

RESEARCH ARTICLE

Proteomic analysis for Type I interferon antagonism of Japanese encephalitis virus NS5 protein

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Japanese encephalitis virus (JEV) nonstructural protein 5 (NS5) exhibits a Type I interferon (IFN) antagonistic function. This study characterizes Type I IFN antagonism mechanism of NS5 protein, using proteomic approach. In human neuroblastoma cells, NS5 expression would suppress IFN β -induced responses, for example, expression of IFN-stimulated genes PKR and OAS as well as STAT1 nuclear translocation and phosphorylation. Proteomic analysis showed JEV NS5 downregulating calreticulin, while upregulating cyclophilin A, HSP 60 and stress-induced-phosphoprotein 1. Gene silence of calreticulin raised intracellular Ca²⁺ levels while inhibiting nuclear translocation of STAT1 and NFAT-1 in response to IFN β , thus, indicating calreticulin downregulation linked with Type I IFN antagonism of JEV NS5 via activation of Ca²⁺/calcineurin. Calcineurin inhibitor cyclosporin A attenuated NS5-mediated inhibition of IFN β -induced responses, for example, IFN-sensitive response element driven luciferase, STAT1-dependent PKR mRNA expression, as well as phosphorylation and nuclear translocation of STAT1. Transfection with calcineurin (vs. control) siRNA enhanced nuclear translocation of STAT1 and upregulated PKR expression in NS5-expressing cells in response to IFN β . Results prove Ca²⁺, calreticulin, and calcineurin involvement in STAT1-mediated signaling as well as a key role of JEV NS5 in Type I IFN antagonism. This study offers insights into the molecular mechanism of Type I interferon antagonism by JEV NS5.

Received: January 2, 2013

Revised: September 8, 2013

Accepted: October 11, 2013

Keywords:

Calreticulin / Interferon / Japanese encephalitis virus / Microbiology / Non-structural protein 5 / STAT1



Additional supporting information may be found in the online version of this article at the publisher's web-site

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Abbreviations: CsA, cyclosporine A; DEN, dengue; IFN, interferon; JEV, Japanese encephalitis virus; MEM, minimum essential medium; NS5, nonstructural protein 5; Q-TOF, quadruple-TOF; TBEV, tick-borne encephalitis; WNV, West Nile; YF, yellow fever

1 Introduction

Japanese encephalitis virus (JEV) is a member of the *Flaviviridae* family comprising important human pathogens: dengue (DEN), yellow fever (YF), St. Louis encephalitis, West Nile (WNV), and tick-borne encephalitis (TBEV) virus [1, 2]. JEV

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infection involves the nervous system, for example, basal ganglia, brainstem, cerebellum, cerebral cortex, spinal cord, and thalamus [3–5]. JEV causes severe CNS disease—for example, poliomyelitis-like acute flaccid paralysis, aseptic meningitis, encephalitis [6]—with 30% fatality rate. Around half the survivors show severe neurological sequelae [6]. About 50 000 Japanese encephalitis cases with 10 000 deaths occur annually in East and Southeast Asia, along with northern Australia [1, 7].

The single, long ORF of JEV genome encodes structural proteins capsid (C), membrane (prM/M), and envelope (E) plus seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5) [2]. Mature viral proteins arise from proteolytic processing of this single polyprotein by viral NS2B-NS3 and variant cellular proteases [2]. NS5, multifunctional protein containing RNA-dependent RNA polymerase and methyltransferase activity, is involved in viral RNA replication [8]. Short hairpin RNA targeting JEV NS5 gene reduces levels of viral RNA genome and proteins, specifically and efficiently inhibiting JEV replication [9]. JEV NS5 protein suppresses STAT1 nuclear translocation and tyrosine phosphorylation of Tyk2 and SATA1 that arise in response to IFN by blocking IFN-stimulated (where IFN is interferon) JAK-STAT signaling [10]. WNV NS5 protein prevents phosphorylated STAT1 accumulation and blocks IFN-dependent gene expression, then rescues growth of a highly IFN-sensitive virus in the presence of IFN treatment [11]. DEN NS5 inhibits IFN- α , not IFN- γ , signaling via binding with and blocking phosphorylation of STAT2 [12, 13]. TBEV NS5 inhibits STAT1 phosphorylation in response to IFN via binding interaction with post synaptic density protein-95/drosophila disc large tumor suppressor-1/zonula occludens-1 protein scribble (hScrib) affecting IFN Type I and II mediated JAK-STAT signaling [8, 14, 15]. Studies demonstrate flavivirus NS5 proteins as potent IFN antagonists of variant mechanisms.

Clinical, neurophysiological, and radiological examination of Japanese encephalitis patients has indicated the presence of JEV antigens and genomes in the basal ganglia, brainstem, cerebellum, cerebral cortex, spinal cord, and thalamus [3–5]. TE671 neural cells derived from medulloblastoma serve to rate pathogenesis of the measles virus [16, 17]. JEV NS2B-NS3 protease demonstrably induces TE671 human medulloblastoma apoptosis [18, 19]. IFN-stimulated gene 15 (ISG15) overexpression in TE671 cells caused phosphorylation of IRF-3 (Ser396), JAK2 (Tyr1007/1008), and STAT1 (Tyr701 and Ser727), along with activation of STAT1-dependent genes, such as IRF-3, IFN- β , IL-8, PKR and OAS, correlating with inhibited JEV replication [20]. This study uses proteomic analysis of TE671 cell expressing JEV NS5 to identify unique protein profiling involved in IFN antagonism of JEV NS5 protein. Specific inhibitors of calreticulin/calnexin pathway show reversal of STAT1-mediated Type I IFN-induced response in NS5-expressing cells, correlating with inhibitory effects on JEV replication in cells and virus-induced apoptosis.

2 Materials and methods

2.1 Viruses and cells

This study used JEV strain T1P1, as described earlier [21]. BHK-21 cells used for plaque assays were maintained in the minimum essential medium (MEM) with 10% FBS. Human TE671 medulloblastoma cells for transfection with empty vector and NS5-expressing vector were grown in MEM with 2 mM L-glutamine, 1 mM sodium pyruvate, and 10% FBS.

2.2 Expression of recombinant JEV NS5 protein and immunofluorescent staining

JEV NS5 Flag-tagged pCR3.1 and its empty control vectors were supplied by Dr. Yi-Ling Lin (Genomics Research Center, Academia Sinica, Taiwan), as described in a prior report [21]. TE671 cells at 60–90% confluency in 6-well plates were transfected with a mixture of GenePorter reagent and recombinant vector containing NS5 gene or empty vector for 5 h, then maintained in 2 mL of MEM containing 20% bovine serum for 2 days, as described previously [22]. Transfected cell line was selected with a long-term incubation of MEM containing 10% FBS and 800 $\mu\text{g}/\text{mL}$ of G418. To detect NS5 expression in TE671 cells by immunofluorescent assay, transfected cells were fixed with cold methanol, incubated with diluted mouse anti-Flag tag mAb for 2 h, followed by FITC-conjugated anti-mouse IgG antibodies for 2 h. Cells were subsequently stained with DAPI (Sigma) for 10 min. After three washings in PBS, photographs of cells were taken by immunofluorescent microscopy. To analyze subcellular localization of STAT1 and NFAT-1, transfected cells were stained with primary antibodies anti-STAT1 or NFAT-1 (Cell Signaling), followed by incubation with secondary antibodies FITC-conjugated anti-mouse IgG plus DAPI, as described above. To quantify nuclear translocalization of STAT1 and NFAT-1, green fluorescence intensity of 100 random cells and their nuclei in images were measured by Image Pro 6 software (Media Cybernetics), results expressed as fluorescence intensity ratio of nuclear area to cell area in counted cells.

2.3 Western blot

To assess NS5 expression, lysates of NS5-expressing and vector control cell lines were resolved by SDS-PAGE and transferred to NC, as we previously reported [23]. Resulting blots were blocked with 5% skim milk and reacted with anti-Flag tag mAb as probe; immune complexes were detected with HRP-conjugated goat anti-mouse IgG antibodies, followed by ECL detection (Amersham Pharmacia Biotech). To rate protein profiling in response to IFN β , both NS5-expressing and vector control cells were harvested postincubation with

or without 1000 U/mL IFN β for 2 days prior to Western blot with properly diluted antibodies: mouse anti-Flag tag, anti-cyclophilin A (Cell Signaling), anti-calreticulin, and anti- β -actin antibodies (Invitrogen). For analyzing signal transduction in response to IFN β and cyclosporin A (CsA), both NS5-expressing and vector control cells were harvested postincubation with or without 1000 U/mL IFN β and 10 μ g/mL CsA for 30, 60, and 120 min prior to Western blot with rabbit anti-STAT1 and antiphospho STAT1 (Tyr701) antibodies (Cell Signaling).

2.4 Transient transfection, dual-luciferase reporter assay, and RNA interference

For IFN signaling pathway assay, dual-luciferase reporter system with *cis*-reporter plasmid pISRE-Luc containing IFN-sensitive response element (ISRE) and internal control reporter pRLuc-C1 was carried out, as we reported earlier [23]. JEV NS5-expressing and empty vector control cells were transfected with dual *cis*-reporter plasmids using GenePorter reagent, then treated with or without 1000 U/mL IFN β (Merck-Serono). After 4-h incubation, activity of firefly and Renilla luciferase was measured by dual Luciferase Reporter Assay System (Promega) and TROPIX TR-717 Luminometer (Applied Biosystems). For silencing calreticulin and calcineurin, NS5-expressing and vector control cells were transfected with control siRNA, ON-TARGETplus Human CALR (811) siRNA (SMARTpool L-008197-00-0005), or ON-TARGETplus Human PPP3CA (5530) siRNA (SMARTpool L-008300-00-0005). After 2-day incubation, cells underwent further experiments, for example, mRNA expression of ISGs by real-time PCR and subcellular localization of STAT1 and NFAT-1.

2.5 Quantification of mRNA levels of IFN β -induced genes, using real time RT-PCR

NS5-expressing and vector control cells were transfected with control, calreticulin, and calcineurin siRNA, then treated with 1000 U/mL IFN β or 10 μ g/mL CsA for 4-h incubation. Total RNAs from lysate of each experiment were isolated by a PureLink Micro-to-Midi Total RNA Purification System Kit (Invitrogen), 1000 ng of total RNA for cDNA synthesis using oligo dT primer and SuperScript III reverse transcriptase kit (Invitrogen). Two-step RT-PCR with SYBR Green I gauged mRNA levels of IFN β -induced genes. Primer pairs included 5'-CAACCAGCGGTTGACT TTTT-3' and 5'-ATCCAGGAAGGCAAAGTAA-3' for PKR, 5'-GATGTG GTTAG GTTTATAGCTG-3' and 5'-TTGGG GGTTAGGTTTT CTGCC TTT-3' for OAS, 5'-CT GCTGCGGCCCTTGTTA TT-3' and 5'-CATGGGCTGGACCTGACGGTGA AG-3' for ISG15, 5'-CCTGCCACCCAAGAAGATAAAG-3' and 5'-GTT CCCACTCTCCGTCCATC-3' for calreticulin, 5'-GATGCT GGTAATGTC CTCAAC-3' and 5'-CACACTCTCACTCT CTCTCTG-3' for calcineurin, 5'-TGCT GCCTCCAAGAA

CACAA-3' and 5'-TGTAGAACTGCCGG AGCACA-3' for IL-4, and 5'-AGCCACATCGCTCAGACAC-3' and 5'-GCCCA ATACGACCAA ATCC-3' for housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Real-time PCR reaction contained 2.5 μ L of cDNA (reverse transcription mixture), 200 nM of each primer in SYBR Green I master mix (LightCycler TaqMan Master, Roche Diagnostics); it was performed by amplification protocol consisting of 1 cycle at 50°C for 2 min, 1 cycle at 95°C for 15 s and 60°C for 1 min. PCR product was quantified by ABI PRISM 7700 sequence detection system (PE Applied Biosystems), relative fold changes of PKR and OAS mRNA normalized by GAPDH mRNA.

2.6 2DE, in-gel digestion, and nanoelectrospray MS

2DE, protein spot analysis, in-gel digestion, nanoelectrospray MS, data interpretation, and database search/gel electrophoresis were performed as we previously reported [24]. Briefly, vector control and NS5-expressing cells with or without 2-day incubation of 1000 U/mL IFN β were harvested and dissolved in lysis buffer containing 8 M urea, 4% CHAPS, 2% pH 3–10 nonlinear IPG buffer (GE Healthcare), and Complete, Mini, EDTA-free protease inhibitor mixture (Roche). A 100 μ g of protein sample of each lysate in rehydration buffer (8 M urea, 2% CHAPS, 0.5% IPG buffer pH 3–10 nonlinear, 18 mM dithiothreitol, 0.002% bromophenol blue) was applied to nonlinear Immobiline DryStrips (17 cm, pH 3–10; GE Healthcare). After IEF, gel strips were incubated for 30 min in equilibration solution I (6 M urea, 2% SDS, 30% glycerol, 1% DTT, 0.002% bromophenol blue, 50 mM Tris-HCl, pH 8.8), then for 30 min in equilibration solution II (6 M urea, 2% SDS, 30% glycerol, 2.5% iodoacetamide, 0.002% bromophenol blue, 50 mM Tris-HCl, pH 8.8). Gel strips were subsequently transferred to run 12% PAGE. Gels were fixed in 40% ethanol and 10% glacial acetic acid for 30 min, stained with AgNO₃ solution for 20 min, and finally scanned by a GS-800 imaging densitometer with PDQuest software version 7.1.1 (Bio-Rad). Data from three independent lysates under each experimental condition were used to correct spot intensity graphs and statistical analysis (Microsoft Excel). In silver-stained gels, each spot of interest showing statistically significant 1.5-fold rise or fall in spot intensity was sliced, put into the microtube, and washed twice with 50% ACN in 100 mM ammonium bicarbonate buffer (pH 8.0) for 10 min at room temperature. In-gel digestion method described previously [24] served to recover peptides from gel spots for nanoelectrospray MS. Proteins in spots of interest were identified by Ultimate Capillary LC system (LC Packings, Amsterdam) along with QSTARXL quadrupole-TOF (Q-TOF) mass spectrometer (Applied Biosystem/MDS Sciex, Foster City, CA). Nanoelectrospray MS and database search were described earlier [25]. Protein function and subcellular location were annotated using Swiss-Prot (<http://us.expasy.org/sprot/>), proteins categorized according to biological process and pathway via PANTHER classification (<http://www.pantherdb.org>) described previously [24].

2.7 Detecting intracellular Ca²⁺ and subcellular localization of STAT1 and NFAT-1 in siRNA transfected cells

Vector control and NS5-expressing cells were transfected with control or calreticulin siRNA (SMARTpool L-008197-00-0005, Thermo Scientific) in FBS-free media overnight, harvested, washed twice with PBS, then incubated with 10 mg/mL FLUO3/AM (Sigma) at 37°C for 30 min in darkroom. After last wash with PBS, cells were analyzed by flow cytometry (excitation at 506 nm and emission at 526 nm; Becton Dickinson FACS Calibur). For subcellular localization assay, siRNA transfected cells were treated with or without 1000 U/mL IFN β for 4 h, then tested by immunofluorescent staining with rabbit anti-STAT1 or anti-NFAT-1 antibodies (Cell Signaling) described above.

2.8 Effects of calcineurin inhibitor and siRNA on IFN β -induced, STAT1-mediated responses

Since IFN β induced apoptosis of TE671 cells, vector control and NS5-expressing cells were used for test their susceptibility to IFN β , then treated singly or with both 10 μ g/mL CsA (calcineurin inhibitor) and 1000 U/mL IFN β for 48 h. IFN β -induced cytopathic effect of both cell types was photographed by light microscopy. Both types were transfected with control and calcineurin siRNA (SMARTpool L-008300-00-0005, Thermo Scientific). Effect of CsA and calcineurin siRNA on cells in response to IFN β was analyzed by assay for ISRE-driven promoter activity, ISGs gene expression, phosphorylation, and subcellular location of STAT1, performed as described above.

2.9 Extracellular virus yield reduction assays

TE671 cells were infected with JEV at an multiplicity of infection (MOI) of 0.5 in the presence or absence of 500 U/mL IFN β and/or 1000 ng/mL CsA. At 48 h post infection, cultured supernatants were harvested to calculate virus yield. BHK-21 cell monolayers were incubated with serial dilution of cultured supernatant at 37°C for 1 h and overlaid with MEM containing 1.1% methylcellulose. After 72 h incubation, monolayers were stained with naphthol blue-black dye and viral plaques counted.

3 Results

3.1 IFN β antagonistic ability of JEV NS5 in TE 671 human medulloblastoma cells

Since JEV NS5 protein showed Type I IFN antagonistic ability in BHK-21 cells [21], we further characterized JEV NS5

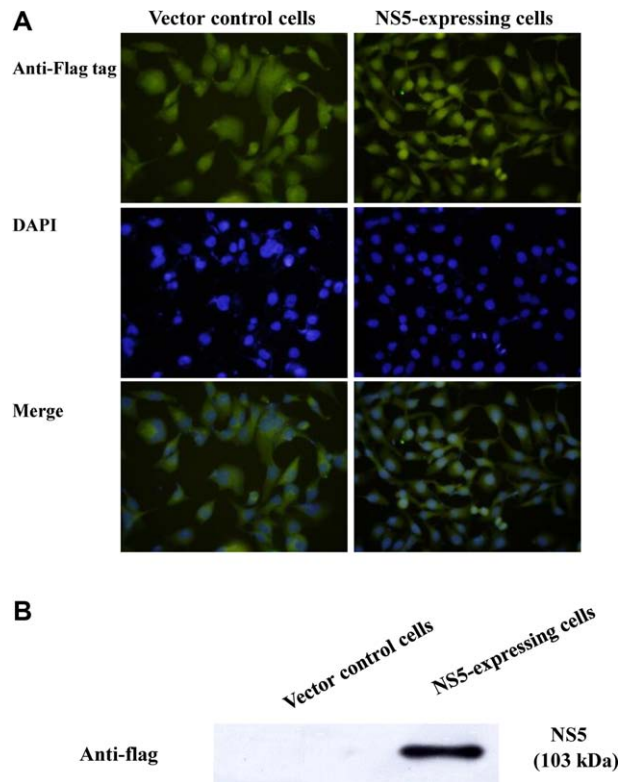


Figure 1. Expression of JEV NS5 protein in TE671 human medulloblastoma cells. Cells transfected with pCR3.1-Flag (control vector; left) or pCR3.1-JEV NS5-Flag (right) were selected by 2-wk incubation with G418. Expression of Flag-epitope tagged protein was examined by fluorescence microscopy (A). Also, Western blot to detect expressed NS5 was conducted via anti-Flag antibody (B). Lysates from cells transfected with pCR3.1-Flag (lane 1) or pCR3.1-JEV NS5-Flag (lane 2) were analyzed by 10% SDS-PAGE prior to blotting, resulting blots probed with anti-Flag tag antibody.

inhibitory effect on IFN β -induced response in TE671 human medulloblastoma cells. Recombinant plasmid pCR3.1-JEV NS5 (expressing JEV NS5 with Flag tags) or empty control vector (expressing Flag tags), generated as the prior report [21], were first transfected into TE671 cells, JEV NS5 expression detected by immunofluorescent staining (Fig. 1A) and Western blot (Fig. 1B). Immunofluorescent staining with anti-Flag antibodies and DAPI found vector-derived Flag-tag in both empty vector- and pCR3.1-JEV NS5-transfected cells (Fig. 1A). In particular, most vector-derived Flag-tag fusion proteins in pCR3.1-JEV NS5-transfected cells were located in nucleus, consistent with prior reports in which NS5 protein of DEN-2, YF and JEV was detected in nuclei of mammalian cells [26–28]. Western blot of lysates with anti-Flag-tag antibodies revealed 103-kDa band in pCR3.1-JEV NS5-transfected (Fig. 1B) but not in empty vector-transfected cells, proving JEV NS5 stably expressed in human medulloblastoma cells.

Antagonistic ability of JEV NS5 on IFN β -induced ISRE-driven promoter activity was analyzed by dual luciferase

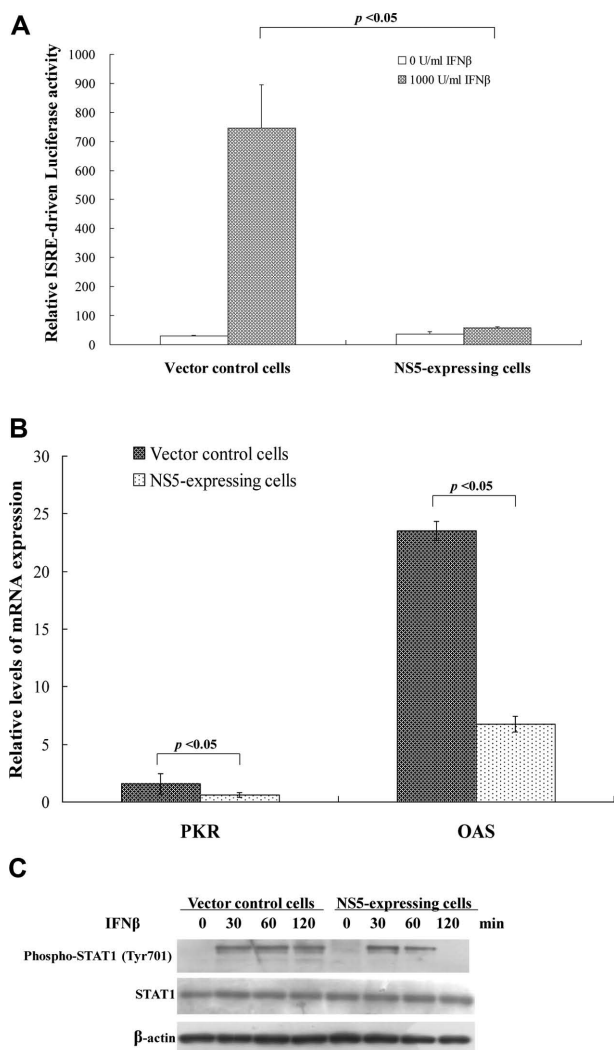


Figure 2. Inhibitory effects of JEV NS5 protein on IFN β -induced responses. (A) To analyze ISRE promoter activity, vector control and NS5-expressing cells were transiently cotransfected with reporter plasmid containing firefly luciferase under control of ISRE and internal control reporter pRLuc-C1. After 4-h IFN β treatment, firefly luciferase and renilla luciferase were measured; firefly luciferase activity normalized to renilla luciferase activity is reported. (B) To analyze ISRE-driven gene expression, vector control and NS5-expressing cells were treated with or without 1000 U/mL IFN β for 8 h; relative levels of PKR and OAS mRNAs were gauged by quantitative real-time PCR, relative fold levels of PKR or OAS mRNA presented as ratio of PKR or OAS mRNA/GAPDH mRNA. (C) To analyze STAT1 phosphorylation, Western blot of lysates from cells treated with IFN β for 0, 30, 60, or 120 min was performed by anti-phospho-STAT1 (Tyr701) and anti- β actin antibody as internal control.

reporter assay. IFN β induced significant increase of ISRE-driven firefly luciferase activity in vector control cells, 20 times higher than in NS5-expressing cells (Fig. 2A). Meanwhile, quantitative real-time RT-PCR assays indicated induction of ISRE-driven genes PKR and OAS by IFN β as ap-

proximately 2.5- and 3.5-fold lower in NS5-expressing cells than in vector controls, respectively (Fig. 2B). Western blot with antiphospho-Tyr701 STAT1 antibodies also indicated JEV NS5, reducing IFN β -induced phosphorylation of STAT1 at Tyr701 (Fig. 2C, lanes 6–8). Results demonstrated JEV NS5 inhibiting IFN β -induced responses as well as showing its Type 1 IFN antagonistic activity in human medulloblastoma cells. Results concurred with a previous finding: NS5 blocked STAT1 nuclear translocation and tyrosine phosphorylation in BHK21 cells treated with Type I IFN [21].

3.2 Proteomic analysis of IFN β antagonism by JEV NS5

To identify key proteins inhibiting IFN β -induced STAT1-mediated signaling, protein expression in vector control and NS5-expressing cells with or without IFN β was analyzed by 2D electrophoresis and nanoscale capillary LC/ESI Q-TOF MS to differentiate regulated protein spots (Supporting Information Fig. 1). Table 1 shows unique protein profile of NS5-expressing cells compared to vector controls. JEV NS5 expression upregulated inosine-5'-monophosphate dehydrogenase 2, 60 kDa HSP (Hsp60), heterogeneous nuclear ribonucleoprotein D0, HSP 27 (Hsp27), stress-induced phosphoprotein 1 (STIP1), peroxiredoxin-1, and cyclophilin A (Table 1); it downregulated D-3-phosphoglycerate dehydrogenase, heterogeneous nuclear ribonucleoprotein H3, T-complex protein 1 subunit beta, thioredoxin, prohibitin, and calreticulin in human medulloblastoma cells (Table 1).

To confirm expression profile of vector control and NS5-expressing cells treated with/without IFN β , spots in 2D gels, such as thioredoxin, cyclophilin A, and calreticulin, were enlarged, identified using MS/MS analysis and quantified relative expression by Western blotting and real-time PCR (Fig. 3A to E). Two peptide peaks of thioredoxin (Spot ID 10) with a MASCOT score of 86 and sequence coverage of 19% in MS analysis were sequenced by Q-TOF MS analysis and identified to match within thioredoxin residues 86–94 and 98–105 (Fig. 3B and C). Lysates of both cells treated with varied IFN β concentrations were analyzed by Western blot with monoclonal antibodies against cyclophilin A and calreticulin (Fig. 3D), indicating IFN β treatment increased 18-kDa immuno-reactive bands as cyclophilin A in NS5-expressing, but not vector control cells. On the other hand, IFN β treatment slightly reduced protein levels of calreticulin in NS5-expressing compared to vector control cells. Relative calreticulin mRNA levels in both cells were measured by real-time PCR (Fig. 3E). NS5-expressing cells had nearly 40% decrease of calreticulin mRNA compared to vector controls. Results confirmed data of 2D/MS.

Functional classification of these regulatory proteins by PANTHER system indicated upregulated proteins as involved in catalytic (IMPDH2, CYPA, PRDX1), isomerase (CYPA), peroxidase (PRDX1), RNA-binding (HNRNPD), and antioxidant (PRDX1) activities; downregulated proteins exhibited

Table 1. Up- and down-regulated proteins in NS5-expressing cells compared to vector control cells

Spot ID	PANTHER gene ID	Protein identification	MASCOT score	MW/pI	Numbers of peptides identified	Sequence coverage (%)	Molecular function	Biological process	Fold change (±SD)
Upregulated proteins in NS5-expressing cells compared to vector control cells									
1	3615	Inosine-5'-monophosphate dehydrogenase 2 (IMPDH 2)	451	55.8/6.44	11	35	Oxidoreductase	Purine metabolism	4.16 ± 0.27↑
4	3329	60 kDa HSP (Hsp60)	1145	61/5.7	15	52		Protein folding, protein complex assembly	1.71 ± 0.01↑
5	3184	Heterogeneous nuclear ribonucleoprotein D0 (hnRNP D0)	96	38.4/7.62	2	6	RNA splicing factor, transesterification, structural constituent of ribosome poly(A) RNA binding	mRNA splicing	3.42 ± 0.05↑
6	3315	HSP beta-1 (HSPB1, Hsp27-1)	458	22.8/5.98	5	66	Structural molecule	Protein folding, stress response	1.60 ± 0.32↑
8	5052	Peroxiredoxin-1 (PRDX1)	192	22.1/8.27	4	13	Oxidoreductase peroxidase	Antioxidation and free radical removal	3.28 ± 0.18↑
9	10963	Stress-induced-phosphoprotein 1 (STIP1)	70	62.6/6.40	6	11		Stress response	2.08 ± 0.11↑
12	5478	Cyclophilin A (CYPA)	119	18.09/7.82	11	68	Isomerase	Protein folding, nuclear transport, immunity and defense	1.72 ± 0.08↑
Downregulated proteins in NS5-expressing cells compared to vector control cells									
2	26227	D-3-phosphoglycerate dehydrogenase (3-PGDH)	424	56.6/6.29	13	33	Oxidoreductase	Amino acid biosynthesis	0.01 ± 0.04↓
3	3189	Heterogeneous nuclear ribonucleoprotein H3 (HNRPH3)	94	36.9/6.37	3	13	Structural constituent of ribosome, nucleic acid binding	mRNA splicing	0.12 ± 0.09↓
7	10576	T-complex protein 1 subunit beta (TCP-1-beta)	129	57.5/6.01	7	20		Chaperonin	0.60 ± 0.06↓
10	7295	Thioredoxin (MTRX)	86	11.7/4.82	2	19	Oxidoreductase	Electron transport, sulfur redox metabolism, other intracellular signaling cascade, stress response, apoptosis, cell proliferation and differentiation	0.27 ± 0.11↓
11	5245	Prohibitin (PHB)	72	29.8/5.57	7	37		DNA replication, cell cycle control, cell proliferation and differentiation	0.12 ± 0.07↓
13	811	Calreticulin precursor (CALR)	101	63.89/4.3	8	24	Calcium ion binding	Protein folding	0.51 ± 0.12↓

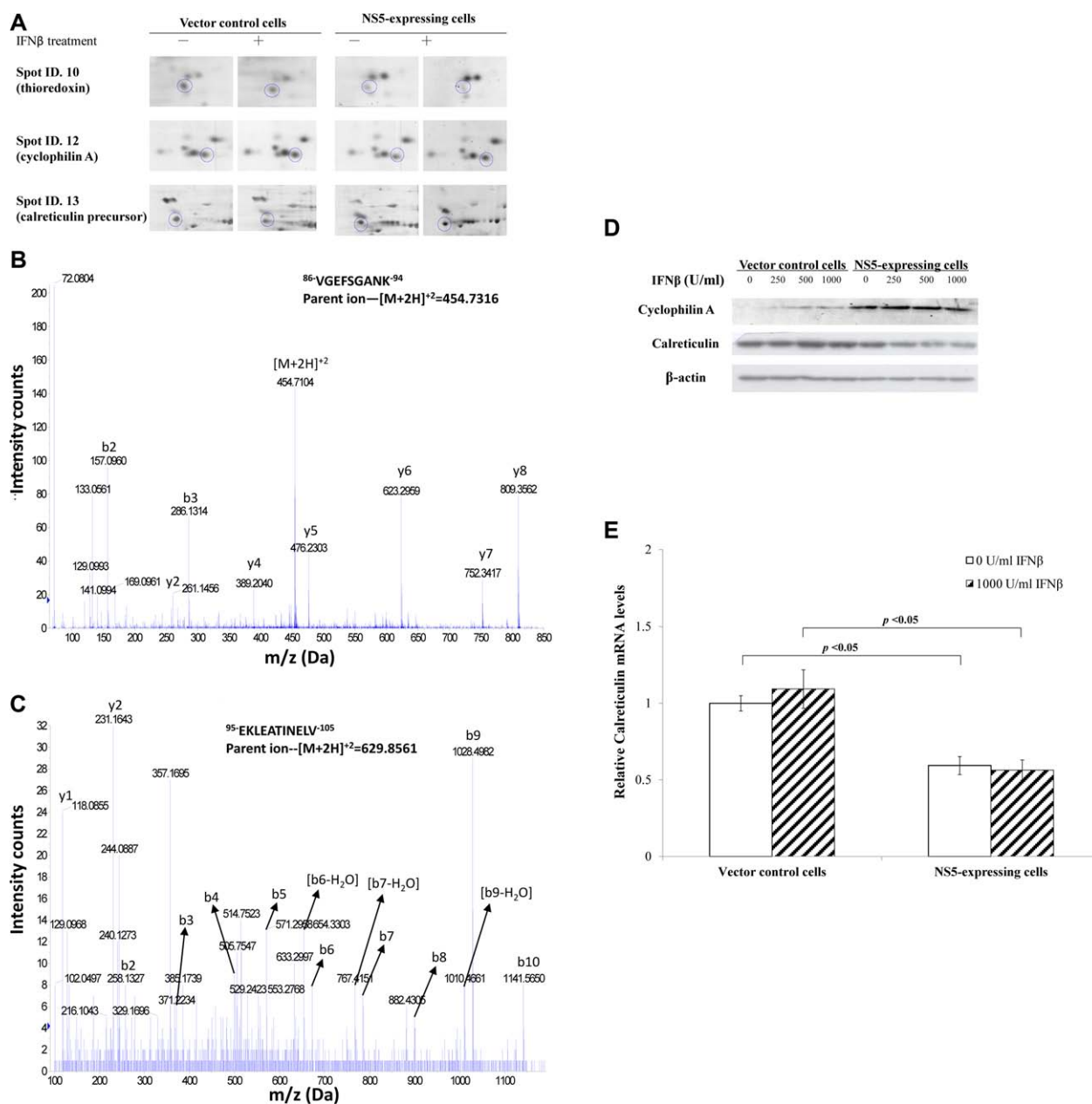


Figure 3. Western blot and MS/MS analysis of protein profiling in NS5-expressing and vector control cells in response to IFN β . (A) Enlarged images of cyclophilin A and calreticulin protein spots in 2D gels from vector control and NS5-expressing cells in response to IFN β treatment. (B, C) Nano-electrospray mass spectrum of two charged ion peaks for thioredoxin (spot ID 10) was shown. (D) To analyze cyclophilin A and calreticulin protein levels, vector control and NS5-expressing cells in the presence or absence of IFN β were harvested for Western blot with anti-cyclophilin A, anti-calreticulin, and anti- β -actin antibodies. (E) To quantify relative calreticulin mRNA levels, total RNA extracts from both cells were analyzed by quantitative PCR and normalized by GAPDH mRNA, presented as relative ratio.

calcium ion binding activities (CALR), catalytic (MTRX, 3-PGDH), and structural molecule (HNRNPH3; Table 1). Since cyclophilin A forms the complex with calcineurin and calreticulin is an ER Ca^{2+} storage protein [29], cyclophilin A upregulation and calreticulin downregulation could link with increase of intracellular Ca^{2+} and acti-

vation of calcineurin-dependent pathway. Moreover, calcineurin is a Ca^{2+} /calmodulin-dependent serine/threonine phosphatase that desphosphorylates NFAT1, STAT3, and STAT6 in innate immune response [30–32]. Therefore, calreticulin/ Ca^{2+} /calcineurin pathway might be involved in Type I IFN antagonism by JEV NS5.

To test functional role of calreticulin in Type I IFN antagonism of JEV NS5 protein, calreticulin in vector control and NS5-expressing cells was silenced using siRNAs (Fig. 4A to E). Intracellular Ca^{2+} levels in cells transfected with both control and calreticulin siRNA were stained with FLUO3/AM, then analyzed by flow cytometry (Fig. 4A). Intracellular Ca^{2+} levels were 4.9-fold higher in NS5-expressing than vector control cells posttransit transfection with control siRNA. Importantly, calreticulin silence by siRNA caused sharp (1.8- and 5.5-fold) increase of Ca^{2+} levels in NS5-expressing and vector control cells, respectively. Immunofluorescent analysis indicated IFN β treatment increasing expression and nuclear localization of STAT1 in vector controls transfected with control siRNA; calreticulin silence decreased STAT1 in nuclei of vector controls (Fig. 4B). By contrast, NS5 expressing cells with or without calreticulin silence showed no significant change of expression and subcellular localization of STAT1 in response to IFN β (Fig. 4C). To elucidate effect of calreticulin silence on calcineurin-NFAT signaling, NFAT-1 nuclear localization in both types of cells was analyzed by immunofluorescent staining with anti-NFAT-1 antibodies (Fig. 4D and E). IFN β treatment induced nuclear translocation of NFAT-1 in vector controls; calreticulin silence definitely reduced NFAT-1 nuclear localization in response to IFN β , indicating downregulation and gene silence of calreticulin linked with increase of intracellular Ca^{2+} and reduction of STAT1 and NFAT-1 nuclear localization, as well as activating Ca^{2+} -calcineurin-NFAT signaling.

3.3 Attenuating IFN β antagonistic ability of JEV NS5 by calcineurin inhibitor CsA

To test the role of Ca^{2+} -dependent calcineurin in Type I IFN antagonism of JEV NS5, CsA (calcineurin inhibitor) and IFN β -induced response in each type of cell were correlated (Fig. 5); IFN β treatment caused death of vector control, but not NS5-expressing cells (Fig. 5A). Combined treatment of IFN β and CsA induced death of both cell types, implying that calcineurin inhibition by CsA attenuates IFN β antagonistic activity of JEV NS5 protein. In dual-luciferase reporter system, single CsA treatment activated ISRE-driven promoter in NS5-expressing cells, not vector controls (Fig. 5B). Combined treatment of CsA and IFN β induced higher ISRE-driven promoter activity than single CsA or IFN β treatment in NS5-expressing cells (Fig. 5B). Single CsA treatment had no effect on ISRE-driven promoter activity in vector controls; combined treatment of CsA and IFN β showed less ISRE-driven promoter activity than single IFN β treatment in vector controls.

Transcriptional factor IRF-3 bonded with ISRE of ISG15 promoter, then directly induced ISG15 mRNA expression, while STAT1 was involved in PKR gene regulation [33,34]. To analyze association of IRF-3 and STAT1 with CsA effect on activation of ISRE-driven promoter, relative mRNA fold change of IRF-3 dependent gene ISG15 and STAT1 dependent gene

PKR was quantified by real-time PCR. Single IFN β treatment induced 20.7-fold upregulation of PKR mRNA in vector controls, only 4.7-fold increase in NS5-expressing cells. Combined treatment of CsA and IFN β had a similar level of PKR mRNA in vector control cells; PKR mRNA expression rose by 9.1-fold in NS5-expressing cells (Fig. 5C). Relative PKR mRNA fold change indicated similar pattern as ISRE-driven promoter activity in response to single or combined treatment (Fig. 5B and C). Single IFN β treatment raised mRNA levels of ISG15 in NS5-expressing cells more than in vector controls; combined treatment with CsA and IFN β upregulated ISG15 mRNA expression, similar to single IFN β treatment (Fig. 5D). Results suggest calcineurin inhibitor CsA reducing Type I IFN antagonism of JEV NS5 via STAT1-dependent signal pathways.

3.4 Influence of calcineurin inhibitor CsA on NS5-suppressed phosphorylation and nuclear translocation of STAT1 in response to Type I IFN

Combined treatment with CsA and IFN β reduced Type I IFN antagonistic activity of JEV NS5; we hypothesized calcineurin involvement in NS5-induced IFN β antagonism on STAT1-mediated signaling pathway. Tyrosine phosphorylation and nuclear translocation of STAT1 were subsequently examined in vector control and NS5-expressing cells with or without single or double CsA and IFN β treatment (Fig. 6). Single treatment of IFN β yielded marked increase of STAT1 phosphorylation at Tyr701 in vector controls (Fig. 6A, lanes 2–4); tyrosine phosphorylated STAT1 in NS5-expressing cells was lower than those in vector controls 30-, 60-, and 120-min postsingle treatment of IFN β (Fig. 6A, lanes 10–12). Combined treatment with CsA and IFN β elevated phosphorylation level of STAT1 at Tyr701 in NS5-expressing cells compared to single treatment (Fig. 6A, lanes 14–16 vs. 10–12). Importantly, confocal imaging analysis with anti-STAT1 antibodies revealed combined treatment of CsA and IFN β enhancing IFN β -induced STAT1 nuclear translocation in NS5-expressing cells (Fig. 6B and C), supporting elevation of STAT1 phosphorylation at Tyr701 in NS5-expressing cells by combined IFN β and CsA treatment. Meanwhile, CsA alone induced a slight amount of STAT1 translocation to nuclei in both cell types, implying calreticulin downregulation activating Ca^{2+} /calcineurin signals, associated with inhibition of STAT1-mediated signaling in NS5-induced IFN β antagonism.

3.5 Enhancing IFN β -induced response by siRNA-mediated silence of calcineurin

Besides calcineurin inhibitor CsA, siRNA-mediated gene silence was performed to affirm the hypothesis, with calcineurin activation linked to suppression of STAT1-mediated signaling, as responsible for a crucial mechanism of Type I

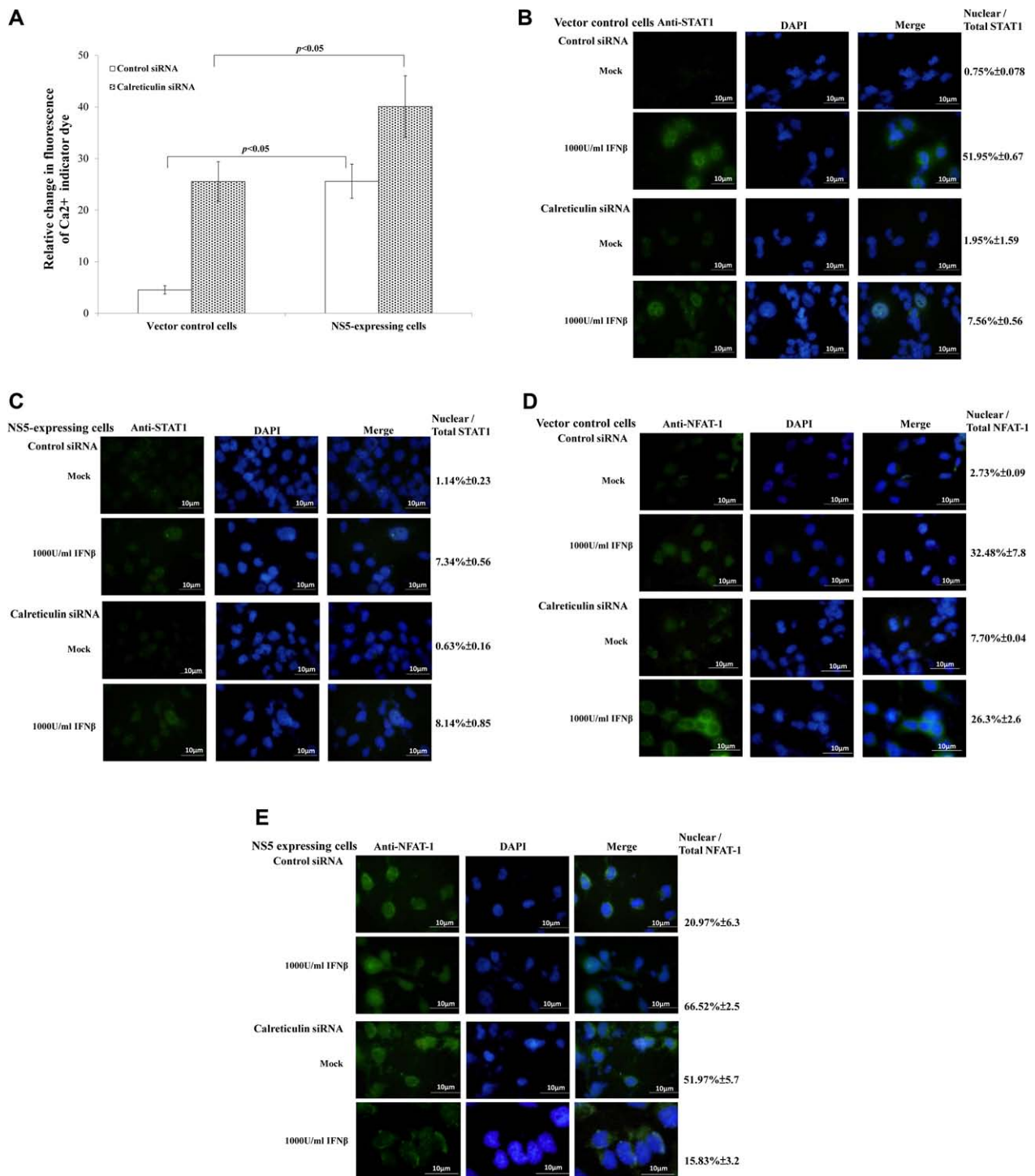


Figure 4. Functional characterization of calreticulin with siRNA-mediated gene silencing. (A) To detect intracellular Ca²⁺, cells transfected with control or calreticulin siRNA were harvested 4 h posttreatment with or without IFN β , stained with FLUO3/AM and analyzed by flow cytometry. To analyze subcellular localization of STAT1 (B, C) and NFAT-1 (D, E), vector control (B, D) and NSS-expressing cells (C, E) transfected with control or calreticulin siRNA were tested by immunofluorescent staining with anti-STAT1 or anti-NFAT-1 antibodies.

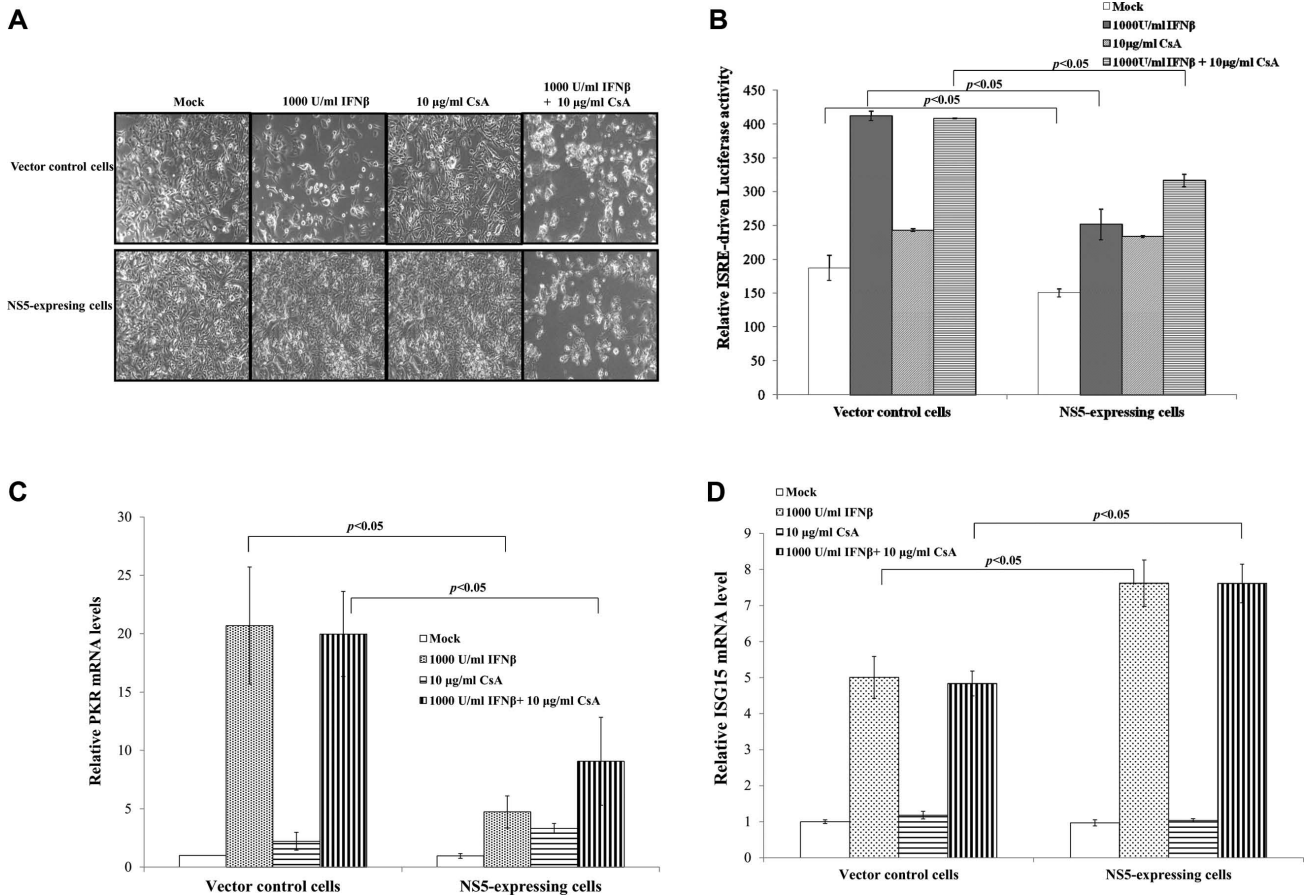


Figure 5. Effects of cyclosporin A (CsA) on NS5-modulated suppression of IFN β -induced response. (A) For cytopathic effect assays, cells were treated singly or with both CsA and IFN β for 48 h, imaging analyzed by light microscopy. (B) To analyze ISRE-driven promoter activity, vector control and NS5-expressing cells were transiently cotransfected with dual-luciferase reporters, treated singly or with both CsA and IFN β for 4 h. Firefly and renilla luciferase were measured, with relative firefly luciferase activity normalized to renilla luciferase activity. Relative mRNA levels of PKR (C) and ISG15 (D) were measured by quantitative PCR and normalized by GAPDH mRNA, presented as relative ratio.

IFN antagonism by JEV NS5 (Supporting Information Fig. 2; Fig. 7A to D). Silencing of calcineurin significantly increased STAT1 nuclear translocation in NS5-expressing cells stimulated by IFN β . Control siRNA did not reduce Type I IFN antagonistic activity of JEV NS5 (Fig. 7B); calcineurin silencing induced twofold increase of PKR mRNA over control siRNA in NS5-expressing cells stimulated by IFN β (Fig. 7C). Calcineurin-dependent expression of IL-4 was tested in NS5-expressing and vector control cells (Fig. 7D). Due to calcineurin activation by JEV NS5 (Figs. 5 and 6), relative IL-4 mRNA level in NS5-expressing cells was nearly 50% higher than vector controls in response to IFN β (Fig. 7D). siRNA silencing of calcineurin significantly decreased IL-4 mRNA in both types of cells, even stimulated with IFN β . Results confirmed activation of Ca²⁺/calcineurin signaling as a critical mechanism of Type I IFN antagonism by JEV NS5.

3.6 Inhibition of in vitro JEV replication by single and combined treatment of cyclosporine A (CsA) and IFN β

To analyze protein levels of calreticulin, TE671 cells were infected with JEV at MOIs of 0.1, 0.5, 1 and 2, then harvested 48 h post infection; lysate used Western blot with anti-calreticulin monoclonal antibodies (Fig. 8A). Calreticulin was downregulated in infected cells by viral dose-dependent manner, correlating with downregulation of calreticulin by NS5 protein. Inhibitory effect of single and combined treatment of CsA and IFN β was subsequently determined in vitro by viral replication (Fig. 8B). Single treatment of CsA (1000 ng/mL) significantly reduced JEV plaque titers by 4.3-fold 48 h post infection; single treatment of IFN β (500 U/mL) significantly reduced JEV plaque titers by 24.0-fold 48 h post infection. Combined treatment of CspA and IFN β potently

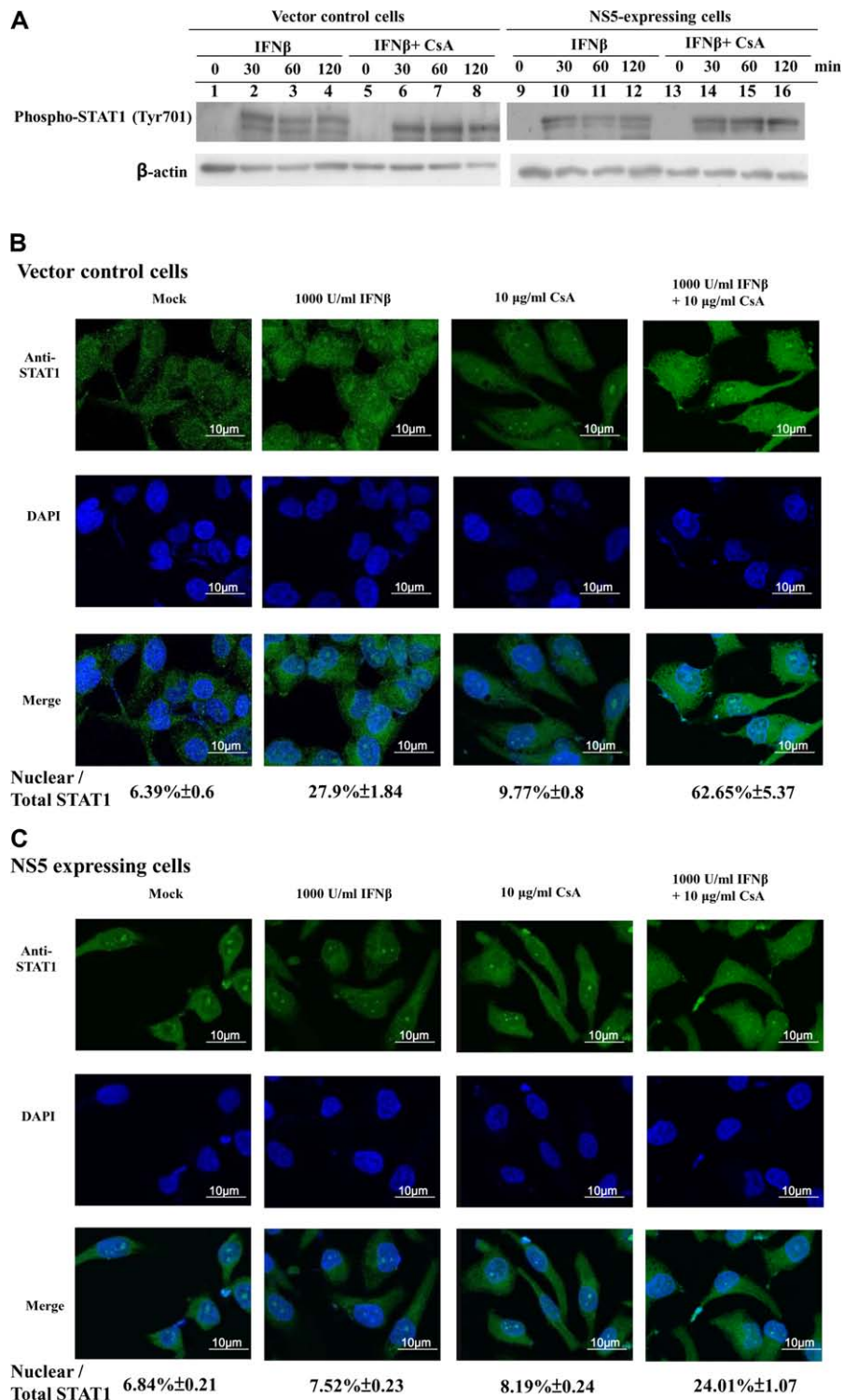


Figure 6. Effect of CsA on IFN β -induced phosphorylation and nuclear translocation of STAT1 in NS5-expressing cells. (A) To analyze tyrosine phosphorylated STAT1, vector control and NS5-expressing cells were treated singly or with both IFN β and cyclosporine A for 0, 30, 60, or 120 min. Lysates were subjected to Western blot, probed with anti-phospho-STAT1 (Tyr701). For sub-cellular location of STAT1, vector control (B) and NS5-expressing (C) cells were treated singly or with both CsA and IFN β for 24 h, then washed, fixed, and reacted with anti-STAT1 and FITC-conjugated anti-mouse IgG antibodies. Finally, cells were stained with DAPI for 10 min, imaging analyzed by immunofluorescent microscopy.

inhibited JEV plaque titers by 63.4-fold at 48 h post infection. Annexin V-FITC/propidium iodide apoptosis staining indicated ratio of late apoptotic/necrotic cells (Annexin V-FITC positive/propidium iodide positive) at 48 h post infection as 3.2% in mock controls, 28.7% in JEV-infected cells, 13.2%

in infected cells with single IFN β treatment, 11.1% in infected cells treated with single CsA, and 6.5% in those treated with both CsA and IFN β (Fig. 8C). Analysis ruled out indirect side effect of combined treatment on apoptosis reducing viral replication. Higher anti-JEV efficacy by combined CsA

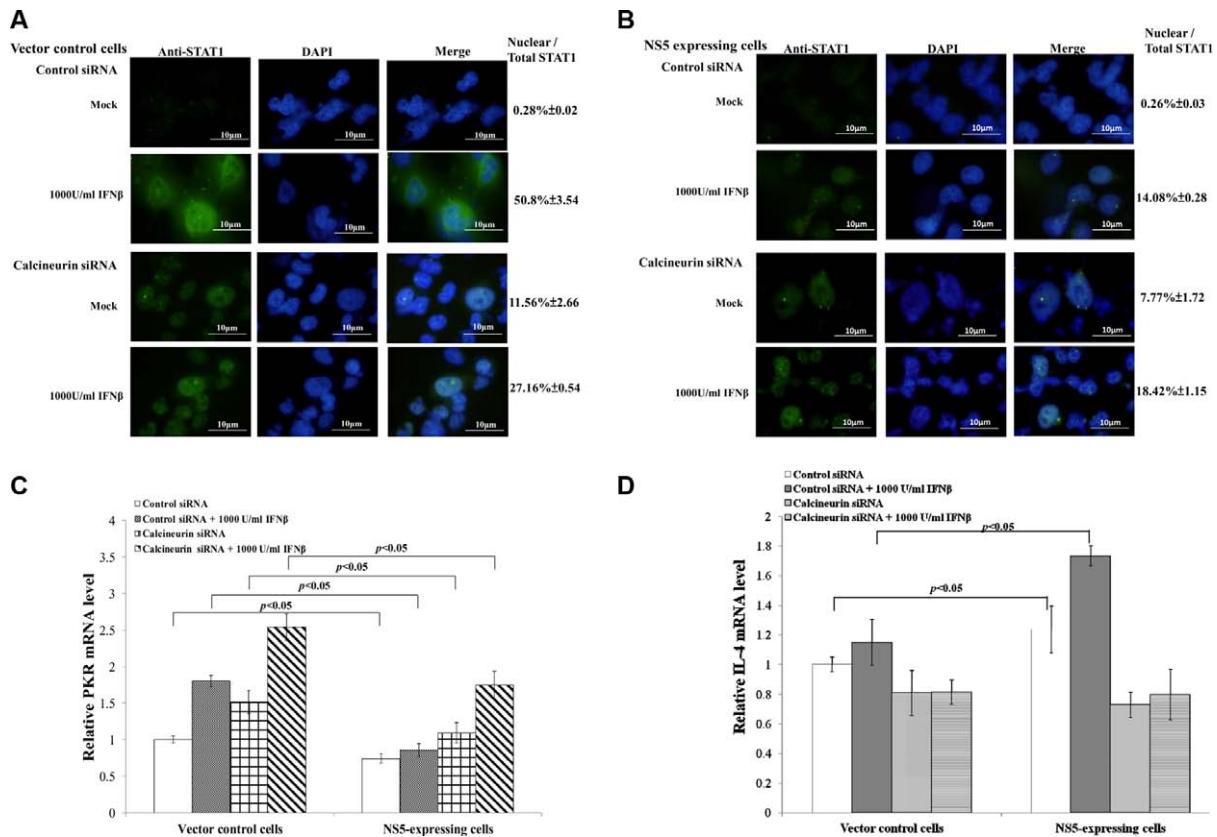


Figure 7. Functional analysis of calcineurin with siRNA-mediated gene silencing. To analyze subcellular localization of STAT1, vector control (A) and NS5-expressing cells (B) transfected with control or calcineurin siRNA were tested using immunofluorescent staining with anti-STAT1 antibodies. Relative mRNA levels of PKR (C) and IL-4 (D) were measured by quantitative PCR and normalized by GAPDH mRNA, presented as relative ratio.

and IFN β versus single treatment concurred with recovery of IFN β -induced STAT1 phosphorylation in NS5-expressing cell by combined treatment of CsA and IFN β .

4 Discussion

JEV induces no Type I IFN response (vital to pathogenesis) in cell culture. This study dealt with JEV NS5, previously reported as antagonistic toward members of Type I IFN pathway. We first demonstrated that JEV NS5 expression in human medulloblastoma cells inhibits (a) IFN β -induced ISRE promoter activities, (b) IFN β -stimulated gene expression, and (c) suppression of IFN β -induced nuclear translocation and phosphorylation of STAT1 (Fig. 2). Significant suppression of STAT1 phosphorylation at Tyr701 was responsible for Type I IFN antagonism of JEV NS5 proteins, in agreement with prior studies: WNV and TBEV NS5 proteins suppressing tyrosine phosphorylation of STAT1 and DEN NS5 protein suppressing tyrosine phosphorylation of STAT2, blocking formation of STAT1/STAT1 homo- or heterodimers in Type I IFN-induced JAK/STAT signaling pathway [8, 10–13, 35]. Recently, interaction of TBEV NS5 with PDZ protein scribble (hScrib) was

associated with JAK-STAT interference, vital for an antagonist of IFN response [15].

Proteomics approach to identify NS5-induced protein profiling indicated downregulation of calcineurin plus upregulation of cyclophilin A, Hsp60, and STIP1, as linked with activation of calcineurin (Fig. 3, Table 1), suggesting the key of host cellular factors in Type I IFN antagonistic function of JEV NS5 protein. Calcineurin is a Ca²⁺-binding chaperone in ER, as a key upstream regulator of calcineurin in Ca²⁺-signaling cascade [36]. NS5 downregulated Ca²⁺-binding chaperone calcineurin, confirmed by Western blot and quantitative real-time PCR (Fig. 3D and E), as correlating with increased release of Ca²⁺ from ER into cytosol (Fig. 4A), as well as gene silencing of calcineurin raising intracellular Ca²⁺ levels in controls. Promoter of human calcineurin gene comprised AP-1, Sp1, and H4TF-1 sites, plus four CCAAT sequences, binding with c-Jun/c-Fos, specific protein 1 (Sp1), H4TF-1, CAAT/enhancer-binding protein transcriptional factors [37, 38] regulated by p38 MAPK and ERK1/2. Western blot indicated JEV NS5 protein suppressing phosphorylated levels of ERK1/2, not p38 MAPK, in response to IFN β (Supporting Information Fig. 3); JEV NS5 protein affects transcriptional activation of calcineurin promoter by reducing ERK1/2 phosphorylation.

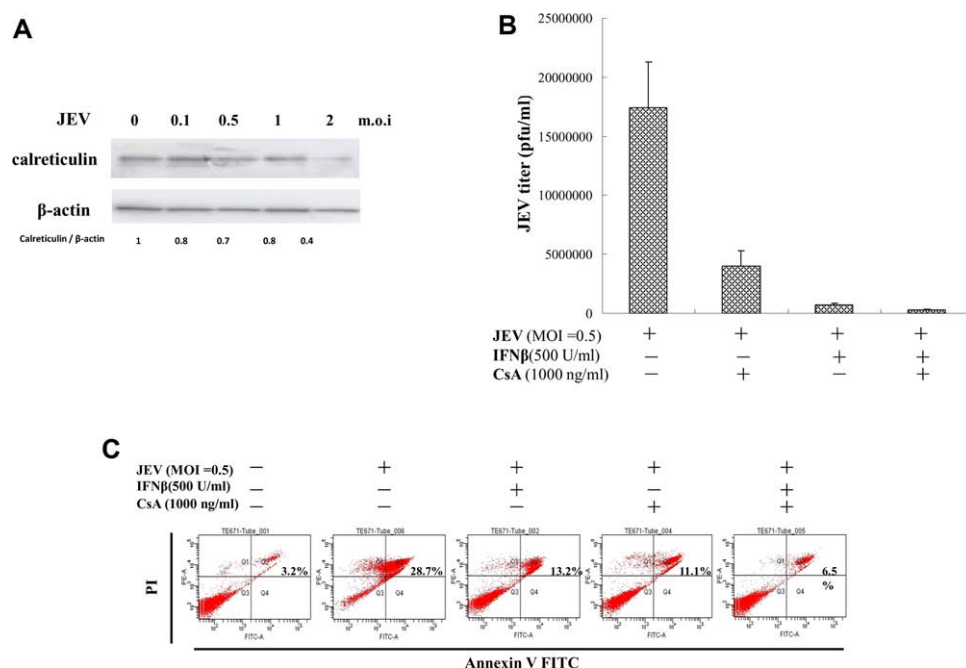


Figure 8. Effect of cyclosporine A on JEV replication in human medulloblastoma cells. (A) To analyze calreticulin expression, TE671 cells were infected with JEV at MOIs of 0.1, 0.5, 1, and 2, then harvested 2 days post infection. Lysates subjected to Western blot were probed with anticalreticulin. (B) TE671 cells were infected with JEV at the MOI of 0.5 and treated with IFN β alone or both IFN β and cyclosporine A for 48-h and cultured supernatants harvested to determine virus yields. BHK-21 cell monolayers were incubated with serial dilution of cultured supernatant at 37°C for 1 h, then overlaid with MEM medium containing 1.1% methylcellulose. After 3 days of incubation, BHK-21 cell monolayers were stained with naphthol blue-black dye and viral plaques counted. (C) For analysis effect of CsA on apoptosis, JEV-infected, TE671 cells were infected with JEV at MOI of 0.5 and treated with IFN β alone or with IFN β plus CsA for 2 days, then harvested, stained with both Annexin V-FITC and PI, and analyzed for percent of cells in apoptosis, using flow cytometry.

Downregulation and siRNA-mediated silence of calreticulin reduced the nuclear translocation of NFAT-1 in response to IFN β (Fig. 4D and E), indicating activation of calcineurin by calreticulin downregulation and silence. Among upregulated proteins, cyclophilin A is involved in virus replication and virion maturation [39], significantly interacting with calcineurin and inhibited its phosphatase activity in the presence of CsA [40,41]. Hsp60 is one calcineurin-interacting protein [42]. STIP1, an Hsp70/ Hsp90-organizing protein, as a cochaperone binds to both Hsp70/Hsp90, correlated with calcineurin regulation [43]. SARS-CoV NSP1 overexpression strongly induced production of interleukin 2 by activating calcineurin/NFAT pathway; calcineurin inhibitor CspA strongly inhibited pan-coronavirus replication in vitro [41]. Proteomics results and the above literature survey led us to hypothesize calcineurin as involved in Type I IFN antagonism of JEV NS5. Because induction of ISG15 was IRF-3-dependent, PKR expression is STAT1-dependent in response to Type I IFN [33, 34], IFN β upregulated expression of ISG15, but not PKR in NS5-expressing cells (Fig. 5C and D), implying NS5 specifically inhibited STAT1-mediated signaling. Combined CsA and IFN β treatment as well as siRNA-mediated calcineurin silence restored IFN β -induced STAT1-mediated responses in NS5-expressing cells, for example., PKR mRNA expression, STAT1 phosphorylation at Tyr701, nuclear translocation

of STAT1 (Figs. 5–7). These affirm Type I IFN antagonism of JEV NS5 correlating with downregulation of calreticulin in which activated Ca²⁺-dependent calcineurin phosphatase inhibits STAT1-mediated signaling. HBV-induced upregulation of calreticulin was identified as antagonizing Type I IFN responses: inhibiting STAT1 phosphorylation and reducing PKR mRNA expression [44]. Calreticulin, an ER Ca²⁺-binding chaperone, might be involved in viral pathogenesis, offering a potential target for development of antiviral agents.

Combined treatment of CsA and IFN β proved more effective in suppressing JEV replication than single treatment (Fig. 8). Interestingly, single treatment with CsA slightly induced ISRE-driven promoter activation, STAT1 nuclear translocation in both cell types, and inhibition of JEV replication in vitro by 4.3-fold (Figs. 5B, 6B and C, and 8B). This indicated inhibition of calreticulin/calcineurin pathway by CsA eliciting antiviral innate immune responses. CsA with distinct immunosuppressive and anti-inflammatory mechanisms from IFN antiviral mechanisms sharply reduced IFN antagonistic function of JEV NS5 via inactivation of calcineurin-mediated pathways. Single and combined treatment of CspA analogs and IFNs should be further tested in vitro and in vivo for evaluating antiviral efficacy.

Cyclophilin A directly bonded with WNV RNA and NS5 while facilitating viral replication, serving as a component of

the replication complex [35]. Replication of WNV, DEN, and YF viruses in cyclophilin A knockdown cells was less efficient; transsupplying of wild peptidyl-prolyl isomerases domain of cyclophilin A rescued viral replication in cyclophilin A knockdown cells [35]. In HCV replication complex, cyclophilin A was recruited to maintain optimal conformation between RNA-dependent RNA polymerase and template binding [45]. A chaperone system was likewise required for viral replication assembly and RNA binding, for example, interaction of HSP 90 with herpes simplex virus Type 1 polymerase [46] and hepatitis B virus reverse transcriptase [47]. Cyclophilin A was suggested as a molecular chaperone to keep flavivirus replication complexes in active conformation. In our laboratory, coimmunoprecipitation assays also proved direct interaction of JEV NS5 with cyclophilin A (data not shown). Likewise, overexpression of cyclophilin A induced IFN β production, linked with suppressed rotavirus replication via activation of peptidyl-prolyl isomerases independent but JNK-dependent signaling [48]. Cyclophilin A could thus be crucial for maintaining JEV NS5 functional structure.

Type I IFN antagonism of JEV NS5 protein correlated with suppressing phosphorylation and nuclear translocation of STAT1, linking with calreticulin downregulation and activation of Ca²⁺/calcineurin-signaling pathway. Treatment with calcineurin-specific inhibitor or siRNA increased tyrosine phosphorylation and STAT1 nuclear translocation, up-regulating STAT1-dependent ISG gene expression in NS5-expressing cells responded to IFN β . This study indicated correlation between Type I IFN antagonistic activity and proteome profiling induced by JEV NS5 protein, highlighting a pivotal role of JEV NS5 protein in Type I IFN antagonism.

We thank China Medical University and National Science Council, Taiwan, for financial support (CMU101-S-24, CMU98-P-03, NSC99-2628-B-039-006-MY3, and NSC98-2324-B-039-006).

The authors have declared no conflict of interest.

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