01$.

3.3.4 IL-6 Is Required for the Control of WNV and JEV Load in the Periphery and Brain of Mice

We measured the viral titers in the serum and brains of WT and IL-$6^{-/-}$ mice at different time points following subcutaneous infection with WNV NY99 or JEV Nakayama strain by performing plaque assays. We detected significantly higher WNV or JEV titers in the serum of IL-$6^{-/-}$ mice compared to WT mice on day 3. The viral load decreased from day 3 to 6 in both groups; however, it remained significantly higher in IL-$6^{-/-}$ mice compared to the WT mice. We observed low viremia on day 8; however, the difference between IL-$6^{-/-}$ mice and WT mice was not significant **(Figure 6A, 6B)**.

Next, we examined the viral load in the brains harvested on days 3, 6, and 8 after WNV or JEV infection using plaque assay. WNV was not found in brains on day 3. WNV was detected on day 6 in both sets of mice, with no significant difference between the groups. However, WNV loads were significantly higher in the brains of IL-$6^{-/-}$ mice than in WT mice on day 8 **(Figure 6C)**. Furthermore, IL-$6^{-/-}$ mice exhibited significantly higher JEV loads in brains than WT mice at all time points **(Figure 6D)**. These data suggest that IL-6 controls WNV and JEV replication in both the periphery and the brains of mice.

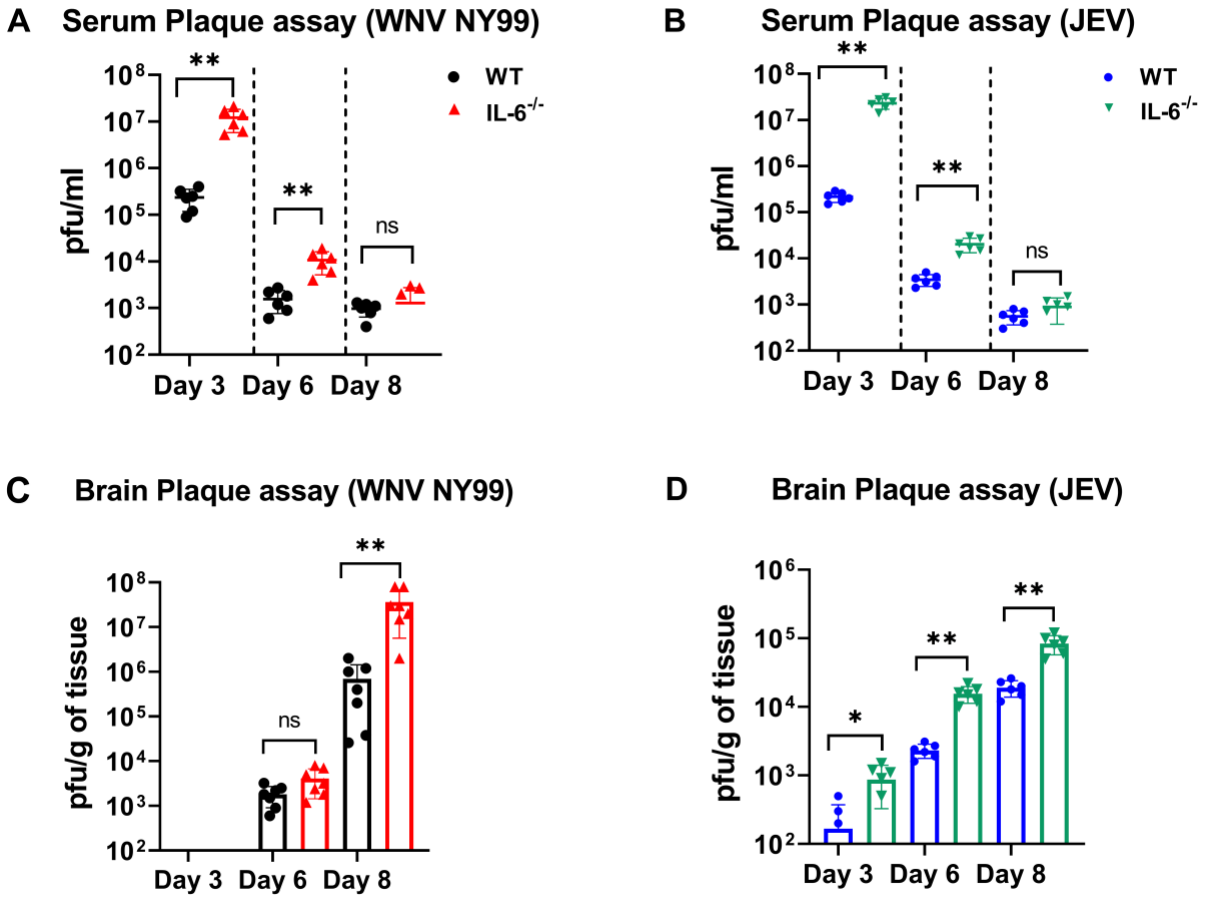

Figure 6: Evaluation of virus titers in WT and IL-6$^{-/-}$ mice. Virus titers were determined in the serum on days 3, 6, and 8 after infection with **(A)** WNV NY99 or **(B)** JEV Nakayama by performing plaque assay, and results are expressed as PFU/mL ± SD **(C, D)** Virus titers in brain homogenates were assessed on days 3, 6, and 8 following WNV NY99 or JEV Nakayama infection and expressed as PFU/g of tissue ± SD. Each data point corresponds to an individual mouse (n = 6–7 mice per group). *p < 0.05, **p < 0.01.

3.3.5 Innate Immune Responses in WT and IL-6$^{-/-}$ Mice after WNV Infection

It is known that WNV infection causes significant upregulation of numerous cytokines and chemokines **[158]**. WNV-induced proinflammatory mediators protect hosts from lethal WNV infection **[159]**. However, sustained activation and dysregulation of these host factors can also result in fatal WNV disease **[121]**. To evaluate the role of IL-6 in modulating key cytokines and chemokines in WNV-infected mice, first, we assessed the concentrations of these proteins in the

serum of WT and IL-6$^{-/-}$ mice on days 2 and 4 using a multiplex immunoassay. Interestingly, the protein levels of IL-1β, IL-10, IL-12, IL-15, TNF-α, GM-CSF, G-CSF, MIP-2 (CXCL2), and RANTES (CCL5) were significantly lower in IL-6$^{-/-}$ mice than WT mice **(Figure 7)**. There are evidence that IL-6 induces several cytokine and chemokine pathways in the periphery **[90, 160]**. Therefore, the absence of IL-6 might have impaired cytokine and chemokine induction in the periphery of IL-6$^{-/-}$ mice. Interestingly, the IP-10 (CXCL10) protein level was significantly elevated in IL-6$^{-/-}$ mice than in WT mice.

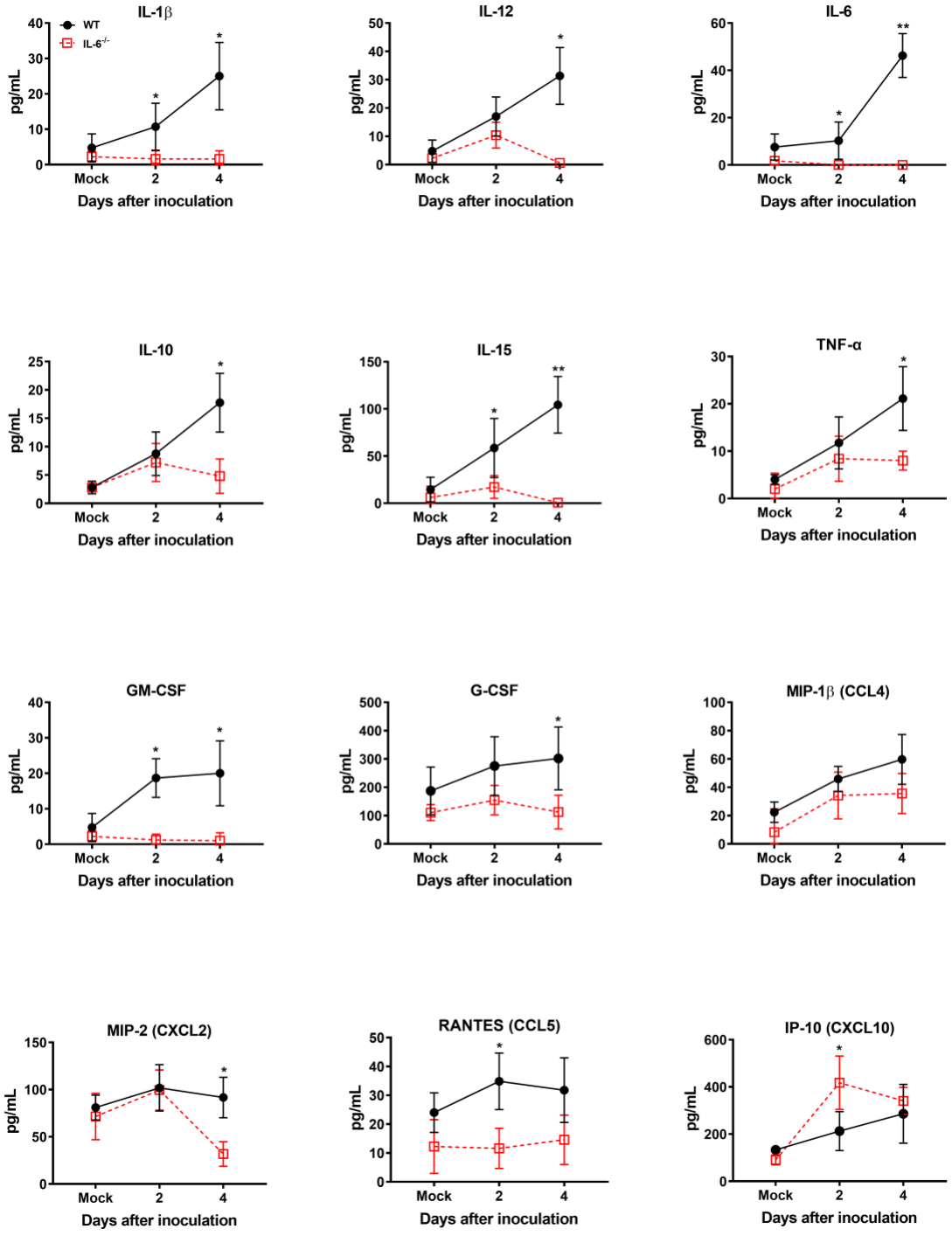

Figure 7: Analysis of cytokine and chemokine protein levels in the serum of WT and IL-6$^{-/-}$ mice after infection with WNV NY99. Cytokine and chemokine levels were assessed in the serum of WNV NY99-infected WT and IL-6$^{-/-}$ mice on days 2 and 4 following infection by performing a multiplex immunoassay. The data are expressed as the mean concentration (pg/mL) ± SD (n = 6–7 mice per group). *$p < 0.05$, **$p < 0.01$.

Type I interferon (IFN-I) response is essential for protecting the brain from neurotropic flaviviruses **[161-164]**. We next investigated whether IL-6 deficiency affects IFN-I activity in mice following WNV NY99 infection. We performed qRT-PCR to measure the expression levels of IFN-α and IFN-β in brain homogenates collected from WT and IL-6$^{-/-}$ mice at 3-, 6-, and 8 days after infection. Our data demonstrated a significant reduction in the expression of IFN-α **(Figure 8A)** and IFN-β **(Figure 8B)** genes in IL-6$^{-/-}$ brains compared to WT brains at day 8.

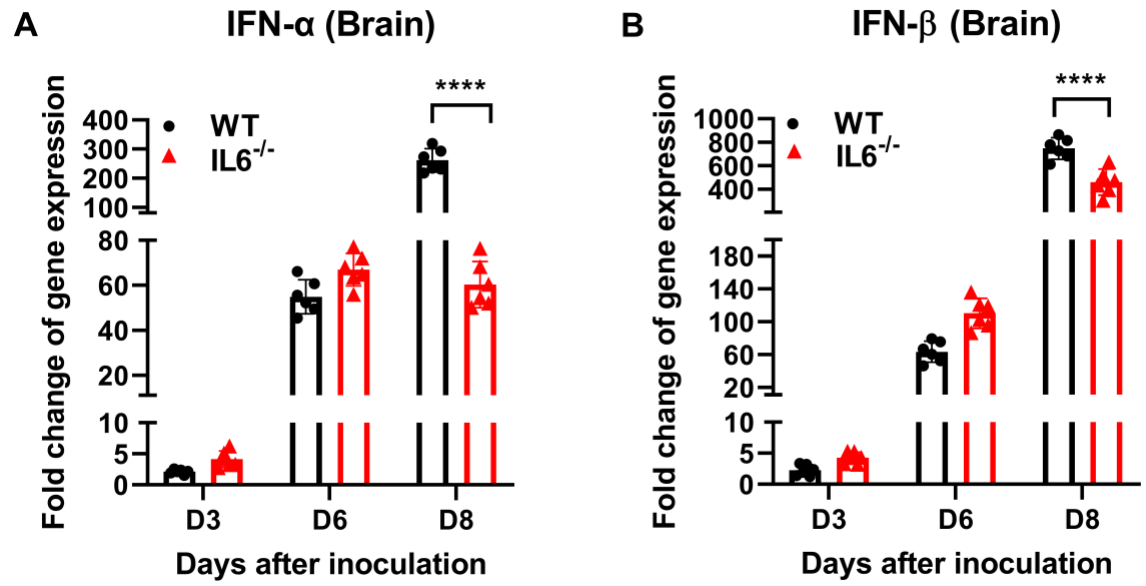

Figure 8: Type I interferon expression in the brains of WT and IL-6$^{-/-}$ mice after WNV NY99 infection. Total RNA was extracted, and cDNA templates were generated from brain homogenates of WT and IL-6$^{-/-}$ mice at days 3, 6, and 8 after infection. Quantification of **(A)** IFN-α and **(B)** IFN-β gene expressions were conducted using qRT-PCR. Expression levels were normalized to the housekeeping gene, GAPDH, and fold changes in infected cells relative to mock-infected controls were determined. Data is presented as mean ± SD from three independent experiments conducted in duplicate (n = 6 mice per group). ****$p < 0.0001$.

Next, we measured the protein levels of essential cytokines and chemokines in the brain homogenates of WT and IL-6$^{-/-}$ mice on days 3, 6, and 8 upon WNV infection **(Figures 9 and 10)**. The multiplex immunoassay revealed that the protein concentrations of chemokines, CCL2, CCL3, and CXCL2, were significantly higher in the brains of IL-6$^{-/-}$ mice than WT mice on days

6 and 8 **(Figure 9)**. For CCL4, CCL5, and CXCL10, the brains of IL-6$^{-/-}$ mice had higher protein levels on day 6. Interestingly, the CXCL1 protein, known for recruiting and activating neutrophils at the infected tissue site **[165]**, was substantially elevated in IL-6$^{-/-}$ mice at all time points.

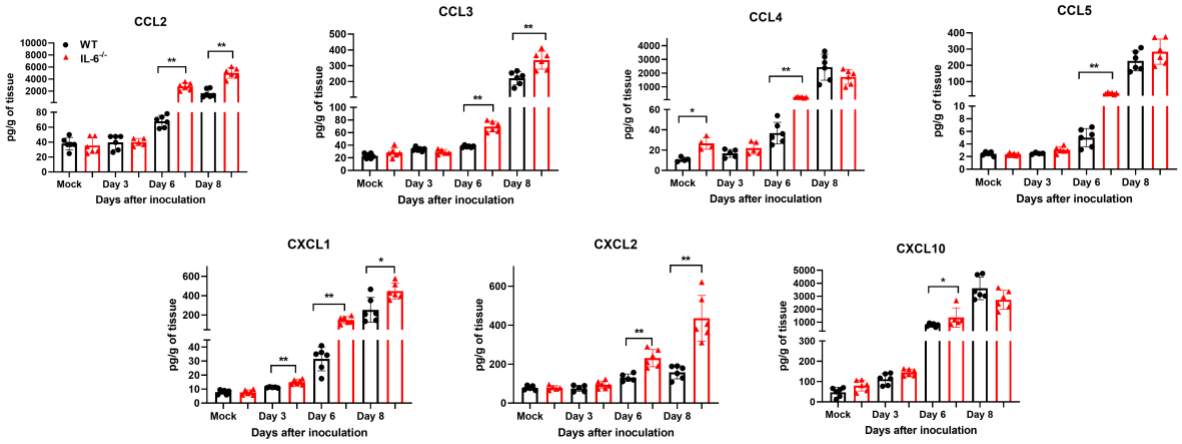

Figure 9: Analysis of chemokine concentrations in murine brains following infection with WNV NY99. Multiplex immunoassay was conducted to measure the protein levels of chemokines in the brains of WT and IL-6$^{-/-}$ mice on days 3, 6, and 8 after WNV NY99 infection. The results were presented as the mean concentration (pg/g) of tissue ± SD (n = 6–7 mice per group). *$p < 0.05$, **$p < 0.01$.

Similarly, protein concentrations of IL-1β and IL-12 (p70) were significantly higher in IL-6$^{-/-}$ mice brains on days 6 and 8 **(Figure 10)**. Notably, TNF-α protein level was significantly elevated in the brains of IL-6$^{-/-}$ mice than WT mice only at day 6. However, the IL-17 protein, which activates local chemokine production **[166]**, was significantly increased in IL-6$^{-/-}$ mice brains compared to WT mice brains at all time points. As expected, IL-6 protein concentration was significantly reduced in IL-6$^{-/-}$ mice brains. Interestingly, protein levels of IL-9, GCSF, and IL-10 were significantly decreased in the brains of IL-6$^{-/-}$ mice. These three cytokines have anti-inflammatory functions **[167, 168]** and prevent excess infiltration of phagocytic cells to the infected tissue sites **[169]**. Our findings in this experiment highlight the role of IL-6 in modulating

the immune response to flavivirus infection in mice by differential regulation of type I interferon, cytokines, and chemokines in the periphery and brain.

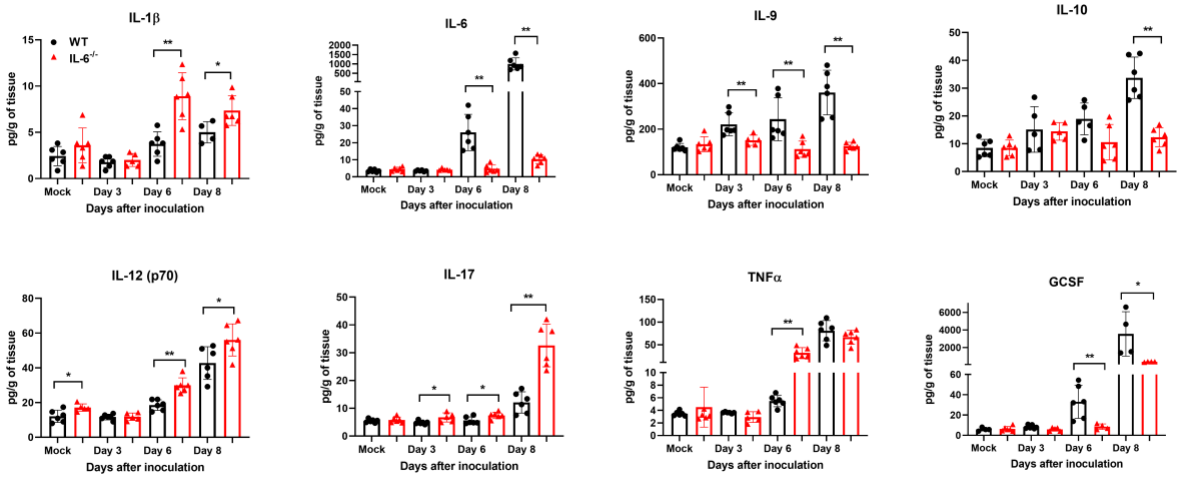

Figure 10: Analysis of cytokine concentrations in murine brains following infection with WNV NY99. Multiplex immunoassay was conducted to measure the protein levels of cytokines in the brains of WT and IL-6$^{-/-}$ mice on days 3, 6, and 8 after WNV NY99 infection. The results were presented as the mean concentration (pg/g) of tissue ± SD (n = 6–7 mice per group). *p < 0.05, **p < 0.01.

3.4 Discussion

IL-6 is a cytokine exerting pleiotropic effects. In the CNS, IL-6 is a neurotropic factor produced by neurons and glial cells, promoting neuronal survival, nerve regeneration, glial cell activation, and astrocyte proliferation while suppressing demyelination **[170-180]**. Several studies have shown that impaired IL-6 function enhances susceptibility to infection with various pathogens. For instance, IL-6 deficiency increased mortality in mice after infection with HSV **[114, 148]** and influenza virus **[112, 113]**. Conversely, when WT mice were infected with a genetically modified rabies virus carrying the IL-6 gene, they exhibited greater resistance to viral infection than the parental virus **[118]**. Additionally, infections with bacteria, such as *Streptococcus pyogenes* **[181]**, *Streptococcus pneumoniae* **[182]**, *M. tuberculosis* **[183]**, and *T.*

cruzi **[184]**, were more severe in IL-6$^{-/-}$ mice at similar inoculation titers than WT mice. However, the effects of IL-6 on flavivirus infection have not been analyzed.

WNV and JEV preferentially infect myeloid cells (lymphoid dendritic cells and macrophages) from peripheral tissues and neuronal cells in vivo **[56, 119, 120]**. However, the specific impact of IL-6 on flavivirus-infected myeloid and neuronal cells has never been studied before. Our study using human neuronal cells demonstrated significantly increased virus titers in WNV or JEV-infected cells when IL-6 was neutralized, indicating a restrictive function of IL-6 in flavivirus replication. To aid our in vitro human data, we infected primary mouse cells derived from the peripheral tissues and brain with a lethal or non-lethal WNV strain or JEV Nakayama strain. Similarly, we found significantly higher viral yields in the cells derived from IL-6$^{-/-}$ mice compared to cells from WT mice. These observations suggest that IL-6 limits flavivirus production in both peripheral and CNS cells. Our results align with previous investigations that have shown adverse effects of IL-6 neutralization on virus clearance in cell-based studies involving hepatitis B virus **[116]** and *Listeria monocytogenes* infection **[185]**. It has also been demonstrated that cells lacking similar cytokines, such as TNF-α and IL-1β, are more susceptible to WNV infection **[186, 187]**.

Mice infected with flavivirus exhibit severe neurological complications that closely resemble human diseases, rendering mice an excellent model for unraveling the molecular mechanisms of WNV or JEV infection **[188, 189]**. Therefore, we used C57BL/6J (WT) and IL-6$^{-/-}$ mice for our experiments. Our in vivo studies revealed that the peripheral inoculation of the lethal NY99 strain of WNV or JEV Nakayama strain caused 100% mortality in IL-6$^{-/-}$ mice, highlighting a significant difference in survival rates compared to WT mice. Interestingly, these findings contrast with a previous work, which implied that WNV infection does not cause any

significant difference in survival between WT and IL-6$^{-/-}$ mice **[190]**. To further dissect the pathogenesis of WNV in IL-6$^{-/-}$ mice, we subcutaneously inoculated WT and IL-6$^{-/-}$ mice with WNV Eg101, a non-lethal strain **[191, 192]**. Consistent with our WNV NY99 data, IL-6$^{-/-}$ mice exhibited a substantial difference from WT mice in mortality rates (60% vs 0%). Collectively, our survival data indicated a protective role for IL-6 against the pathogenic effects of WNV or JEV infection in mice. Subsequent plaque assay analyses showed that the viral titers were significantly higher in the serum and brains of WNV or JEV-infected IL-6$^{-/-}$ mice than those of WT mice. These results align with prior studies that reported increased virus titers in IL-6$^{-/-}$ mice after infection with influenza virus **[112, 113]**, HSV-1 **[114]**, vaccinia virus **[117]**, *E. coli* **[193]**, *S. pneumoniae* **[182, 194]**, and *Candida albicans* **[195]**. Moreover, it is also known that animals lacking essential cytokine functions, such as TNF-R1$^{-/-}$ and Il-1r$^{-/-}$ mice, exhibit significantly increased mortality and elevated WNV titers in the CNS compared to WT mice **[187, 196]**.

IFN-I activation is a vital defense against viral infections **[197]**. Mice lacking the IFN-α/β receptor (IFN-αβR$^{-/-}$) exhibit widespread tissue invasion, uncontrolled viral replication, and increased mortality compared to WT mice following WNV infection **[161]**. Experiments with IFN-β receptor-deficient mice (IFN-β$^{-/-}$) demonstrated that IFN-β controls WNV pathogenesis in mice by limiting infection in a cell and tissue-specific manner **[198]**. Furthermore, studies conducted in vitro have shown that treating primary neurons with IFN-β before and after WNV infection boost neuronal survival **[161]**. Moreover, impaired IFN-I responses rendered neurons and astrocytes more susceptible to TBEV (Tick-borne encephalitis virus) infection **[162, 163]**, and increased the spread of Langat virus (LGTV) within the CNS **[164]**.

It is established that basal IFN-I activity facilitates IL-6 expression **[199-202]**. Evidence shows that cells lacking the IFNAR1 receptor (IFNAR1$^{-/-}$) provide fewer docking sites for STAT1

and STAT3, thus impairing the STAT1/3 pathway and, consequently, affecting IL-6 signaling **[203]**. However, the interplay between IFN-I and IL-6 during flavivirus infection is yet to be explored. Our in vivo data demonstrated that expression levels of IFN-α and IFN-β in the brain homogenates were initially increased on days 3 and 6 but significantly decreased on day 8 in IL-$6^{-/-}$ mice compared to the WT mice. These findings indicate that the lack of IL-6 gene restricted the antiviral response induced by type I IFN in mouse brains after flavivirus infection.

IL-6 initiates signaling cascade events mainly via the JAK/STAT3 activation pathway **[204]**. These phenomena stimulate the transcription of diverse downstream genes, including cytokines, chemokines, receptors, adaptor proteins, and protein kinases **[96]**. To evaluate the role of IL-6 in modulating the immune response after flavivirus infection, we measured the levels of key cytokines after IL-6 neutralization in human neuronal cells. Interestingly, despite having higher viral loads, we observed a significant depletion in the expression of cytokines, TNF-α and IL-1β, in the infected cells. Next, we measured the protein levels of cytokines and chemokines in WT and IL-$6^{-/-}$ mice using multiplex immunoassay. Similarly, our results showed a significantly lower production of key cytokines and chemokines, such as IL-1β, IL-10, IL-12, IL-15, TNF-α, GM-CSF, G-CSF, MIP-2 (CXCL2), and RANTES (CCL5), in the serum of IL-$6^{-/-}$ mice, even though the serum had high virus titers. It may be due to the impaired IL-6-mediated activation of cytokine/chemokine signaling pathways in the periphery. IL-6 is a master regulator and modulates the secretion of other cytokines **[90, 160]**. Therefore, the absence of IL-6 might have suppressed the cytokine and chemokine induction in the periphery of IL-$6^{-/-}$ mice. Interestingly, we found that the protein levels of some cytokines (IL-1β, IL-12, and IL-17) and chemokines (CCL2, CCL3, CXCL1, and CXCL2) were significantly elevated in the brains of IL-$6^{-/-}$ mice than in WT mice. Conversely, IL-9, IL-10, and GCSF protein levels were significantly decreased in the brains of IL-

$6^{-/-}$ mice, which have anti-inflammatory roles **[167, 168, 205]**. The elevated production of some cytokines and chemokines in the brains of IL-$6^{-/-}$ mice could be attributed to the increased immune cell infiltration and the diminished ability to control virus replication due to decreased type I IFN levels. Elevated levels of these inflammatory molecules in the brain correlate with the increased mortality observed in the IL-$6^{-/-}$ mice. Overall, our data indicate the pivotal role of IL-6 in modulating the immune response to flavivirus infection in both human neuronal cells and mice. Further mechanistic studies are needed to unravel the complex interactions between IL-6 and type I IFN and key cytokines in the periphery and CNS of flavivirus-infected mice.

In conclusion, our findings offer novel insights into the role of IL-6 during flavivirus replication and dissemination. Our report shows, for the first time, that lack of IL-6 increases the severity of WNV or JEV infection both in vitro and in vivo. Future mechanistic studies on the function of IL-6 during neurotropic flavivirus infection will significantly impact the development of much-needed therapeutic interventions to improve disease outcomes.

(This work has been published in *Frontiers in Cellular and Infection Microbiology*) **[206]**

4 DECIPHERING GLOBAL GENE EXPRESSION PATTERNS ASSOCIATED WITH SEVERE WEST NILE VIRUS INFECTION IN IL-6 KNOCKOUT MICE BRAINS THROUGH RNA-SEQ ANALYSIS

The impact of interleukin-6 (IL-6) deficiency on the gene expression landscape during West Nile virus (WNV) infection in murine brains was investigated using RNA-seq technology. A comparative analysis revealed a notably higher number of differentially expressed genes in IL-6 knockout (IL-$6^{-/-}$) mice brains at various time points following WNV NY99 infection, in contrast to wild-type (WT) mice. Among the top upregulated genes in IL-$6^{-/-}$ mice, several were implicated in inflammatory responses, cell death, and interferon regulatory pathways. Moreover, we observed a subdued activation of interferon-stimulated genes (ISGs) that indicated a reduced antiviral response, potentially accounting for the increased WNV load observed in IL-$6^{-/-}$ mice. The absence of the IL-6 gene in mice also prompted a surge of proinflammatory cytokine and chemokine genes within the brain during WNV infection while inhibiting gene expressions involved in immunoregulatory roles. Furthermore, the most activated canonical pathways of infected IL-$6^{-/-}$ mice brains underscored elevated recruitment and activation of immune cells, possibly resulting in heightened inflammation. Similarly, our comprehensive study of key functional networks of biological importance unveiled increased numbers and enhanced chemotaxis of immune cells within the brains of infected IL-$6^{-/-}$ mice. Our examination of differentially expressed upstream regulators in the IL-$6^{-/-}$ group highlighted the increased activation of signaling pathways inducing inflammatory mediators. We also noticed the downregulation of immune checkpoint regulator genes that negatively modulate inflammation and NF-κB and MAPK pathways. An examination of differentially expressed pattern recognition receptors and neuroinflammation-associated genes in IL-$6^{-/-}$ mice brains corroborated these

findings and revealed an upregulation of antigen presentation, immune cell activation, leukocyte recruitment, and NF-κB pathway, but downregulation of genes crucial for stimulating interferon and antiviral responses. In summary, this study provides novel insights into the specific role of IL-6 in shaping the global gene expression patterns in response to WNV NY99 infection in mice brains. The findings shed light on the complex interplay between IL-6 and the intricate molecular mechanisms underlying severe neurotropic WNV infection.

4.1 Introduction

West Nile virus (WNV) continues to be a significant public health concern due to its widespread distribution and potential to cause severe neurological infections, such as meningitis, acute flaccid paralysis (AFP), myelitis, and encephalitis, in humans **[1, 55]**. The pathogenesis of WNV-induced neurological diseases involves a complex interplay among viral factors, host immune responses, and the blood-brain barrier's integrity. The virus initially infects skin-resident dendritic cells and then disseminates to regional lymph nodes, eventually reaching the bloodstream and potentially crossing the blood-brain barrier to infect the CNS. Once in the brain, WNV can directly damage neurons and trigger an immune response that leads to inflammation **[207-209]**. Although WNV is the leading cause of arboviral encephalitis in the USA **[210]**, no specific antiviral drug has been approved for WNV infections, which highlights the critical need for a better understanding of the pathogenesis of WNV-induced neurological complications to develop targeted therapies.

Cytokines and chemokines, the crucial components of the host immune response, play a pivotal role in determining the outcome of WNV infection **[121]**. Interleukin-6 (IL-6), a multifunctional cytokine, exerts pleiotropic effects on immune cell activation, inflammation, and tissue repair and emerges as a crucial modulator that influences the balance between viral control

and immune-mediated damage **[90, 93]**. Recent studies have underscored the significance of IL-6 in virus infections, revealing its contribution to modulating disease progression and severity **[96]**. However, the specific role of IL-6 in influencing WNV infection outcomes is largely unknown and requires an in-depth investigation into the underlying molecular mechanisms.

Our data previously revealed that human neuronal cells with blocked IL-6 and primary murine neuronal cultures deficient in the IL-6 gene produced significantly higher virus titers than their counterparts with functional IL-6 activity and wild-type (WT) murine neurons after WNV infection. We also found that IL-6 knockout (IL-$6^{-/-}$) mice exhibited increased morbidity and mortality than WT mice when infected with either lethal or non-lethal strains of WNV. Additionally, higher viral loads were observed in the serum and brain of IL-$6^{-/-}$ mice at different time points of the infection. Subsequently, we explored innate immune responses in WT and IL-$6^{-/-}$ mice after WNV infection, where reduced levels of key cytokines and chemokines were observed in the serum of IL-$6^{-/-}$ mice during early infection but elevated proinflammatory cytokines and chemokines were seen in the brain later, along with suppressed anti-inflammatory cytokines. Furthermore, mRNA expression of IFN-α and IFN-β was significantly lower in the infected IL-$6^{-/-}$ mice. Collectively, these results provide the first evidence of the important role of IL-6 in limiting the severity of WNV infections in mice **[206]**.

To further delineate the specific effect of IL-6 deficiency during the progression of neurotropic WNV infection in mice, we showcased a comprehensive RNA-seq analysis of transcriptional changes in the WNV-infected brains of IL-$6^{-/-}$ mice. RNA-seq analysis offers an unbiased and highly accurate measurement of global shifts in transcriptional activity, allowing us to identify potential transcriptomic signatures associated with severe infection outcomes. Furthermore, using RNA-seq enables us to explore the intricate dynamics of gene expression

patterns over the course of infection **[124, 125]**. In this study, we examined and characterized the immune responses of WT and IL-6$^{-/-}$ mice to the lethal WNV NY99 infection, focusing on individual genes associated with interferon, cytokine, and chemokine signaling, pattern recognition, neuroinflammation, as well as most activated canonical pathways, functional networks of biological importance, and upstream regulators. Overall, we uncovered early and late transcriptional events that shaped the host response to WNV neurotropic infection in mice in the presence and absence of IL-6, providing insights into the temporal modulation of immune pathways and cellular processes by IL-6.

Our results in this study will not only shed light on the significance of IL-6 in WNV-induced neuropathogenesis but also enhance our understanding of the overall molecular mechanisms governing severe neurotropic WNV infection. Ultimately, these insights could pave the way for novel therapeutic interventions to ameliorate the clinical outcomes of WNV and other similar neurotropic flavivirus infections. In the subsequent sections, we detail the experimental methodologies, results, and implications of our analysis.

4.2 Materials and Methods

4.2.1 Animal Infection Experiment and Brain Collection

C57BL/6J (wild-type) mice and IL-6$^{-/-}$ mice (strain 002650) were procured from the Jackson Laboratory (Bar Harbor, ME, USA). The breeding and genotyping of these mice took place within the animal facility at Georgia State University. All experiments involving WNV infection were conducted in a laboratory equipped with a biosafety level-3 (ABSL-3) environment. The study adhered to the guidelines outlined by the National Institutes of Health and the Institutional Animal Care and Use Committee (IACUC). The Georgia State University IACUC validated the research protocol under reference number A21067.

WT and IL-6$^{-/-}$ mice, aged eight weeks, were transferred to the ABSL-3 facility, and allowed to acclimate to the environment before initiating experiments. These mice were subjected to footpad inoculation with either PBS or WNV NY99 (100 PFU). On days 3, 6, and 8 after infection, the mice were anesthetized using isoflurane and perfused with cold 1xPBS. Their brains were harvested and rapidly frozen in 2-methyl butane (Sigma, St. Louis, MO, USA) for further analysis.

4.2.2 Determination of Infectious Virus Titers by Plaque Assays

Portion of brain tissues harvested from infected mice were weighed and homogenized using zirconium beads in a bullet blender (Next Advance, Averill Park, NY, USA), followed by centrifugation and titration. Virus titers in tissue homogenates were quantified by plaque assay using Vero E6 cells. The tissue homogenates were initially serially diluted in 10-fold increments using 1 x M199 Vero media and then applied to Vero E6 cell monolayers for one hour. After incubation, the infected cells were overlaid with a mixture of 2% agarose and 2 x M199 Vero media in a 1:1 ratio. Subsequently, the cells were further incubated for 48 hours and underwent a second overlay with 1.5% neutral red dye to visualize plaque formation.

4.2.3 RNA Extraction and Quantitative RT-PCR

Following tissue homogenization and lysis using RLT buffer, total RNA isolation from the rest of the brain samples was performed using a Qiagen RNeasy Mini kit (Qiagen, Germantown, MD, USA), adhering to the manufacturer's instructions. The quality and quantity of the extracted total RNA were assessed using a Nanodrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) based on the criteria: wavelength absorbance ratio A260/280 ~2.0 and A260/230 ~2.0. cDNA was synthesized from extracted RNA utilizing an iScript^{TM} cDNA synthesis kit (Biorad, Catalog #1708891). The produced cDNA was diluted using RNAse-free

water, and 2 µL of this cDNA was employed for quantitative RT-PCR (qRT-PCR) utilizing SsoAdvanced™ Universal SYBR® Green Supermix (Biorad, Catalog # 1725271) to determine the expression levels of key cytokines. The fold changes in the mRNA expressions of IL-1β, TNF-α, CCL2, and CCL3 in the infected tissues were calculated against mock-infected tissues after normalization to GAPDH gene. The primer sequences used for qRT-PCR are listed in Table 3.

Table 3: Primer sequences employed for qRT-PCR.

Gene (Accession No.)	**Primer Sequence (5′-3′)**
IL-1β (NM_000576)	
Forward	AGCACCTTCTTTCCCTTCATC
Reverse	GGACCAGACATCACCAAGC
TNF-α (NM_013693)	
Forward	CCAGTCTGTATCCTTCTAA
Reverse	TCTTGTGTTTCTGAGTAGT
CCL2 (NM_011333)	
Forward	TCACCTGCTGCTACTCATTCACCA
Reverse	TACAGCTTCTTTGGGACACCTGCT
CCL3 (NM_011337)	
Forward	ATTCCACGCCAATTCATC
Reverse	ATTCAGTTCCAGGTCAGT

4.2.4 Gene Expression Analysis Using RNA-seq

Whole transcriptome sequencing of brain samples was conducted to investigate gene expression profiles. The Illumina platform was utilized to sequence libraries constructed by the

TruSeq Stranded mRNA Library Prep Kit, generating 150-bp paired-end reads (Illumina, San Diego, CA, USA). Starting with a minimum of 0.5µg of total extracted RNA per sample, first, DNase treatment was applied to eliminate DNA contamination, and mRNAs were purified using appropriate RNA purification kits. Then, RNA fragmentation was performed, followed by cDNA reverse transcription and adapter ligation. These fragments were amplified by PCR and fragments of suitable sizes (200-400 bp) were selected. Paired-end sequencing was executed on cDNA ends, producing approximately 40 million reads per sample using the FastQC v0.11.7 (http://www.bioinformatics.babraham.ac.uk/ projects/fastqc/). Raw reads were subjected to quality control analysis for total bases, reads, GC content, and other statistics. The low-quality reads, adaptors, contaminant DNA, and PCR duplicates were removed. The HISAT2 aligner was then used to align the processed reads to the reference genome, and transcripts were assembled using StringTie **[211, 212]**. Expression profiles were represented as read counts and the fragments per kilobase of transcript per million mapped reads (FPKM) values based on transcript length and depth of coverage.

4.2.5 RNA-seq Data Analysis Using Ingenuity Pathway Analysis (IPA)

The RNA-seq FPKM values obtained from mice brains were first subjected to trimming of low-quality sequences. Differential gene expression analysis was conducted for each group of infected mice, considering log2 fold change values outside the range of ± 2 and false discovery rate (FDR) or adjusted p-value less than 0.05. Qiagen's Ingenuity® Pathway Analysis (IPA®) software (Qiagen Redwood City; www.qiagen. com/ingenuity) was utilized to generate canonical pathways, functional networks, and upstream regulators using the identified dataset containing significantly differentially expressed genes (DEGs) **[213-215]**. Upon uploading the selected DEGs, the IPA software provided z-scores to illustrate these pathways' activation levels in both

groups of mice. Furthermore, comprehensive gene analyses within activated pathways associated with pattern recognition and neuroinflammation were demonstrated through log ratio calculations **[124, 125]**.

4.2.6 Statistical Analysis

GraphPad Prism 7.0 was used to perform a Mann-Whitney test and a two-way analysis of variance (ANOVA) to measure the *p* values for plaque assay and qRT-PCR as described previously **[147]**. Genes with FDR values of less than 0.05, determined through Fisher's exact test, and log2 fold changes outside the range of ± 2 were categorized as significantly differentially expressed genes. Statistical comparisons between pathways and pathway-specific genes were performed using metrics like z-score and log ratio to determine activation levels, as mentioned in the previous section.

4.3 Results and Discussion

4.3.1 Alterations in Cellular Gene Expression Patterns in IL-6-Deficient Mice Brains During WNV NY99 Infection

To identify the role of IL-6 in the global gene expression patterns associated with WNV NY99 infection, we employed RNA-seq analysis. This study encompassed transcriptomic profiles from the brains of WT and IL-$6^{-/-}$ mice exposed to the lethal WNV NY99 strain. In our earlier research, IL-$6^{-/-}$ mice exhibited pronounced neurological symptoms such as ataxic gait, tremors, and paralysis, starting on the sixth day following WNV NY99 footpad inoculation **[206]**. Notably, viral presence was detected in the brains of both groups by the sixth day; however, IL-$6^{-/-}$ mice displayed significantly higher viral loads than WT mice on day 8 **(Fig. 11A)**. Also, our previous investigation revealed elevated levels of proinflammatory cytokines and chemokines in the IL-$6^{-/-}$ mice brain on days 6 and 8, along with suppressed anti-inflammatory cytokines **[206]**.

Collectively, these earlier findings underscored the specific function of IL-6 in viral replication, host immune response, and disease progression. To further follow up, we delved into understanding the longitudinal transcriptomic changes during WNV NY99 infection of mice in the absence of IL-6 gene expression. RNA harvested from the brains of mock-infected and WNV NY99-infected WT and IL-6$^{-/-}$ mice were subjected to RNA-seq.

A higher number of differentially expressed genes (log2 fold change outside the range of ±2) was observed in the brains of WNV-infected IL-6$^{-/-}$ mice **(Fig. 11B)**. This finding correlated with the significantly increased brain viral load observed in these mice compared to WT mice. Specifically, on days 3, 6, and 8 post-infection, 108, 244, and 158 genes were upregulated, and 58, 430, and 57 genes were downregulated in IL-6$^{-/-}$ mice. In contrast, WT mice showed 74, 132, and 182 upregulated genes and 3, 20, and 30 downregulated genes at the corresponding time points. The number of upregulated genes exceeded the downregulated genes in both virus-infected groups, except for day 6 in IL-6$^{-/-}$ mice, which showed more downregulated than upregulated genes. Notably, IL-6$^{-/-}$ brains exhibited a higher number of downregulated genes at all time points compared to WT mice. Furthermore, on days 3 and 6, the number of upregulated genes in IL-6$^{-/-}$ mice was higher than in WT mice.

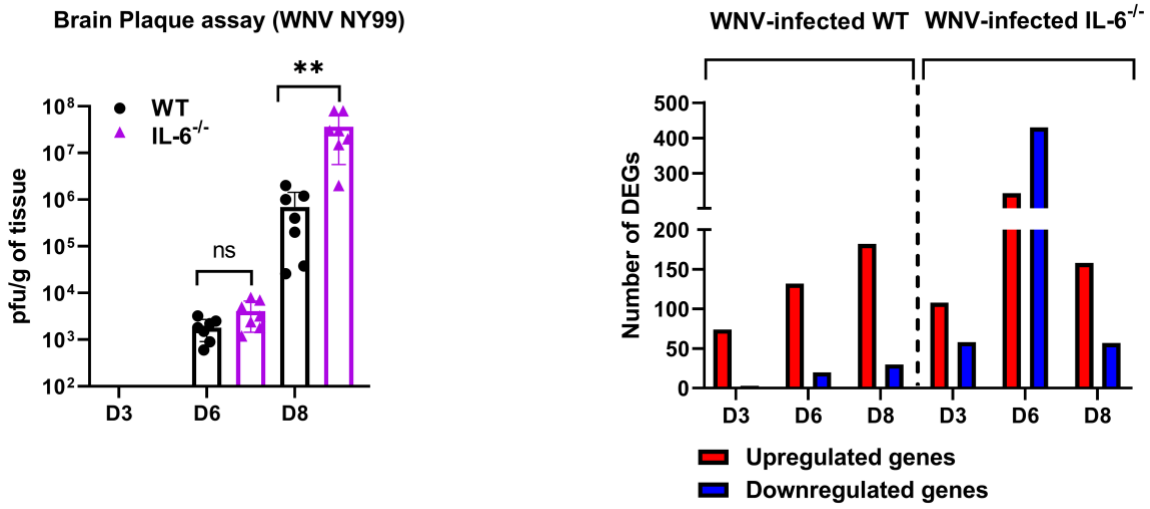

Figure 11: Changes in gene expression patterns in the brain tissues of WNV NY99-infected WT and IL-6$^{-/-}$ mice determined by RNA-Seq analysis. Eight-week-old mice were subjected to footpad inoculation with either PBS (mock) or 100 PFU of WNV NY99. Brain tissues (n = 6-7 per group) were collected post-infection on days 3, 6, and 8. **(A)** The viral load in the brain was evaluated by plaque assay using Vero E6 cells and was expressed as PFU per gram of tissue. The data represents the mean ± SD, with statistical significance denoted by (**$p < 0.01$). **(B)** The bar graph showcases the number of differentially expressed genes (DEGs) in the brains of WT and IL-6$^{-/-}$ mice following WNV NY99 inoculation, with an adjusted p-value (FDR) below 0.05 and log2 fold change outside the range of ±2.

Genes involved in inflammatory responses, innate antiviral immunity, and cell death activation were the most differentially expressed in IL-6$^{-/-}$ mice after WNV NY99 infection **(Table 4)**. These include increased expression of chemokine genes responsible for recruiting myeloid cells, lymphocytes, and NK cells **[216-218]**. Also, cell death-associated genes, Gzma and Zbp1, were markedly upregulated, indicating enhanced activation of cell death pathways such as pyroptosis and necroptosis **[219, 220]**. Moreover, Irf7 was highly expressed, suggesting augmented regulation of type I IFN genes (IFN-α and IFN-β) and IFN-stimulated gene (ISG) transcriptions in IL-6$^{-/-}$ mice **[221]**. The increase in the expression of cell death-associated genes and IFN pathway-regulating genes occurred in a time-dependent manner in the IL-6$^{-/-}$ brains. Interestingly, WT mice exhibited elevated expression of Cd69 **(Table 5)**, a gene associated with

the activation of regulatory T cells (Tregs) and the inhibition of Th-17 cell differentiation in mice and humans **[222-225]**. Notably, the Cd69 gene was not found among the upregulated genes in the knockout group, implying comparatively less T-cell regulation in IL-$6^{-/-}$ mice following WNV NY99 infection.

Table 4: Top upregulated DEGs in WNV NY99-infected IL-$6^{-/-}$ mice brains.

IL-6 knockout mice brain					
D3 genes	**Log2FC**	**D6 genes**	**Log2FC**	**D8 genes**	**Log2FC**
H2-Q9	5.94575458	Hcrt	10.0208234	Gzma	10.1227536
Ms4a4b	5.2753945	Pmch	8.01624267	Lilr4b	10.0660886
Gm12185	5.06503144	Gm12185	7.52824875	Slfn4	9.87989878
Zbp1	5.02284838	Ccl8	7.09105926	Acod1	9.20114497
Pydc3	4.8877948	Zbp1	6.93768616	Slfn1	9.10348872
Gzma	4.85650828	Isg15	6.49902094	Ifi205	9.06682513
Ifi27l2a	4.77999053	Oas1g	6.45130254	Il12b	9.00638689
Slfn4	4.64778843	Ccl12	6.33317534	Sell	8.96267369
Rab19	4.5842515	Irf7	6.27781276	Pydc4	8.70492755
Irf7	4.55833458	Usp18	6.15841881	Tgm1	8.58123783

Table 5: Top upregulated DEGs in WNV NY99-infected WT mice brains.

WT mice brain					
D3 genes	**Log2FC**	**D6 genes**	**Log2FC**	**D8 genes**	**Log2FC**
Pydc4	5.35663443	H2-Q6	7.37328823	Cd69	10.0604168
Phf11a	5.26110012	Phf11a	6.58885028	Plac8	9.58733582
4930415O20Rik	5.01949733	Pydc4	6.46850932	Cxcl11	9.02263544
Klrg1	4.70472714	Usp18	5.61543886	Ccl2	8.69599261
Slfn4	4.59701081	Plac8	5.5060188	Usp18	8.49199948
Usp18	4.37998778	Mnda	5.24880613	Isg15	8.26017361
Zbp1	4.26121956	Slfn1	5.23861492	Gm4841	7.76255219
F830016B08Rik	4.20700698	Isg15	5.18417566	Rsad2	7.75166941
Ifi204	4.1387899	Zbp1	5.08398958	Phf11d	7.62990575
Irf7	4.01215222	Klrg1	4.99791615	Ifi44	7.54027475

4.3.2 Interferon Signaling-Associated Genes

Numerous DEGs in WNV NY99-infected IL-6$^{-/-}$ mice were associated with IFN signaling, a critical component of the host's innate defense against WNV **[226]**. The paracrine and autocrine release of IFN make cells resistant to viral infections by triggering the activation of ISGs **[227]**. We found that ISGs such as IFIT3, IRF9, IFIH1, ISG15, IFNG, and IFNA4 were downregulated in the absence of IL-6 gene **(Fig. 12A)**. Among these ISGs, IFIT3 and IRF9 are known to confer an antiviral state by inhibiting virus replication, post-transcriptional modification, and virus maturation **[228, 229]**. Additionally, IFIH1 and ISG15 activate type I and II IFNs **[230, 231]**, as evidenced by the lower expression of IFNG (type II IFN) in IL-6$^{-/-}$ mice. IFNG primes

macrophages to produce nitric oxide (NO), contributing to the host's antiviral action **[232]**. Moreover, IFNA4, produced by macrophages, possesses antiviral and anti-inflammatory properties **[233]**. Downregulation of STAT2 and STAT1 in the IL-6$^{-/-}$ mice further supports the implication of impaired cellular antiviral responses through reduced ISG transcription, and IFNG and IFNA4 activation, respectively **[234-236]**.

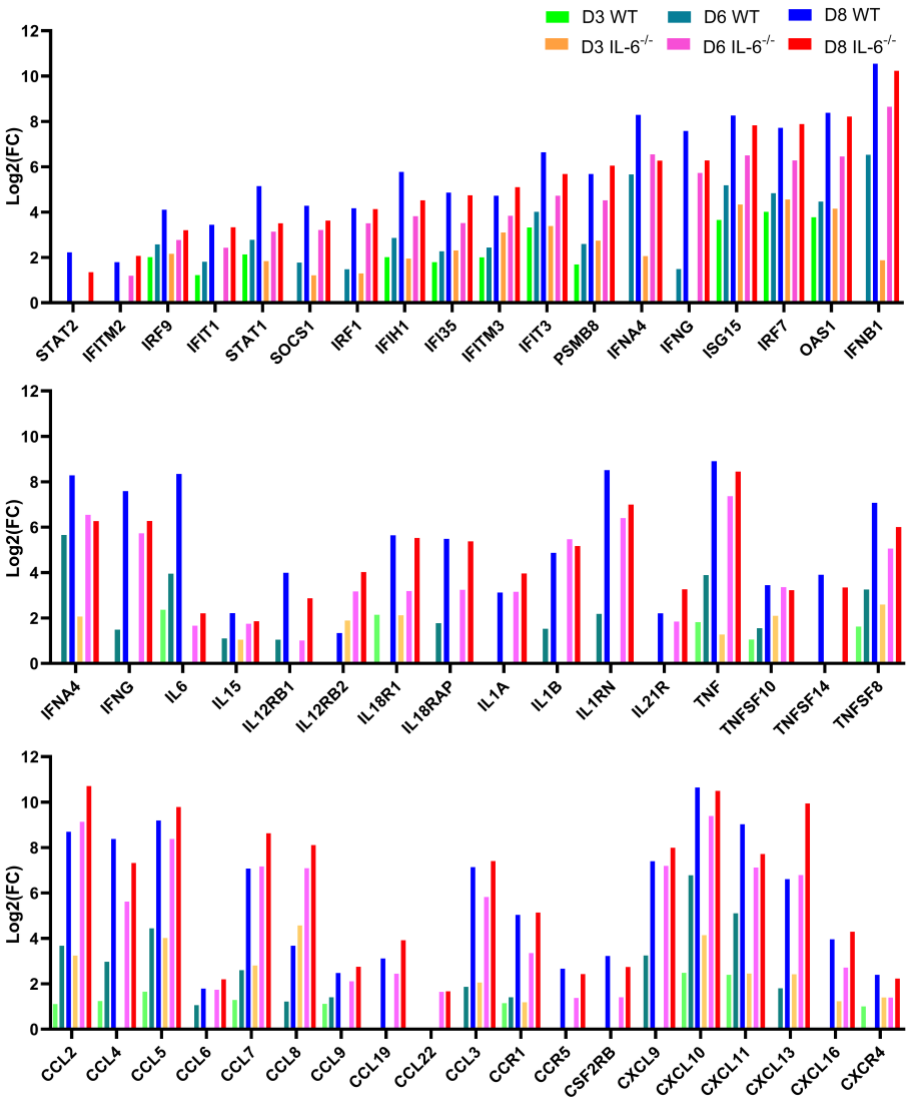

Figure 12: Assessing differential expression of genes associated with interferon, cytokine, and chemokine signaling activation. The expression levels of **A)** interferon, **B)** cytokine, and **C)** chemokine signaling-associated genes were analyzed that demonstrated

significant variance in the brains of WNV NY99-infected WT and IL-6$^{-/-}$ mice relative to the mock-infected brain samples collected from both groups of mice.

4.3.3 Genes Related to Cytokine Activation

Prior studies have highlighted the substantial upregulation of multiple cytokines and chemokines in mice brains during WNV NY99 infection **[125, 237, 238]**. However, the role of the IL-6 gene in mediating the expression of these cytokines and chemokines during WNV infection was unexplored. In our analysis of differentially expressed cytokine genes in WT and IL-6$^{-/-}$ mice, we observed downregulation of IL-6, IL-15, interleukin-1 receptor antagonist protein (IL-1RN), tumor necrosis factor (TNF), TNF superfamily member 8 (TNFSF8), and TNFSF14 in the brains of IL-6$^{-/-}$ mice at a later stage of WNV infection **(Fig. 12B)**. As expected, IL-6, responsible for stimulating acute phase protein synthesis and various aspects of the host immune response **[90, 91, 93, 131]**, is the most downregulated gene in the IL-6$^{-/-}$ mice. IL-15, known for regulating the activation and proliferation of NK cells and T cells, and IL-1RN, which protects against immune dysregulation triggered by IL-1 during virus infection, were both downregulated **[239, 240]**. Additionally, downregulated genes, TNFSF8 and TNFSF14, regulate systemic inflammation by modulating immunoglobulin (Ig) class switching and inducing apoptosis **[241-243]**. Therefore, the absence of the IL-6 gene in mouse brains potentially inhibited cytokine gene expressions involved in regulatory roles. However, IL-1A, IL-1B, and IL-21R genes were upregulated in IL-6$^{-/-}$ mice **(Fig. 12B)**. IL-1α activates NF-κβ and three MAPK pathways, producing proinflammatory cytokines such as IL-1β and TNF-α **[244]**. Furthermore, IL-1β promotes Th-17 differentiation of T-cells and exacerbates damage during acute tissue injury **[245]**. Lastly, IL-21R significantly contributes to T, B, and NK cell proliferation and differentiation **[246]**. Hence, the upregulation of these cytokine genes likely contributed to an augmented inflammatory response in the IL-6$^{-/-}$ mouse brains at a later stage of WNV NY99 infection.

4.3.4 Genes Linked to Chemokine Signaling

Our results demonstrated that chemokine pathway-associated genes, including chemokine (C-C motif) ligand 2 (CCL2), CCL3, CCL5, CCL6, CCL7, CCL8, CCL19, C-X-C motif chemokine 9 (CXCL9), and CXCL13 were upregulated in the IL-$6^{-/-}$ group than the WT group **(Fig. 12C)**. These chemokines promote the activation and recruitment of immune cells, such as monocytes, macrophages, polymorphonuclear leukocytes (neutrophils, eosinophils, and basophils), dendritic cells, NK cells, CD4+ T cells, CD8+ T cells, and B cells, to the inflammation sites **[247-254]**. To be precise, CCL2, CCL3, and CCL5 facilitate the trafficking of inflammatory monocytes, CD4+ and CD8+ T cells, and NK cells to the brain during WNV infection **[255, 256]**. Interestingly, CXCL11, a chemotactic factor for Tregs, was downregulated in the IL-$6^{-/-}$ mice in our experiment **[257]**. Therefore, our data implied that the lack of IL-6 gene in mice contributed to reduced recruitment of regulatory T cells to the brains while triggering an enhanced inflammatory chemokine response during WNV NY99 infection.

4.3.5 Validation of RNA-seq Data Using qRT-PCR

qRT-PCR was conducted on the brain homogenates collected from WNV-infected mice (n = 6-7 in each group) and mock-infected mice (n = 4) to authenticate the gene expression changes of a selected number of DEGs observed in RNA-seq analysis. Like RNA-seq data, IL-1β, TNF-α, CCL2, and CCL3 genes were upregulated in the brains of WNV NY99-infected WT and IL-$6^{-/-}$ mice compared to mock-infected mice. When examining expression patterns among the infected samples, we observed that IL-1β, CCL2, and CCL3 expression were significantly upregulated in IL-$6^{-/-}$ mice on days 6 and 8 compared to WT mice. Significantly elevated TNF-α expression was also evident in IL-$6^{-/-}$ mice brains compared to WT mice at day 6; however, its expression was downregulated by day 8. **(Fig. 13)**. These findings supported our previous demonstration of

significant changes in the protein levels of various cytokines and chemokines in WNV NY99-infected IL-6$^{-/-}$ mice brains on days 6 and 8 **[238]**.

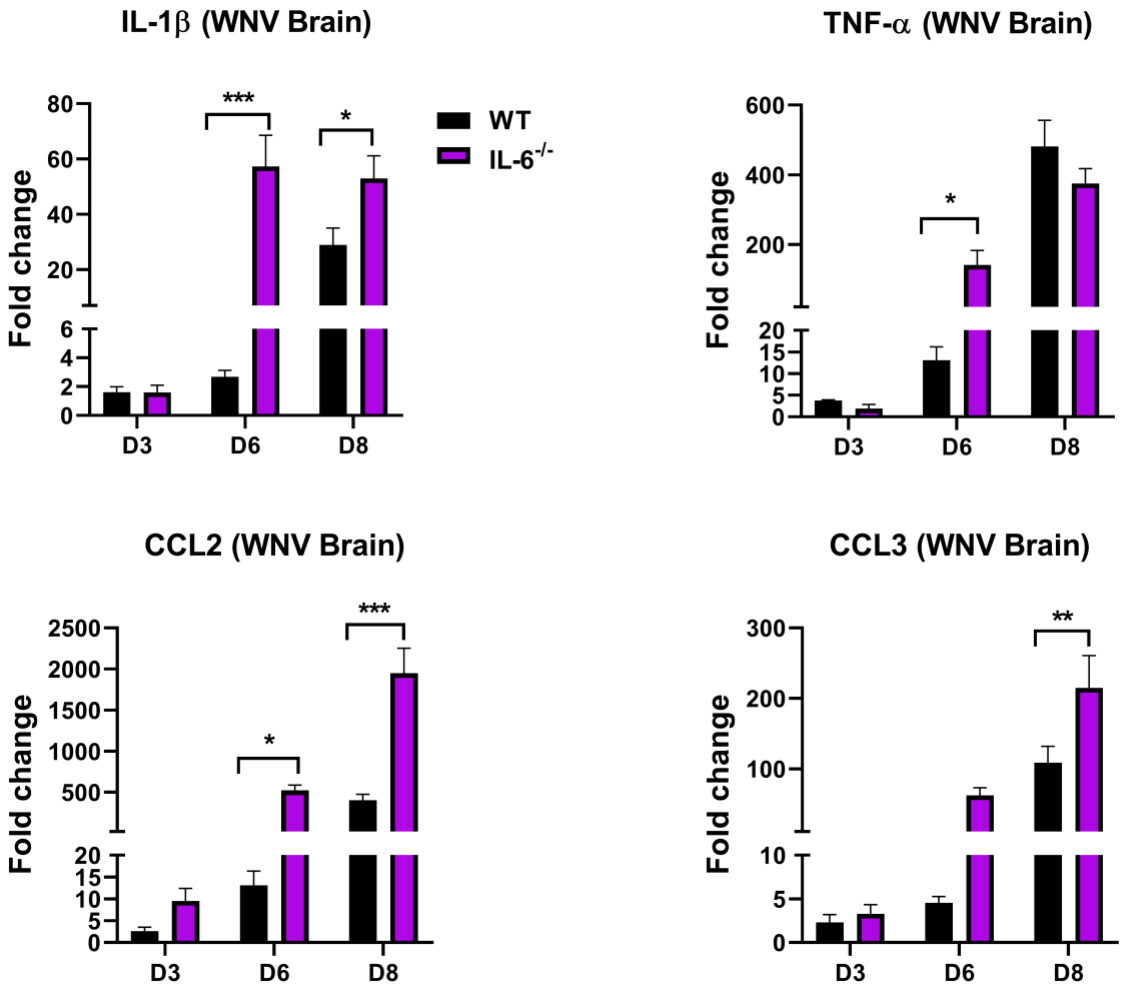

Figure 13: Verifying the expression patterns of selected DEGs using qRT-PCR. qRT-PCR was performed on RNA isolated from the brain homogenates of mock-infected and WNV NY99-infected WT and IL-6$^{-/-}$ mice on days 3, 6, and 8 after infection to determine the gene expression levels of IL-1β, TNF-α, CCL2, and CCL3. The variations in gene expression were initially normalized against the GAPDH gene, followed by calculating the fold change in WNV-infected mice brains in relation to the corresponding mock-infected mice brains. Data represents the mean ± SD and encompasses two independent experiments (n = 6-7 per group). Statistical significance (*p < 0.05, **p <0.01, ***p < 0.001) is indicated.

4.3.6 Functional Analysis of DEGs in WNV-Infected Mice Brains

To explore the biological interactions of DEGs and to pinpoint the relevant key functional networks, significantly altered DEGs derived from RNA-seq analyses were integrated into the Ingenuity Pathway Analysis (IPA) tool following established procedures **[124, 125, 258]** and the most prominently activated networks, with high z-scores, were identified.

We generated a heat map to showcase the most activated canonical pathways in WNV NY99-infected WT and IL-6$^{-/-}$ mice. The topmost activated canonical pathways in the infected IL-6$^{-/-}$ mice were 'Pathogen Induced Cytokine Storm', 'Macrophage Classical Activation', 'Th1', 'TREM1', 'Neuroinflammation', 'Leukocyte Extravasation', 'CD40 Signaling', and 'Natural Killer Cell Signaling' **(Fig. 14A)**. These pathways are responsible for leukocyte recruitment, neutrophil activation, triggering effector functions of macrophages (M1) and B cells, stimulating the cytolytic functions of NK and T cells, and secreting proinflammatory cytokines **[259-267]**. Additionally, the cytokine pathways such as IL-2, -17, -23, and -33 were highly activated, which promote chronic inflammation and generate Th-1 and Th-2 effector cells **[268-272]**. Specifically, IL-33 acts as an 'alarmin' released following cell necrosis to alert the immune system about virus infection, triggering further secretion of cytokines and chemokines **[273]**. The senescence pathway, known to release chemicals triggering inflammation **[274]**, was also activated in the IL-6$^{-/-}$ mice. Interestingly, MIF (macrophage migration inhibitory factor)-mediated regulation of innate immunity was less triggered in these mice **[275]**. Lastly, apoptosis signaling, important for immune regulation **[276]**, was also less activated. These pathways' association with immune responses aligns with the outcomes observed in the analysis of individual genes.

Subsequently, we conducted a comprehensive analysis of key functional networks activated in the brains of WNV NY99-infected IL-6$^{-/-}$ mice **(Fig. 14B)**. The results demonstrated

a notable increase in number and motility of antigen-presenting cells, leukocytes, myeloid cells, and lymphocytes in comparison to the brains of WT mice. Specifically, enhanced chemotaxis and higher quantities of phagocytes and CD4+ T lymphocytes were observed, along with upregulated T cell development and differentiation. These phenomena potentially contribute to the increased activation and secretion of inflammatory cytokines **[277, 278]**, consistent with the elevated hypersensitive response observed in the knockout group. Lastly, we found that virus infection was less inhibited in IL-$6^{-/-}$ mice compared to WT mice, suggesting a possible link to the higher viral load detected in the brains of IL-$6^{-/-}$ mice.

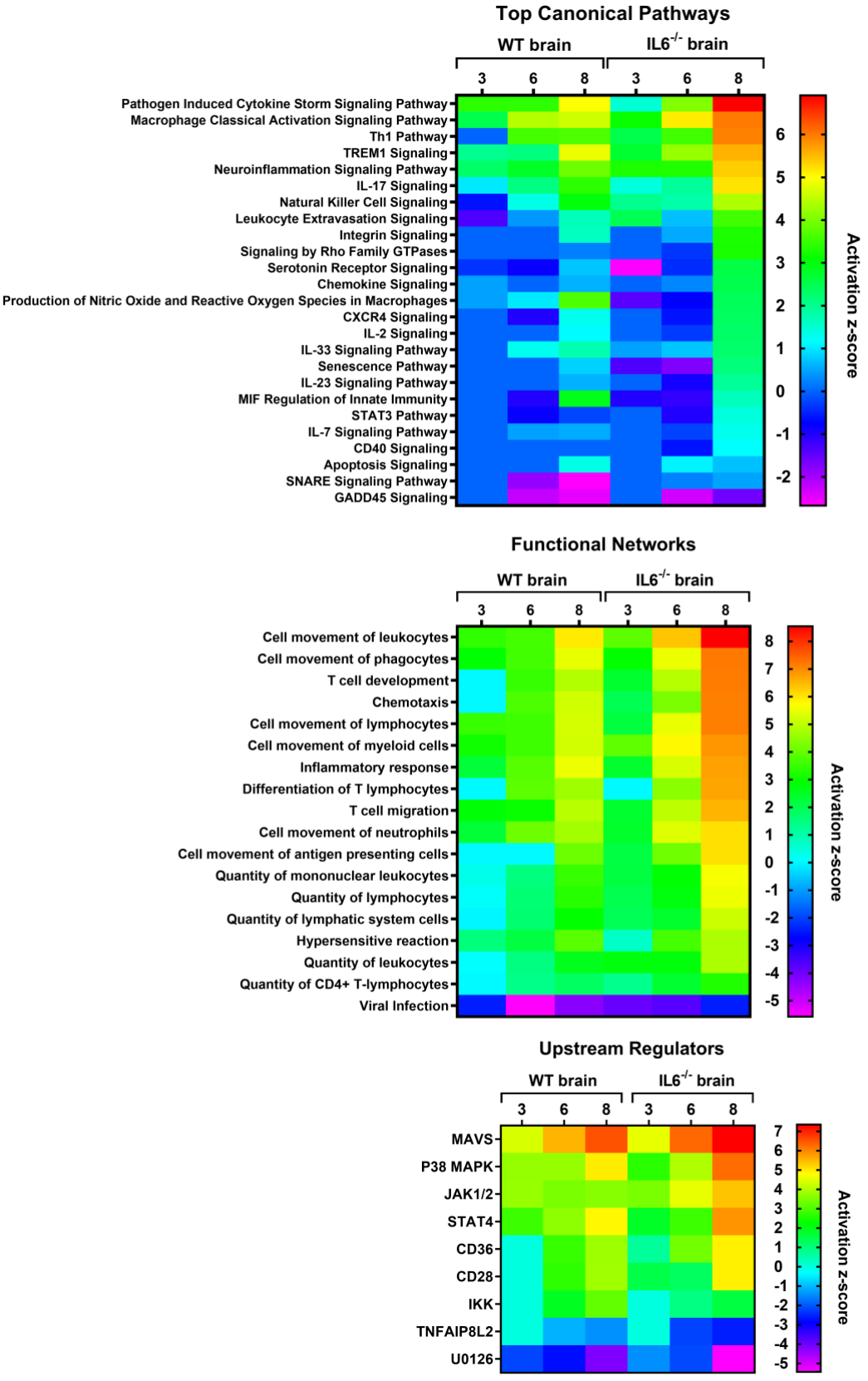
Top Canonical Pathways
WT brain
IL6-/- brain
3 6 8 3 6 8
Pathogen Induced Cytokine Storm Signaling Pathway
Macrophage Classical Activation Signaling Pathway
Th1 Pathway
TREM1 Signaling
Neuroinflammation Signaling Pathway
IL-17 Signaling
Natural Killer Cell Signaling
Leukocyte Extravasation Signaling
Integrin Signaling
Signaling by Rho Family GTPases
Serotonin Receptor Signaling
Chemokine Signaling
Production of Nitric Oxide and Reactive Oxygen Species in Macrophages
CXCR4 Signaling
IL-2 Signaling
IL-33 Signaling Pathway
Senescence Pathway
IL-23 Signaling Pathway
MIF Regulation of Innate Immunity
STAT3 Pathway
IL-7 Signaling Pathway
CD40 Signaling
Apoptosis Signaling
SNARE Signaling Pathway
GADD45 Signaling
6 5 4 3 2 1 0 -1 -2
Activation z-score
Functional Networks
WT brain
IL6-/- brain
3 6 8 3 6 8
Cell movement of leukocytes
Cell movement of phagocytes
T cell development
Chemotaxis
Cell movement of lymphocytes
Cell movement of myeloid cells
Inflammatory response
Differentiation of T lymphocytes
T cell migration
Cell movement of neutrophils
Cell movement of antigen presenting cells
Quantity of mononuclear leukocytes
Quantity of lymphocytes
Quantity of lymphatic system cells
Hypersensitive reaction
Quantity of leukocytes
Quantity of CD4+ T-lymphocytes
Viral Infection
8 7 6 5 4 3 2 1 0 -1 -2 -3 -4 -5
Activation z-score
Upstream Regulators
WT brain
IL6-/- brain
3 6 8 3 6 8
MAVS
P38 MAPK
JAK1/2
STAT4
CD36
CD28
IKK
TNFAIP8L2
U0126
7 6 5 4 3 2 1 0 -1 -2 -3 -4 -5
Activation z-score

Figure 14: Analysis of top canonical signaling pathways, functional networks, and upstream regulators activated by WNV NY99 infections in WT and IL-6$^{-/-}$ mice. The heat maps demonstrate the comparative analysis of **A)** top canonical pathways **B)** functional networks, and **C)** upstream regulators based on the significant DEGs in response to WNV NY99 infection in WT and IL-6$^{-/-}$ mice at 3-, 6-, and 8-days post-infection. The range of activation is depicted as the activation z-score, determined by the IPA tool.

4.3.7 Differentially Expressed Upstream Regulators

Our analysis also revealed differential expression patterns of upstream regulators in the IL-6$^{-/-}$ group **(Fig. 14C)**. We observed an increase in MAVS, p38 MAPK, and JAK1/2 expressions. They are known to orchestrate pathways that lead to the activation of NF-κB, IRF3, and IRF7, subsequently inducing inflammatory mediators like CCL5 and TNF-α **[279-281]**. STAT4 and CD28 were upregulated, possibly contributing to T cell differentiation and activation **[282, 283]**. Moreover, CD36 was more pronounced, which is recognized to promote inflammation in monocytes/macrophages, resulting in the production of CXCL1, CXCL2, CCL5, CCL9, and IL-1β **[284]**. Notably, U0126, a MAPK kinase inhibitor **[285]**, exhibited greater inhibition in IL-6$^{-/-}$ mice, suggesting elevated activation of the MAPK pathway. Lastly, TNFAIP8L2 and IKK, which serve as immune checkpoint regulators negatively modulating inflammation and the NF-κB pathway, respectively **[286, 287]**, displayed downregulation in the IL-6$^{-/-}$ group.

4.3.8 Changes in the Expression of Pattern Recognition Receptors (PRRs)

The host innate immunity consists of a series of PRRs that detect molecular patterns associated with viruses (known as PAMPs) **[288]**. The activation of these PRRs is meticulously controlled at various stages to prevent potential tissue injury from uncontrolled cytokine storm. Furthermore, these receptors coordinate antiviral defense mechanisms through downstream signaling, leading to the synthesis of IFN and ISGs. **[159]**.

We investigated the differential expression of PRRs in WNV NY99-infected mice brains **(Fig. 15A)**. Notably, TLR3, IFIH1, Oas1b, and Oas3 genes were downregulated in the IL-6$^{-/-}$ group, essential in recognizing viral replication intermediates **[289]**, activating type 1 IFN signaling **[290]**, and inducing antiviral protein synthesis **[291]** to inhibit virus replication **[292, 293]**, highlighting their potential relevance to the suppressed antiviral response observed in IL-6$^{-/-}$ brains **[238]**. Conversely, NLRP3, responsible for mediating caspase-1 activation and the secretion of proinflammatory cytokines in response to virus infection **[294]**, was upregulated. CLEC6A (Dectin-2), expressed by myeloid cells, also exhibited increased activity, which mediates the secretion of proinflammatory cytokines **[295, 296]**.

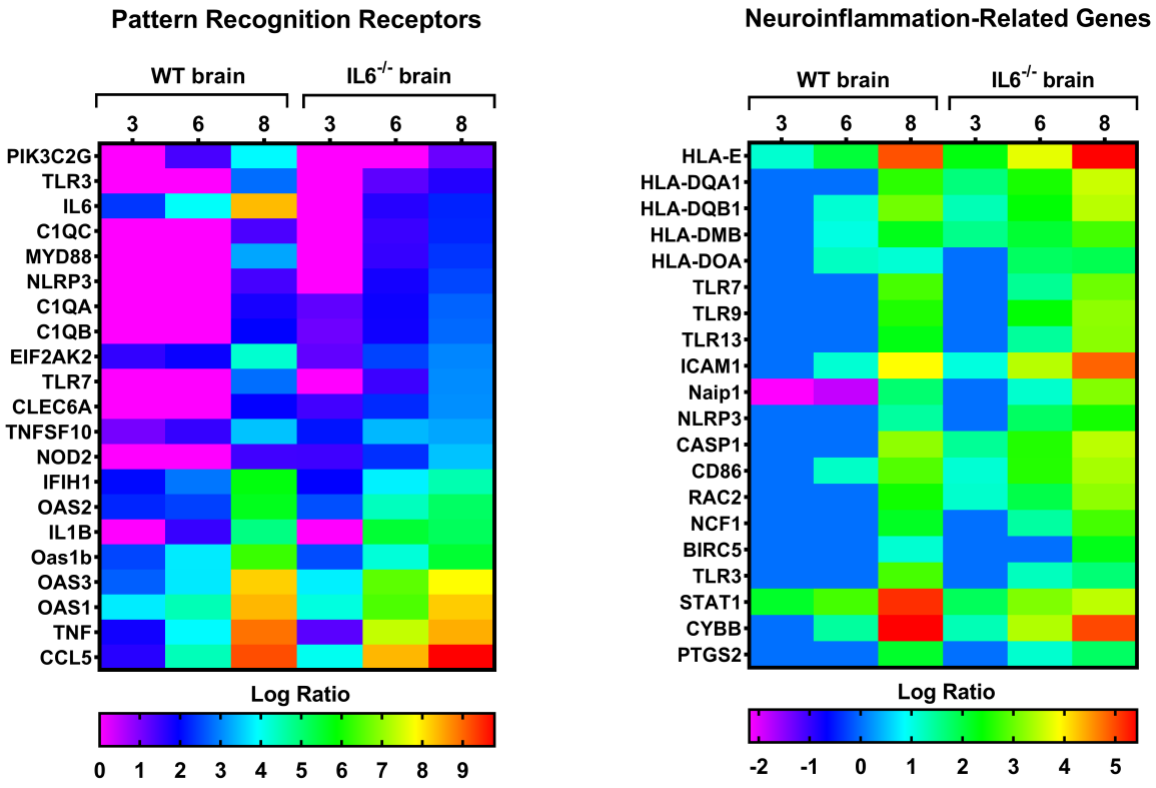

Figure 15: Activation of PRRs and neuroinflammation-associated genes in WNV-infected brain tissues of WT and IL-6$^{-/-}$ mice. Comparative expression of the genes associated with **A)** pattern recognition receptors and **B)** neuroinflammation were determined using IPA. The heat maps present the expression levels as log ratios relative to mock-infected brain samples.

4.3.9 Variations in the Expression of Neuroinflammation-Associated Genes

Our following investigation focused on neuroinflammation-related genes in WT and IL-$6^{-/-}$ mice brains **(Fig. 15B)**. The IL-$6^{-/-}$ mice exhibited upregulation of HLA genes implicated in antigen presentation **[297]**. Similarly, elevated expressions of toll-like receptor 7 (TLR7), TLR9, TLR13, and ICAM1 were noted, which activate the NF-κB pathway, initiating cytokine secretion and promoting leukocyte expansion and recruitment to the site of infection **[298-302]**. Although TLRs play a crucial role in identifying viruses, their excessive activation can lead to a harmful, rather than a protective outcome during CNS infections. Moreover, Naip1 and NLRP3, which mediate caspase-1 activation **[303, 304]**, displayed heightened expression, underscored by upregulated CASP1 itself, that leads to inflammation via pyroptosis induction **[305]**. Interestingly, the involvement of the NLRP3 has been previously demonstrated in the development of Japanese encephalitis **[306]**. Furthermore, upregulation of CD86, RAC2, and NCF1 was observed, entailing functions including T cell activation, neutrophil degranulation, and reactive oxygen species (ROS) release **[307-309]**. Concomitantly, the induction of BIRC5, which is known to dampen the anti-inflammatory response of apoptotic cells, was noted **[310]**. In contrast, TLR3 and STAT1 genes, recognized for activating type I and type II interferons, detecting viral replication intermediates (dsRNA), and orchestrating antiviral protein synthesis, were downregulated in the knockout group **[235, 292]**. CYBB gene expression, pivotal in killing invading viruses via forming the NADPH oxidase enzyme complex **[311]**, was decreased. Lastly, PTGS2, an inflammation regulatory factor **[312]**, displayed reduced expression.

In conclusion, our study offers a comprehensive transcriptome analysis of murine brains infected with lethal WNV NY99 strain in the presence and absence of IL-6 gene. The distinct expression patterns of specific host genes in IL-$6^{-/-}$ mice could account for the altered outcome of

WNV-induced neurotropic infection. However, the investigation is constrained by the lack of over-expression or knockout studies that would provide further insights into the interaction between IL-6 and the implicated genes within the observed signaling pathways, offering potential novel therapeutic targets for managing neurological diseases caused by WNV.

(This manuscript is currently in preparation for submission to a high-impact factor peer-reviewed journal)

5 OTHER SIGNIFICANT CONTRIBUTIONS

5.1 Treating Dengue Infections In Vivo Using mRNA Encoded Cas13

Abstract: Dengue is currently considered one of the top global health threats. Annually, an estimated ~96 million individuals develop dengue disease, ranging from subclinical disease to severe dengue, which can include bleeding and organ impairment. Currently, no approved antiviral agents are available to prevent or treat infections caused by dengue viruses (DENV), and even the newest vaccines have limitations, especially in naive individuals. Here, we present a new paradigm for treating dengue, via the systemic delivery of mRNA encoded Cas13a and guide RNAs, targeted to conserved regions of the DENV 2 and 3 genomes, formulated in lipid nanoparticles (LNP). First, we discovered potent guides against serotype 2 that demonstrated in vitro efficacy. Next, we confirmed that Cas13 enzymatic activity is necessary for specific and effective mitigation of DENV 2 in vitro by qPCR and RNA-seq. We then demonstrated in vitro efficacy against DENV 3 with the same guides. Lastly, we demonstrated the ability of a single dose of LNP formulated, mRNA encoded Cas13a, and guide RNA administered one day post-infection, to greatly mitigate lethal infections of DENV 2 and 3 in relevant mouse models. Overall, these data open the door to the development of a potent pan-DENV drug, and the expansion of this approach to many additional viral infections.

(This manuscript has been submitted to *Nature Microbiology* and is currently under revision)

5.2 SARS-CoV-2 Infects Primary Neurons from Human ACE2 Expressing Mice and Upregulates Genes Involved in the Inflammatory and Necroptotic Pathways

Abstract: Transgenic mice expressing human angiotensin-converting enzyme 2 under the cytokeratin 18 promoter (K18-hACE2) have been extensively used to investigate the pathogenesis and tissue tropism of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection.

Neuroinvasion and the replication of SARS-CoV-2 within the central nervous system (CNS) of K18-hACE2 mice is associated with increased mortality; although, the mechanisms by which this occurs remain unclear. In this study, we generated primary neuronal cultures from K18-hACE2 mice to investigate the effects of SARS-CoV-2 infection. We also evaluated the immunological response to SARS-CoV-2 infection in the CNS of K18-hACE2 mice and mouse neuronal cultures. Our data show that neuronal cultures obtained from K18-hACE2 mice are permissive to SARS-CoV-2 infection and support productive virus replication. Furthermore, SARS-CoV-2 infection upregulated the expression of genes involved in innate immunity and inflammation, including IFN-α, ISG-15, CXCL10, CCL2, IL-6 and TNF-α, in the neurons and mouse brains. In addition, we found that SARS-CoV-2 infection of neurons and mouse brains activates the ZBP1/pMLKL-regulated necroptosis pathway. Together, our data provide insights into the neuropathogenesis of SARS-CoV-2 infection in K18-hACE2 mice.

(This work has been published in *Pathogens*) **[313]**

5.3 SARS-CoV-2 Variants of Concern Infect the Respiratory Tract and Induce Inflammatory Response in Wild-Type Laboratory Mice

Abstract: The emergence of new severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) variants of concern pose a major threat to public health, due to possibly enhanced virulence, transmissibility, and immune escape. These variants may also adapt to new hosts through mutations in the spike protein. In this study, we evaluated the infectivity and pathogenicity of SARS-CoV-2 variants of concern in wild-type C57BL/6 mice. Six-week-old mice were inoculated intranasally with a representative virus from the original B.1 lineage, or the emerging B.1.1.7 and B.1.351 lineages. We also infected a group of mice with a mouse-adapted SARS-CoV-2 (MA10). Viral load and mRNA levels of multiple cytokines and chemokines were analyzed in

the lung tissues on day 3 after infection. Our data show that unlike the B.1 virus, the B.1.1.7 and B.1.351 viruses are capable of infecting C57BL/6 mice and replicating at high concentrations in the lungs. The B.1.351 virus replicated to higher titers in the lungs compared with the B.1.1.7 and MA10 viruses. The levels of cytokines (IL-6, TNF-α, IL-1β) and chemokine (CCL2) were upregulated in response to the B.1.1.7 and B.1.351 infection. In addition, robust expression of viral nucleocapsid protein and histopathological changes were detected in the lungs of B.1.351-infected mice. Overall, these data indicate a greater potential for infectivity and adaptation to new hosts by emerging SARS-CoV-2 variants.

(This work has been published in *Viruses*) **[151]**

5.4 Differential Pathogenesis of SARS-CoV-2 Variants of Concern in Human ACE2-Expressing Mice

Abstract: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused the current pandemic, resulting in millions of deaths worldwide. Increasingly contagious variants of concern (VoC) have fueled recurring global infection waves. A major question is the relative severity of the disease caused by previous and currently circulating variants of SARS-CoV-2. In this study, we evaluated the pathogenesis of SARS-CoV-2 variants in human ACE-2-expressing (K18-hACE2) mice. Eight-week-old K18-hACE2 mice were inoculated intranasally with a representative virus from the original B.1 lineage or from the emerging B.1.1.7 (alpha), B.1.351 (beta), B.1.617.2 (delta), or B.1.1.529 (omicron) lineages. We also infected a group of mice with the mouse-adapted SARS-CoV-2 (MA10). Our results demonstrate that B.1.1.7, B.1.351 and B.1.617.2 viruses are significantly more lethal than the B.1 strain in K18-hACE2 mice. Infection with the B.1.1.7, B.1.351, and B.1.617.2 variants resulted in significantly higher virus titers in the lungs and brain of mice compared with the B.1 virus. Interestingly, mice infected with the

B.1.1.529 variant exhibited less severe clinical signs and a high survival rate. We found that B.1.1.529 replication was significantly lower in the lungs and brain of infected mice in comparison with other VoC. The transcription levels of cytokines and chemokines in the lungs of B.1- and B.1.1.529-infected mice were significantly less when compared with those challenged with other VoC. Together, our data provide insights into the pathogenesis of previous and circulating SARS-CoV-2 VoC in mice.

(This work has been published in *Viruses*) **[149]**

5.5 Upregulation of Neuroinflammation-Associated Genes in the Brain of SARS-CoV-2-Infected Mice

Abstract: Neurological manifestations present a significant complication of coronavirus disease 2019 (COVID-19), but the underlying mechanisms are yet to be understood. Recently, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-induced neuroinvasion and encephalitis were observed in K18-hACE2 mice. Our goal in this study was to gain insights into the molecular pathogenesis of neurological manifestations caused by SARS-CoV-2 infection. We used NanoString analysis to investigate the gene expression changes in K18-hACE2 mouse brain at different time points following SARS-CoV-2 infection. At each time point, brain tissue from three infected mice were collected and transcript quantification was performed for each infected mouse. We determined the number of differentially expressed genes (DEGs) in comparison to mock-infected animals. Using Ingenuity Pathway Analysis, we found that the genes that were significantly upregulated were mainly associated with Toll-like receptor (TLR) signaling, RIG-I-like receptor (RLR) signaling, and cell death pathways. However, downregulated genes were associated with neurodegeneration and synaptic signaling pathways. Consistent with the gene expression profiles determined by NanoString, a multiplexed Luminex system showed the

increased expression of multiple inflammatory markers such as IP10 and MCP1. Furthermore, pathway analysis of DEGs indicated a possible link between TLR2 signaling and neuroinflammation pathway in the brain following SARS-CoV-2 infection. In conclusion, this comprehensive analysis of SARS-CoV-2-induced gene expression changes in the brain will provide insights into the neurological symptoms observed in a subset of COVID-19 patients. These results will be useful for the identification of potential SARS-CoV-2 targeted therapies and restriction factors.

(This manuscript has been submitted to *Pathogens* and is currently under revision)

6 SUMMARY AND FUTURE DIRECTIONS

This dissertation presents investigations on the role of the cytokine IL-6 in flavivirus infections, focusing on West Nile virus (WNV) and Japanese encephalitis virus (JEV). IL-6 has diverse effects, including neurotropic functions in the central nervous system (CNS) **[90, 93, 171, 173, 314]**. Previous research has shown that impaired IL-6 function can increase susceptibility to various infections **[96]**, but its impact on flaviviruses remains unclear. The study explores the influence of IL-6 on WNV or JEV replication in human neuronal cells and primary mouse cells from peripheral tissues and the brain. Our experiments reveal significantly elevated virus titers in these flavivirus-infected cells when IL-6 is neutralized or the gene is absent, underscoring the inhibitory role of IL-6 in flavivirus replication. In vivo experiments demonstrate the protective role of IL-6 against WNV and JEV infections, with increased mortality and higher viral titers observed in IL-$6^{-/-}$ mice. Additionally, the study examines the modulation of immune responses by IL-6. We detect a significant reduction in cytokine expression in infected human neuronal cells with neutralized IL-6, even in the presence of high virus titers. Similarly, cytokine and chemokine production are significantly reduced in the serum of IL-$6^{-/-}$ mice despite high viral loads. We also demonstrate that type I interferon (IFN-I) genes are significantly decreased in the brains of IL-$6^{-/-}$ mice. However, some inflammatory molecules are elevated in the brains of these mice, possibly due to increased immune cell infiltration and the diminished ability to control virus replication due to suppressed antiviral response. These phenomena correlate with the increased mortality observed in the IL-$6^{-/-}$ mice. Conversely, the brains exhibit a significant reduction in the levels of cytokines known for their anti-inflammatory functions. These findings highlight the specific function of IL-6 in regulating immune responses during flavivirus infections. To conclude, our study offers novel insights into the role of IL-6 in flavivirus replication and the host immune responses to flavivirus

infection. Our research presents the evidence, for the first time, that the absence of IL-6 increases the severity of WNV or JEV infection both in vitro and in vivo. Additional mechanistic studies are required to elucidate the complex interplays among IL-6, IFNs, and essential cytokines in the periphery and CNS of flavivirus-infected mice. The results will significantly influence the advancement of potential therapeutic approaches to mitigate disease outcomes caused by neurotropic flavivirus infections.

Our next study offers comprehensive transcriptome analysis to delineate how the absence of IL-6 impacts global gene expressions during WNV infection in murine brains. Through RNA-seq, we compare the responses of WT mice and IL-6$^{-/-}$ mice to WNV infection, focusing on key genes and pathways related to immune signaling, interferon response, and neuroinflammation. We observe a higher number of genes with differential expression in IL-6$^{-/-}$ mice during various stages of WNV infection compared to WT mice. Many highly upregulated genes in IL-6$^{-/-}$ mice are associated with inflammatory responses, cell death pathways, and interferon regulation. Furthermore, IL-6 deficiency results in a reduced activation of interferon-stimulated genes (ISGs), suggesting a weakened antiviral response **[315]**, potentially contributing to higher viral loads in IL-6$^{-/-}$ mice. The absence of IL-6 also leads to excessive production of proinflammatory cytokines and chemokines in the brain during WNV infection while suppressing genes with immunoregulatory functions. Additionally, we employ the Ingenuity Pathway Analysis (IPA) tool to identify the most activated canonical pathways and key functional networks involving the differentially expressed genes, revealing the prominent increase in immune cell recruitment and inflammatory pathways among infected IL-6$^{-/-}$ mice. Our analysis of upstream regulators supports these observations, highlighting the activation of signaling pathways that induce inflammation. We also note the downregulation of immune checkpoint regulators and NF-κB and MAPK

pathway suppressors, which negatively modulate inflammation **[316]**. Lastly, our examination of pattern recognition receptors and neuroinflammation-related genes in IL-6$^{-/-}$ mice brains reveals upregulation of antigen presentation, immune cell activation, leukocyte recruitment, and NF-κB pathway, but downregulation of genes crucial for stimulating interferon and antiviral responses. In summary, our study unveils the temporal modulation of immune pathways and cellular processes by IL-6 during WNV neurotropic infection in mice. Future experiments involving gene overexpression or knockout would deliver deeper insights into the interactions between IL-6 and the genes involved in the activated signaling pathways, informing much-needed therapeutic strategies for treating WNV and similar neurotropic flavivirus infections.

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8 APPENDICES

8.1 Auroni, T. T., Arora, K., Natekar, J. P., Pathak, H., Elsharkawy, A. and Kumar, M. (2023). The critical role of interleukin-6 in protection against neurotropic flavivirus infection. *Frontiers in Cellular and Infection Microbiology*, 13, 1275823.

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The critical role of interleukin-6 in protection against neurotropic flavivirus infection

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West Nile virus (WNV) and Japanese encephalitis virus (JEV) are emerging mosquito-borne flaviviruses causing encephalitis globally. No specific drug or therapy exists to treat flavivirus-induced neurological diseases. The lack of specific therapeutics underscores an urgent need to determine the function of important host factors involved in flavivirus replication and disease progression. Interleukin-6 (IL-6) upregulation has been observed during viral infections in both mice and humans, implying that it may influence the disease outcome significantly. Herein, we investigated the function of IL-6 in the pathogenesis of neurotropic flavivirus infections. First, we examined the role of IL-6 in flavivirus-infected human neuroblastoma cells, SK-N-SH, and found that IL-6 neutralization increased the WNV or JEV replication and inhibited the expression of key cytokines. We further evaluated the role of IL-6 by infecting primary mouse cells derived from IL-6 knockout (IL-$6^{-/-}$) mice and wild-type (WT) mice with WNV or JEV. The results exhibited increased virus yields in the cells lacking the IL-6 gene. Next, our *in vivo* approach revealed that IL-$6^{-/-}$ mice had significantly higher morbidity and mortality after subcutaneous infection with the pathogenic WNV NY99 or JEV Nakayama strain compared to WT mice. The non-pathogenic WNV Eg101 strain did not cause mortality in WT mice but resulted in 60% mortality in IL-$6^{-/-}$ mice, indicating that IL-6 is required for the survival of mice after the peripheral inoculation of WNV or JEV. We also observed significantly higher viremia and brain viral load in IL-$6^{-/-}$ mice than in WT mice. Subsequently, we explored innate immune responses in WT and IL-$6^{-/-}$ mice after WNV NY99 infection. Our data demonstrated that the IL-$6^{-/-}$ mice had reduced levels of key cytokines and in the serum during early infection but elevated levels of proinflammatory cytokines and in the brain later, along with suppressed anti-inflammatory cytokines. In addition, mRNA expression of IFN-α and IFN-β was significantly lower in the infected IL-$6^{-/-}$ mice. In conclusion, these data suggest that the lack of IL-6 exacerbates WNV or JEV infection *in vitro* and *in vivo* by causing an increase in virus replication and dysregulating host immune response.

KEYWORDS

West Nile virus, Japanese encephalitis virus, flavivirus, interleukin-6 (IL-6), host pathogen interaction, neuronal cells, mouse models, encephalitis

8.2 Rothan, H. A., Kumari, P., Stone, S., Natekar, J. P., Arora, K., Auroni, T. T., & Kumar, M. (2022). SARS-CoV-2 Infects Primary Neurons from Human ACE2 Expressing Mice and Upregulates Genes Involved in the Inflammatory and Necroptotic Pathways. *Pathogens*, *11*(2), 25.

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Article

SARS-CoV-2 Infects Primary Neurons from Human ACE2 Expressing Mice and Upregulates Genes Involved in the Inflammatory and Necroptotic Pathways

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Abstract: Transgenic mice expressing human angiotensin-converting enzyme 2 under the cytokeratin 18 promoter (K18-hACE2) have been extensively used to investigate the pathogenesis and tissue tropism of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) infection. Neuroinvasion and the replication of SARS-CoV-2 within the central nervous system (CNS) of K18-hACE2 mice is associated with increased mortality; although, the mechanisms by which this occurs remain unclear. In this study, we generated primary neuronal cultures from K18-hACE2 mice to investigate the effects of a SARS-CoV-2 infection. We also evaluated the immunological response to SARS-CoV-2 infection in the CNS of K18-hACE2 mice and mouse neuronal cultures. Our data show that neuronal cultures obtained from K18-hACE2 mice are permissive to SARS-CoV-2 infection and support productive virus replication. Furthermore, SARS-CoV-2 infection upregulated the expression of genes involved in innate immunity and inflammation, including IFN-α, ISG-15, CXCL10, CCL2, IL-6 and TNF-α, in the neurons and mouse brains. In addition, we found that SARS-CoV-2 infection of neurons and mouse brains activates the ZBP1/pMLKL-regulated necroptosis pathway. Together, our data provide insights into the neuropathogenesis of SARS-CoV-2 infection in K18-hACE2 mice.

Keywords: COVID-19; SARS-CoV-2; K18-hACE2 mice; neurons; neuropathogenesis; inflammation; necroptosis

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Citation: Rothan, H.A.; Kumari, P.; Stone, S.; Natekar, J.P.; Arora, K.; Auroni, T.T.; Kumar, M. SARS-CoV-2 Infects Primary Neurons from Human ACE2 Expressing Mice and Upregulates Genes Involved in the Inflammatory and Necroptotic Pathways. *Pathogens* **2022**, *11*, 257. https://doi.org/10.3390/pathogens11020257

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1. Introduction

Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), continues to be a global concern. In addition, several variants of SARS-CoV-2 have been identified that may influence antibody treatment and vaccine efficiency [1–4]. Neurological complications, such as brain fog, loss of taste and smell, changed mental status and anosmia have been reported in some COVID-19 patients [5–9]. Studies have shown presence of meningitis, encephalitis, leukocytes infiltration and neuronal death in COVID-19 patients [6,10]. Evidence of SARS-CoV-2 neuroinvasion in COVID-19 patients' brain autopsies has been demonstrated and the olfactory mucosa has been suggested as a route of entry [4,6,11–13]. Several studies have also reported that neurologic symptoms may result from the exacerbated systemic pro-inflammatory responses without a direct infection of the brain cells [9,13]. Angiotensin-converting enzyme 2 (ACE2), the entry receptor of SARS-CoV-2, has recently been demonstrated to be present in neurons and glial cells of different brain regions [5,14–18]. Studies using brain organoids derived from human pluripotent stem cell (hPSC) have shown the presence of the virus in neuronal cells [19–23]. In addition, anti-ACE2 antibodies can inhibit the SARS-CoV-2 infection of neuronal cells [19].

8.3 Stone, S., Rothan, H. A., Natekar, J. P., Kumari, P., Sharma, S., Pathak, H., Arora, K., Auroni, T. T., & Kumar, M. (2021). SARS-CoV-2 Variants of Concern Infect the Respiratory Tract and Induce Inflammatory Response in Wild-Type Laboratory Mice. *Viruses*, *14*(1), 27.

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Brief Report

SARS-CoV-2 Variants of Concern Infect the Respiratory Tract and Induce Inflammatory Response in Wild-Type Laboratory Mice

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Abstract: The emergence of new severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2) variants of concern pose a major threat to public health, due to possible enhanced virulence, transmissibility and immune escape. These variants may also adapt to new hosts, in part through mutations in the spike protein. In this study, we evaluated the infectivity and pathogenicity of SARS-CoV-2 variants of concern in wild-type C57BL/6 mice. Six-week-old mice were inoculated intranasally with a representative virus from the original B.1 lineage, or the emerging B.1.1.7 and B.1.351 lineages. We also infected a group of mice with a mouse-adapted SARS-CoV-2 (MA10). Viral load and mRNA levels of multiple cytokines and chemokines were analyzed in the lung tissues on day 3 after infection. Our data show that unlike the B.1 virus, the B.1.1.7 and B.1.351 viruses are capable of infecting C57BL/6 mice and replicating at high concentrations in the lungs. The B.1.351 virus replicated to higher titers in the lungs compared with the B.1.1.7 and MA10 viruses. The levels of cytokines (IL-6, TNF-α, IL-1β) and chemokine (CCL2) were upregulated in response to the B.1.1.7 and B.1.351 infection in the lungs. In addition, robust expression of viral nucleocapsid protein and histopathological changes were detected in the lungs of B.1.351-infected mice. Overall, these data indicate a greater potential for infectivity and adaptation to new hosts by emerging SARS-CoV-2 variants.

Keywords: COVID-19; SARS-CoV-2 variants; C57BL/6 mice; host-range; inflammation

check for updates

Citation: Stone, S.; Rothan, H.A.; Natekar, J.P.; Kumari, P.; Sharma, S.; Pathak, H.; Arora, K.; Auroni, T.T.; Kumar, M. SARS-CoV-2 Variants of Concern Infect the Respiratory Tract and Induce Inflammatory Response in Wild-Type Laboratory Mice. *Viruses* **2022**, *14*, 27. https://doi.org/10.3390/v14010027

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1. Introduction

Coronaviruses are a family of positive-sense single-strand RNA viruses. Their large genomes and propensity for mutation have resulted in a diversity of coronavirus strains that are capable of adapting to new hosts. COVID-19, the disease caused by the new beta coronavirus, SARS-CoV-2, has caused significant human and economic burden [1–3]. As of 10 December 2021, the number of confirmed cases worldwide is over 269 million, with 5.29 million deaths. Few therapies are available to treat COVID-19 in humans, and the rapid evolution of SARS-CoV-2 variants threatens to diminish their efficacy [2,4]. The lineage B.1.1.7, first identified in the United Kingdom, and lineage B.1.351, first described in South Africa, have been termed variants of concern because of the greater risk they pose due to their possible enhanced transmissibility, disease severity and immune escape [4–7]. These variants may also adapt to new hosts, in part, through mutations on the receptor-binding domain (RBD) of the spike protein [6,7].

SARS-CoV-2 infection begins with the viral particles binding to the receptors on the host cell surface. The RBD of the spike protein binds to angiotensin-converting enzyme 2 (ACE-2), present on the host cellular surfaces [3,8]. The RBD of the spike protein from the SARS-CoV-2 strain (Wuhan strain, lineage B.1) that started the pandemic does not

8.4 Natekar, J. P., Pathak, H., Stone, S., Kumari, P., Sharma, S., Auroni, T. T., Arora, K., Rothan, H. A., & Kumar, M. (2022). Differential Pathogenesis of SARS-CoV-2 Variants of Concern in Human ACE2-Expressing Mice. *Viruses*, *14*(6), 1139.

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Brief Report

Differential Pathogenesis of SARS-CoV-2 Variants of Concern in Human ACE2-Expressing Mice

Janhavi Prasad Natekar †, Heather Pathak †, Shannon Stone, Pratima Kumari, Shaligram Sharma, Tabassum Tasnim Auroni, Komal Arora, Hussin Alwan Rothan and Mukesh Kumar *

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Abstract: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has caused the current pandemic, resulting in millions of deaths worldwide. Increasingly contagious variants of concern (VoC) have fueled recurring global infection waves. A major question is the relative severity of the disease caused by previous and currently circulating variants of SARS-CoV-2. In this study, we evaluated the pathogenesis of SARS-CoV-2 variants in human ACE-2-expressing (K18-hACE2) mice. Eight-week-old K18-hACE2 mice were inoculated intranasally with a representative virus from the original B.1 lineage or from the emerging B.1.1.7 (alpha), B.1.351 (beta), B.1.617.2 (delta), or B.1.1.529 (omicron) lineages. We also infected a group of mice with the mouse-adapted SARS-CoV-2 (MA10). Our results demonstrate that B.1.1.7, B.1.351 and B.1.617.2 viruses are significantly more lethal than the B.1 strain in K18-hACE2 mice. Infection with the B.1.1.7, B.1.351, and B.1.617.2 variants resulted in significantly higher virus titers in the lungs and brain of mice compared with the B.1 virus. Interestingly, mice infected with the B.1.1.529 variant exhibited less severe clinical signs and a high survival rate. We found that B.1.1.529 replication was significantly lower in the lungs and brain of infected mice in comparison with other VoC. The transcription levels of cytokines and chemokines in the lungs of B.1- and B.1.1.529-infected mice were significantly less when compared with those challenged with other VoC. Together, our data provide insights into the pathogenesis of previous and circulating SARS-CoV-2 VoC in mice.

Keywords: COVID-19; SARS-CoV-2 variants; omicron; ACE2-expressing mice; inflammation

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1. Introduction

SARS-CoV-2 is a positive-sense, single-stranded RNA virus belonging to the Betacoronavirus family [1,2]. Since the emergence of SARS-CoV-2 in late 2019, several new variants of concern (VoC), alpha (B.1.1.7 lineage), beta (B.1.351 lineage), gamma (P.1 lineage), delta (B.1.617.2 lineage), and omicron (B.1.1.529 lineage), have fueled recurring global infection waves. These variants have been termed VoC because of the higher risk due to their possible enhanced transmissibility, disease severity, immune escape, and increased adaptation to new hosts [3–8]. Mutations occurring in the spike protein are of major concern due to the role of this glycoprotein in mediating virus entry and as the major target of neutralizing antibodies [3,9–11]. The lineage B.1.1.7 was first identified in the United Kingdom, lineage B.1.351 was discovered in South Africa, and lineage B.1.617.2 was first described in India. Most recently, the omicron (B.1.1.529) VoC that emerged in South Africa was estimated to have been responsible for the majority of infections worldwide. The B.1.1.7 variant has mutations in the receptor binding domain (RBD) region, including N501Y, 69/70 deletion, and P681H near the S1/S2 furin cleavage site [7,12–14]. The B.1.351 variant has eight mutations, of which the three most notable mutations are K417N, E484K, and N501Y in the