List of 10 Best Publications highlighting the discoveries/contributions

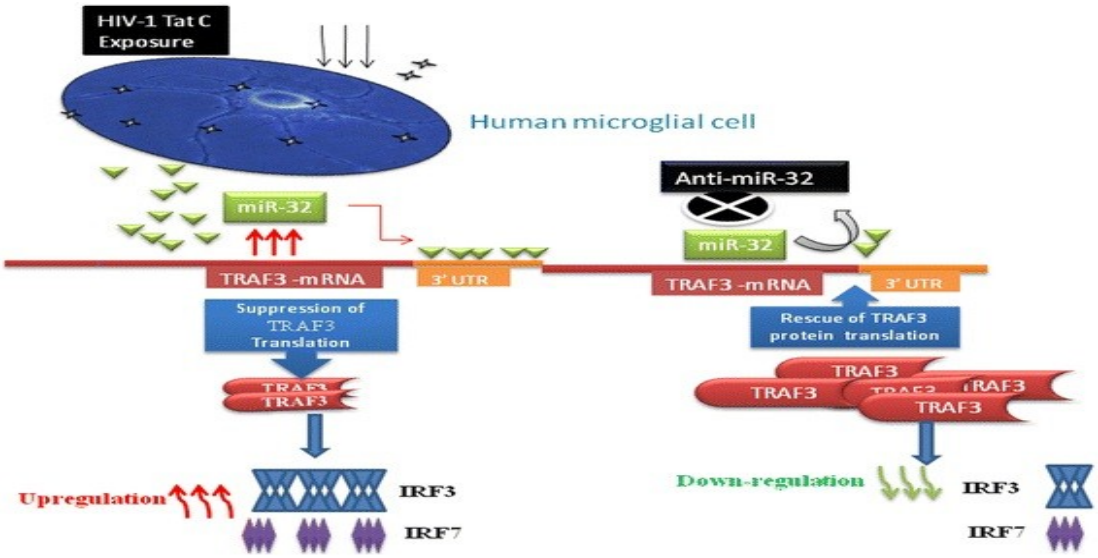
Prof. Sunit K. Singh's area of work is related to the Molecular Virology. His research group is engaged in understanding the molecular pathogenesis or Immune Evasion strategies utilized by Neurotropic Viruses.

Publications related to NeuroAIDS

1. Mishra R, Chhatbar C, Singh SK (2012), HIV-1 Tat C-mediated regulation of tumor necrosis factor receptor-associated factor-3 by microRNA 32 in human microglia, *Journal of Neuroinflammation*, Jun 18;9:131. doi: 10.1186/1742-2094-9-131

HIV is known to enter into brain during early course of infections and leading to NeuroAIDS. HIV-1 Tat protein is known to be associated with neuroinflammation, a condition that develops in almost half of patients infected with HIV-1. HIV-1 Tat can alter glial neuroprotective functions, leading to neurotoxicity within the CNS. HIV-1 Tat is known to be secreted from productively infected cells and can affect neighboring uninfected cells by modulating cellular gene expression in a bystander fashion. We reported the dose dependent increase in the expression pattern of miR-32 in HIV-1 Tat-C exposed human microglial cells. We found that tumor necrosis factor-receptor-associated factor 3 TRAF3) is a direct target for miR-32, and overexpression of miR-32 in human microglial cells decreased TRAF3 both at the mRNA and the protein level. Recovery of TRAF3 protein expression after transfection of anti-miR-32 and the results of the luciferase reporter assay provided direct evidence of TRAF3 regulation by miR-32. We found that the regulation of interferon regulatory factor 3 (IRF3) and IRF7 is controlled by cellular levels of TRAF3 protein in microglial cells, as after overexpression of miR-32 and application of anti-miR-32, expression levels of IRF3 and IRF7 were inversely regulated by

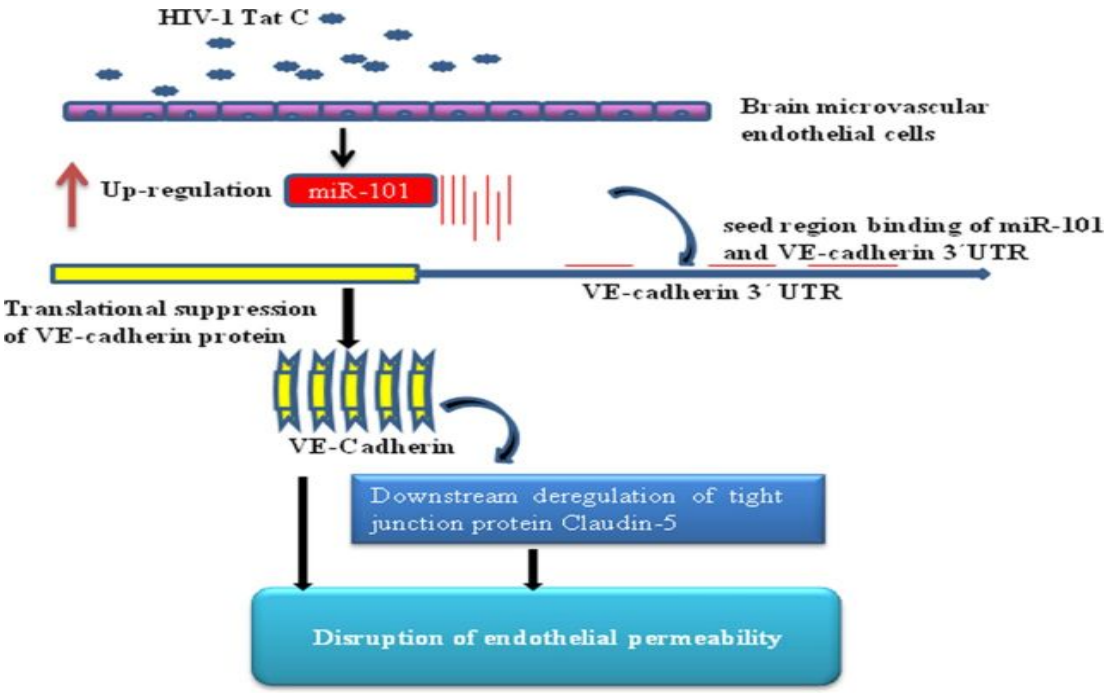
expression levels of TRAF3. Thus, our results suggest a novel miRNA mediated mechanism for regulation of TRAF3 in human microglial cells exposed to HIV-1 Tat C protein. These results may help to elucidate the detrimental neuroinflammatory consequences of HIV-1 Tat C protein in bystander fashion. HIV-1 Tat protein can modulate TRAF3 expression through miRNA mediated pathway and can change the downstream expression of IRF3 and IRF7. This study demonstrates a novel mechanism of HIV-1 Tat C protein-mediated perturbation of miRNA, resulting in dysregulation of cellular TRAF3.



Proposed Model: Proposed model for HIV-1 Tat C-induced, miR-32-mediated post-transcriptional regulation of tumor necrosis factor receptor-associated factor 3 (TRAF3). In response to HIV-1 Tat C exposure of human microglial cells, miR-32 was upregulated, consequently downregulating the protein level of TRAF3 post-transcriptionally by binding to its 3′ untranslated region. The miRNA inhibitor against miR-32, ant-miR-32, reduced the cellular level of miR-32 and rescued the expression level of TRAF3 protein. The cellular expression level of TRAF3 protein had an inverse relationship to the expression level of interferon regulatory factor (IRF)3/7 and this could perturb the expression of inflammatory genes in microglial cells after exposure to HIV-1 Tat C protein.

2. Mishra R, Singh SK (2013), HIV-1 Tat C modulates expression of miRNA-101 to suppress VE-Cadherin in Human Brain Microvascular Endothelial Cells, *The Journal of Neuroscience* 33(14):5992-6000; doi:10.1523/JNEUROSCI.4796-12.2013.

HIV-1 infection leads to the development of HIV-associated neurological disorders. The HIV-1 Tat protein has been reported to exert an adverse effect on blood-brain barrier integrity and permeability. Perturbation in permeability is mainly caused by disruptions in adherens junctions and tight junction proteins. We have identified HIV-1 Tat C-induced disruption of VE-cadherin mediated by miRNA-101 in human brain microvascular endothelial cells (BMVECs). HIV-1 Tat C increased the expression of miR-101, which led to downregulation of VE-cadherin. Overexpression of miR-101 resulted into the suppression of VE-cadherin. Inhibition of miR-101 by the miRNA inhibitor enhanced the expression of VE-cadherin. We have demonstrated that VE-cadherin is a direct target of miR-101 using a luciferase reporter assay, which showed that mutated VE-cadherin 3'UTR and miR-101 co-transfection did not change luciferase activity. By overexpression and knockdown of miR-101, we have demonstrated that the expression level of claudin-5 is governed by the expression of VE-cadherin. These findings demonstrate a novel mechanism for the regulation of barrier permeability by miR-101 via posttranscriptional regulation of VE-cadherin in human BMVECs exposed to the HIV-1 Tat C protein.



Proposed Model: Proposed model for Tat C-mediated downregulation of VE-cadherin and claudin-5 in BMVECs through miR-101. We propose that expression of VE-cadherin is regulated through miR-101 in BMVECs exposed to the HIV-1 Tat C protein. After HIV-1 Tat C treatment, the expression level of miR-101 increases and targets the expression level of VE-cadherin directly, which in turn influences the expression level of claudin-5 and thereby the permeability in BMVECs.

3. Jadhav V; Krause KH; Singh SK, 2014, HIV-1 Tat C modulates NOX2 and NOX4 expressions through miR-17 in Human Microglial Cells, *Journal of Neurochemistry*. Dec; 131(6):803-15

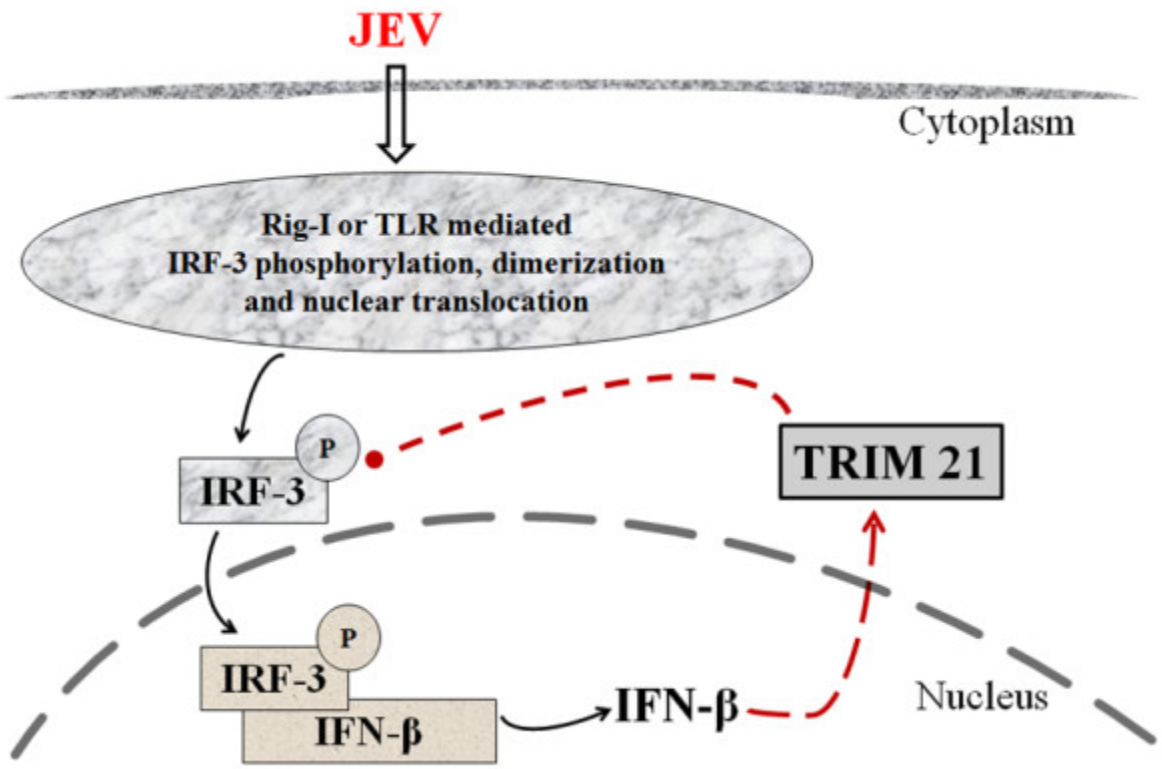
HIV-1 invades CNS in the early course of infection, which can lead to the cascade of neuroinflammation. NADPH oxidases (NOXs) are the major producers of reactive oxygen species (ROS), which play important roles during pathogenic insults. The molecular mechanism of ROS generation via microRNA-mediated pathway in human microglial cells in response to HIV-1 Tat protein has been demonstrated in this study. Over-expression and knockdown of microRNAs, luciferase reporter assay, and site-directed mutagenesis are main molecular techniques used in this study. A significant reduction in miR-17 levels and increased NOX2,

NOX4 expression levels along with ROS production were observed in human microglial cells upon HIV-1 Tat C exposure. The validation of NOX2 and NOX4 as direct targets of miR-17 was done by luciferase reporter assay. The over-expression and knockdown of miR-17 in human microglial cells showed the direct role of miR-17 in regulation of NOX2, NOX4 expression and intracellular ROS generation. We demonstrated the regulatory role of cellular miR-17 in ROS generation through over-expression and knockdown of miR-17 in human microglial cells exposed to HIV-1 Tat C protein. Activated microglial cells mediated neuroinflammatory events are observed in HIV-associated neurological disorders. The reduction in miR-17 levels was observed in microglial cells exposed to HIV-1 Tat C protein. miR-17 regulated the expression of NOX2 and NOX4, which in turn regulated the reactive oxygen species (ROS) production in microglial cells. Increased ROS production led to the activation of microglial cells and increased cytokine production. This study thus demonstrated a novel miR-17-mediated regulatory pathway of ROS production in microglial cells.

Publications related to Japanese Encephalitis

4. **Manocha GD, Mishra R, Sharma N, Kumawat KL, Basu A, Singh SK (2014), Regulatory role of TRIM21 in type-I interferon pathway in Japanese encephalitis virus infected human microglial cells *Journal of Neuroinflammation*, Feb 1;11:24. doi: 10.1186/1742-2094-11-24.**

Japanese encephalitis virus (JEV) infection leads to Japanese encephalitis (JE) in humans. JEV is transmitted through mosquitoes and maintained in a zoonotic cycle. This cycle involves pigs as the major reservoir, water birds as carriers and mosquitoes as vectors. JEV invasion into the central nervous system (CNS) may occur via antipodal transport of virions or through the vascular endothelial cells. Microglial cells get activated in response to pathogenic insults. JEV infection induces the innate immune response and triggers the production of type I interferons. The signaling pathway of type I interferon production is regulated by a number of molecules. TRIM proteins are known to regulate the expression of interferons; however, the involvement of TRIM genes and their underlying mechanism during JEV infection are not known. We reported that JEV infection increased expression of TRIM21 in human microglial cells. JEV induced an innate immune response by increasing production of IFN-β via IRF3 activation and phosphorylation. Overexpression of TRIM21 resulted in downregulation of p-IRF3 and IFN-β, while silencing led to increased production of p-IRF3 and IFN-β in JEV-infected human microglial cells. This study demonstrates TRIM21 as a negative regulator of interferon-β (IFN-β) production mediated by IRF-3 during JEV infection in human microglial cells.



Proposed Model: Model showing a plausible role of TRIM21 as a negative regulator of IRF3 activation and IFN-β production following JEV infection in human microglial cells. JEV infection causes activation of the RIG-1 receptor, initiating a downstream signaling mechanism leading to the activation of IRF-3. Phosphorylated IRF-3 dimerizes and translocates into the nucleus, where it leads to the transcription and production of IFN-β. JEV infection also induces the TRIM21 protein, which negatively regulates IRF-3 phosphorylation, leading to reduced IFN-β production. The upregulation of TRIM21 is proposed to be a feedback mechanism to inhibit the innate immune response in JEV infection.

5. Sharma N, Verma R, Kumawat KL, Basu A, Singh SK (2015), miR-146a suppresses cellular immune response during Japanese encephalitis virus JaOArS982 strain infection in human microglial cells, *Journal of Neuroinflammation*, Feb 18;12:30. doi: 10.1186/s12974-015-0249-0

Japanese encephalitis virus (JEV) infection leads to Japanese encephalitis (JE) in humans. JEV is transmitted through mosquitoes and maintained in a zoonotic cycle. This cycle involves pigs as the major reservoir, water birds as carriers and mosquitoes as vectors. JEV invasion into the central

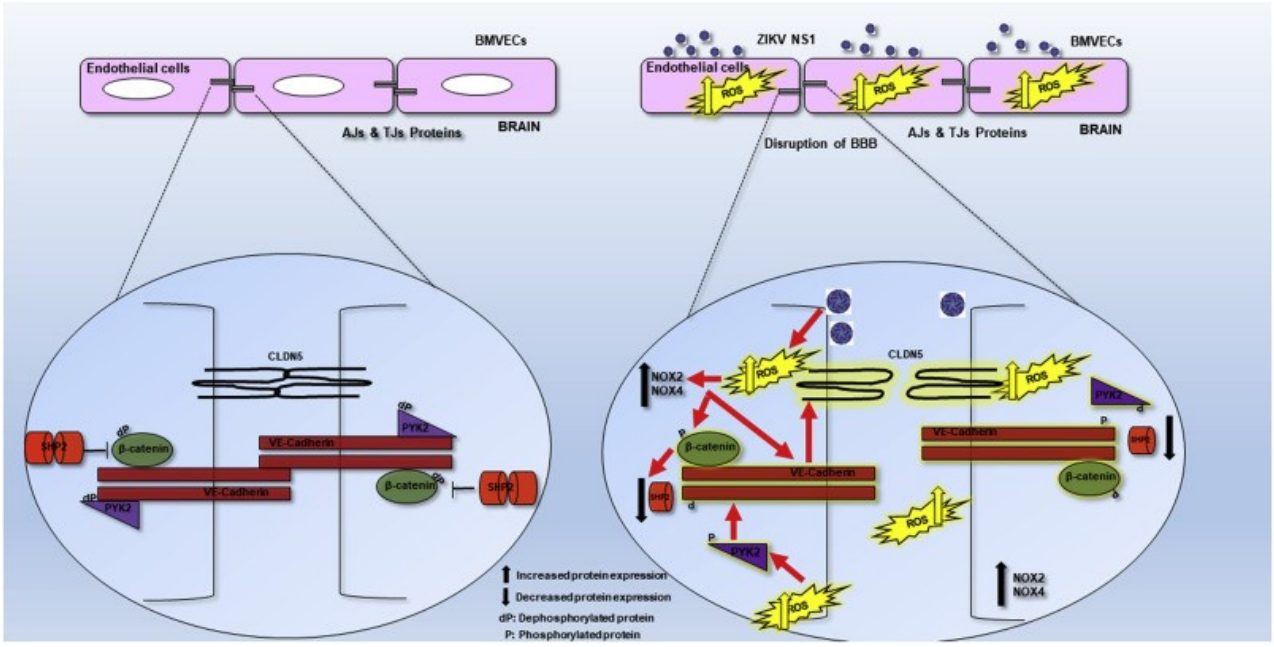
nervous system (CNS) may occur via antipodal transport of virions or through the vascular endothelial cells. Microglial cells get activated in response to pathogenic insults. JEV infection induces the innate immune response and triggers the production of type I interferons. The signaling pathway of type I interferon production is regulated by a number of molecules. TRIM proteins are known to regulate the expression of interferons; however, the involvement of TRIM genes and their underlying mechanism during JEV infection are not known. We reported that JEV infection increased expression of TRIM21 in human microglial cells. JEV induced an innate immune response by increasing production of IFN-β via IRF3 activation and phosphorylation. Overexpression of TRIM21 resulted in downregulation of p-IRF3 and IFN-β, while silencing led to increased production of p-IRF3 and IFN-β in JEV-infected human microglial cells. This report demonstrates TRIM21 as a negative regulator of interferon-β (IFN-β) production mediated by IRF-3 during JEV infection in human microglial cells.

Publications related to ZIKA Virus (ZIKV)

6. Rastogi M, Singh SK, 2020, Zika Virus NS1 affects the Junctional Integrity of Human Brain Microvascular Endothelial Cells, *Biochimie*, 176 (2020), 52-61, 10.1016/j.biochi.2020.06.011.

Zika virus (ZIKV) infection leads to microcephaly in newborns. Flaviviruses are known to secrete NS1 protein extracellularly and its concentration in serum directly co-relate to disease severity. The presence of ZIKV-NS1 near the brain microvascular endothelial cells (BMVECs) affects blood-brain-barrier, which is composed of tight junctions (TJs) and adherens junctions (AJs). Viruses utilize different strategies to circumvent this barrier to enter in brain. The present study demonstrated the mechanism of junctional integrity disruption in BMVECs by ZIKV-NS1

protein exposure. The Transendothelial Electrical Resistance and sodium fluorescein migration assays revealed the endothelial barrier disruption in BMVECs exposed to ZIKV-NS1 at different time (12hr and 24hr) and doses (500 ng/mL, 1000 ng/mL and 1500 ng/mL). The exposure of ZIKV-NS1 on BMVECs led to the phosphorylation of AJs and suppression of TJs through secreted ZIKV-NS1 in a bystander fashion. The activation of NADPH dependent reactive oxygen species activity and redox sensitive tyrosine kinase further increased the phosphorylation of AJs. The reduced expression of the phosphatase led to the increased phosphorylation of the AJs. The treatment with Diphenyleneiodonium chloride rescued the phosphatase and TJs expression and suppressed the expression of kinase and AJs in BMVECs exposed to ZIKV-NS1.

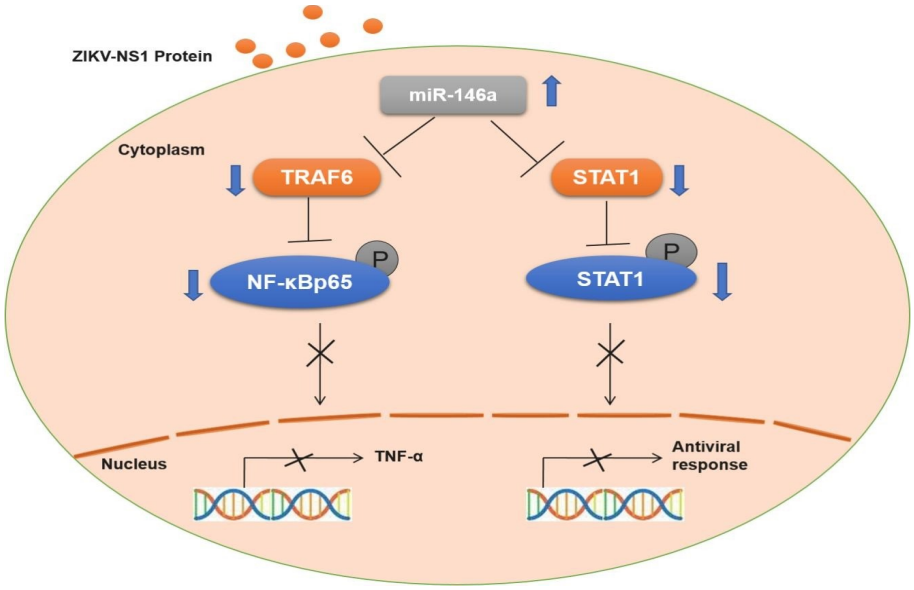


Proposed Model: The Proposed model of the molecular mechanisms involved in the endothelial barrier disruption in BMVECs exposed to ZIKV-NS1.The ZIKV-NS1 treatment activates the intracellular ROS production by NADPH-dependent enzymes (NOX2 and NOX4). The increase in ROS production activates the redox-sensitive tyrosine kinase, PYK2 which phosphorylates the VE-cadherin and β-catenin proteins. The phosphorylation of VE-cadherin promotes the disruption of tight junction protein, CLDN5. The SHP2 is a phosphatase, which maintains the barrier integrity by binding

to the VE-cadherin complex and keeps them in de-phosphorylated state. Upon ZIKV-NS1 treatment, the SHP2 expression decreases thereby further destabilizing the VE-cadherin complex.

7. Shukla A, Rastogi M, Singh SK (2021), Zika virus NS1 suppresses the innate immune responses via miR-146a in human microglial cells, *International Journal of Biological Macromolecules* Dec 15;193(Pt B):2290-2296. doi: 10.1016/j.ijbiomac.2021.11.061

Zika virus (ZIKV) is a positive-single strand RNA virus that belongs to the Flaviviridae family. ZIKV infection causes congenital ZIKV syndrome (CZS) in children and Guillain Barre Syndrome (GBS) in adults. ZIKV infected cells secrete non-structural protein 1 (sNS1), which plays an important role in viral replication and immune evasion. The microglial cells are the brain resident macrophages that mediate the immune responses in CNS. The miRNAs are small non-coding RNAs that regulate the expression of their target genes by binding to the 3'UTR region. The present study highlights the bystander effect of ZIKV-NS1 via miR-146a. The Real-Time PCR, Immunoblotting, overexpression, knockdown studies, and reactive oxygen species measurement have been done to study the immunomodulatory effects of ZIKV-NS1 in human microglial cells. ZIKV-NS1 induced the expression of miR-146a and suppressed the ROS activity in human microglial cells. The up-regulated miR-146a led to the decreased expression of TRAF6 and STAT-1. The reduced expression of TRAF6 in turn led to the suppression of pNF-κBp65 and TNF-α downstream. The miR-146a suppressed the pro-inflammatory and cellular antiviral responses in microglial cells. Our findings demonstrate the bystander role of ZIKV-NS1 in suppressing the pro-inflammatory and cellular antiviral responses through miR-146a in human microglial cells.

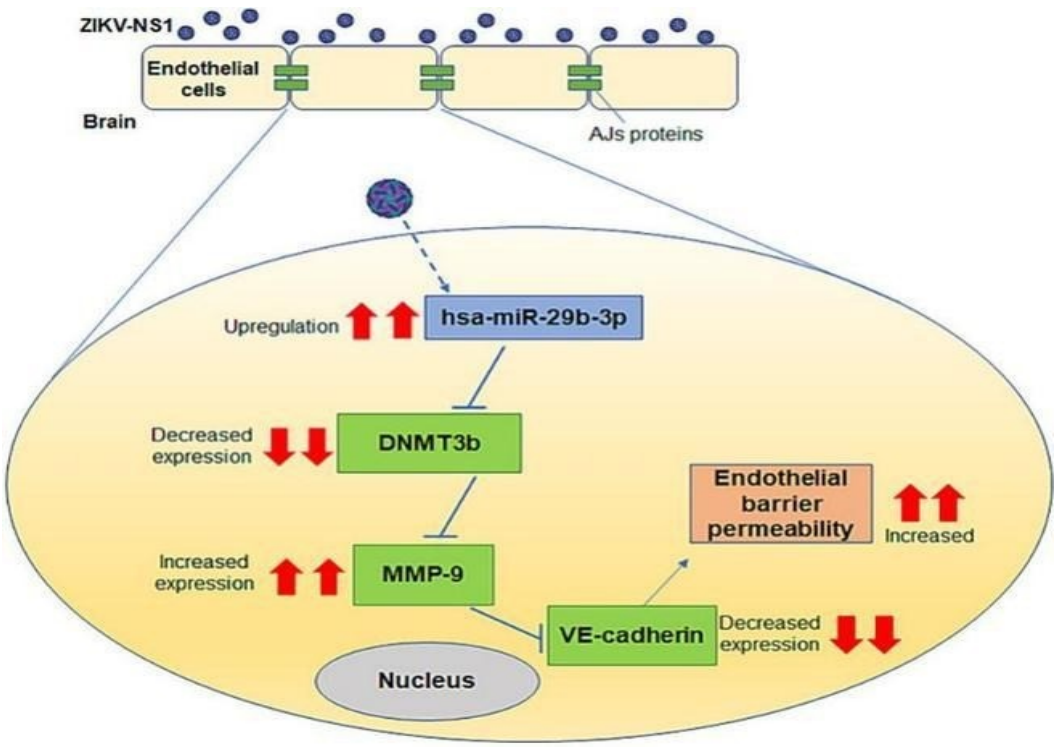


Proposed Model: ZIKV-NS1 mediated suppression of pro-inflammatory and antiviral response via miR-146a. The ZIKV-NS1 protein up-regulates the miR-146a expression in human microglial cells. The up-regulated miR-146a targets TRAF6 and STAT-1 proteins. The reduced expression of TRAF6 and STAT-1 suppresses the pro-inflammatory and antiviral response respectively.

8. Bharadwaj U, Singh SK (2023), Zika Virus NS1 suppresses VE-Cadherin via hsa-miR-29b-3p/DNMT3b/MMP-9 pathway in Human Brain Microvascular Endothelial Cells; *Cellular Signalling* DOI: 10.1016/j.cellsig.2023.110659

Zika virus infection has been reported to cause microcephaly in newborns. ZIKV exploits various strategies to cross the blood-brain barrier. ZIKV NS1 may compromise the barrier integrity of endothelial cells by regulating expression of junctional proteins. MicroRNAs play an important role in post-transcriptional gene regulations. We demonstrated that ZIKV-NS1 affected the adherence junction protein in human brain microvascular endothelial cells via hsa-miR-29b-3p/DNMT3b/MMP-9 pathway. The hCMEC/D3 cells were exposed to ZIKV-NS1 with different

doses (500 ng/mL and 1000 ng/mL) for 24 h. The expression pattern of DNTM3b, MMP-9, and VE-cadherin were studied using immunoblotting and the distribution of DNMT3b and MMP-9 were studied using immunofluorescence. The quantification of hsa-miR-29b-3p was done through qRT-PCR. Direct regulation of DNMT3b by hsa-miR-29b-3p was demonstrated by overexpression of hsa-miR-29b-3p using hsa-miR-29b-3p mimic, and knockdown of hsa-miR-29b-3p by using hsa-miR-29b-3p inhibitors. The ZIKV-NS1 affected the barrier function of endothelial cells through the increased expression of hsa-miR29b-3p, which suppressed the DNMT3b, thus enhanced expression of MMP-9, which finally suppressed the expression of VE-cadherin. These findings suggested that ZIKV-NS1 alters the expression of Adherens Junction protein in human brain microvascular endothelial cells through hsa-miR-29b-3p/DNMT3b/MMP-9 pathway, which compromised the barrier function of human brain microvascular endothelial cells.



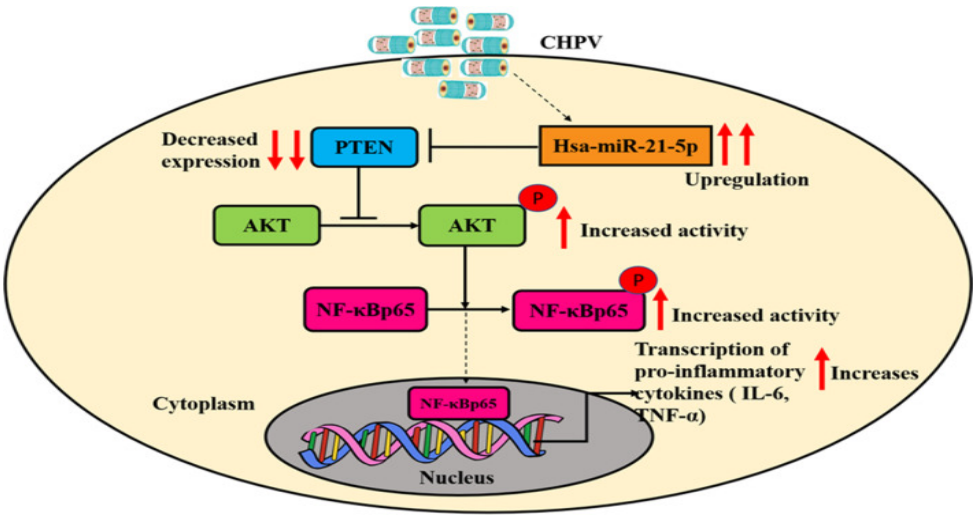
Proposed Model: ZIKV-NS1 facilitated VE-cadherin expression via hsa-miR-29b-3p/DNMT3b/MMP-9 pathway in human brain microvascular endothelial cells. The ZIKV-NS1 triggered the production hsa-miR-29b-3p in human brain microvascular endothelial cells. The hsa-miR-29b-3p negatively affected the DNMT3b, which in turn inhibited the MMP-9. As a result, increased MMP-9 expression suppressed VE-cadherin expression and compromised the endothelial barrier integrity.

Publications related to Chandipura Virus (CHPV) Encephalitis

9. Pandey N, Rastogi M, Singh SK (2021), Chandipura virus dysregulates the expression of hsa-miR-21-5p to activate NF-κB in human microglial cells. *J Biomed Sci*. doi: 10.1186/s12929-021-00748-0.

Chandipura virus (CHPV) is a negative single-stranded RNA virus of the Rhabdoviridae family. CHPV infection has been reported in Central and Western India. CHPV causes acute encephalitis with a case fatality rate of 70 % and mostly affects children below 15 years of age. CHPV infection in brain leads to neuronal apoptosis and activation of the microglial cells. The microRNAs (miRNAs) are small endogenous non-coding RNA that regulate the gene expression. Viral infections perturb the expression pattern of cellular miRNAs, which may in turn affect the expression pattern of downstream genes. This study aims to investigate hsa-miR-21-5p mediated regulation of PTEN, AKT, NF-κBp65, IL-6, TNF-α, and IL-1β, in human microglial cells during CHPV infection. The hsa-miR-21-5p was found to be upregulated during CHPV infection in human microglial cells. This led to the downregulation of PTEN which promoted the phosphorylation of AKT and NF-κBp65. Over-expression of hsa-miR-21-5p led to the decreased expression of PTEN and promoted further phosphorylation of AKT and NF-κBp65 in human microglial cells. However, the inhibition of hsa-miR-21-5p using hsa-miR-21-5p inhibitor

restored the expression. This study supports the role of hsa-miR-21-5p in the regulation of pro-inflammatory genes in CHPV infected human microglial cells.

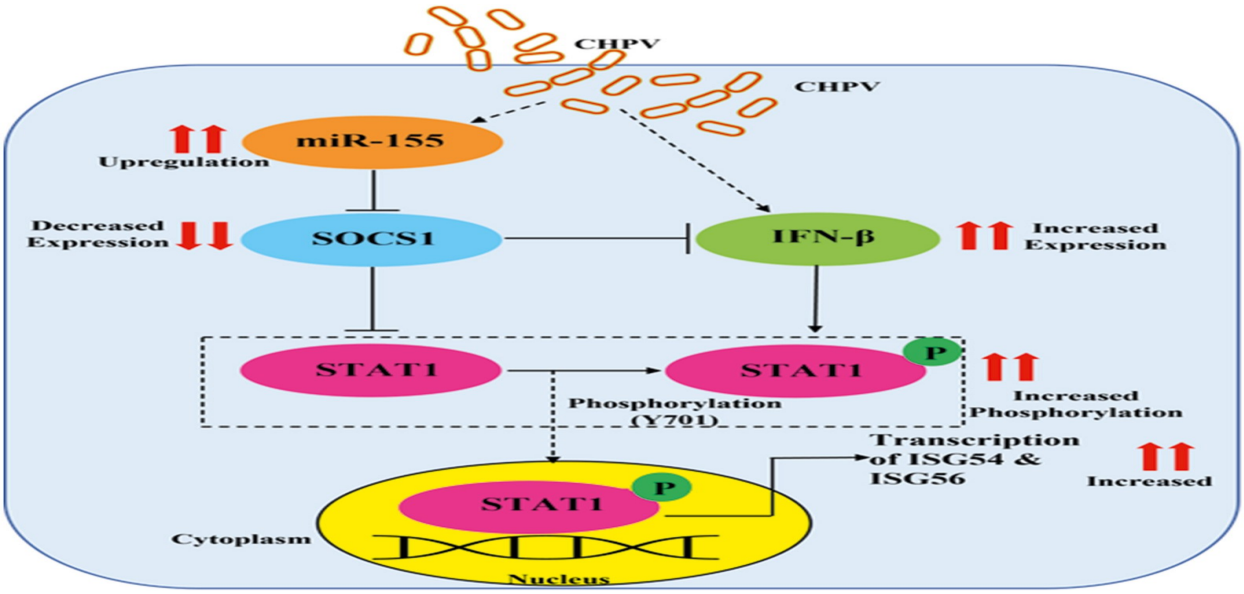


Proposed Model: The CHPV mediated regulation of inflammatory responses by perturbation of hsa-miR-21-5p in human microglial cells. CHPV infection in human microglial cells increases the expression of hsa-miR-21-5p. The microRNA targets and supresses the expression of PTEN which in turns promotes phosphorylation and activation of AKT and NF-κBp65 in human microglial cells

10. Pandey N, Singh SK (2023), MicroRNA-155 triggers a cellular antiviral immune response against Chandipura virus in human microglial cells, *Microbes and Infection*. *2023 Jun 14;105173. doi: 10.1016/j.micinf.2023.105173.*

Chandipura virus (CHPV) belongs to the family Rhabdoviridae and has a single-stranded RNA genome that causes encephalitis among children in India's tropical states. Activation of the antiviral immune response upon viral infection is important for the host's defense. In response to CHPV infection, the brain resident macrophages (microglial cells) control the pathogenic insults. The microRNAs (miRNAs) are 22 nts non-coding RNAs that serve as delicate regulators of their target genes at the post-transcriptional level. In this study, we explored miR-155 mediated antiviral

response in CHPV infected human microglial cells. The gene and protein expression patterns were studied through quantitative real-time PCR (qPCR) and immunoblotting, respectively. Additionally, miRNA target validation was done by overexpression and knockdown of miR-155. We observed an increased expression of miR-155 in CHPV infected human microglial cells. The upregulated miR-155 suppresses the Suppressor of Cytokine Signalling 1 (SOCS1). Reduced SOCS1, in turn, led to enhanced phosphorylation of Signal Transducer and Activator of Transcription 1 (STAT1) and induction of Interferon-β (IFN-β), which promoted the expression of IFN-stimulated gene 54 (ISG54) and IFN-stimulated gene 56 (ISG56). In this study, miR-155 positively modulated the cellular antiviral response by enhancing type I IFN signalling through inhibition of SOCS1 in CHPV infected microglial cells.



Proposed Model: miR-155 mediated regulation of antiviral response in CHPV infected human microglial cells. CHPV infection increases the expression of miR-155 in human microglial cells. This microRNA suppresses SOCS1 expression, which enhances the expression of IFN-β. In human microglial cells, decreased SOCS1 and elevated IFN-β promote the phosphorylation of STAT1 at Y701. This leads to the increased expression of the

interferon stimulated genes ISG54 and ISG56 and creates a strong antiviral state against CHPV in human microglial cells.