รายงานวิจัยฉบับสมบูรณ์

โครงการ

“บทบาทของโปรตีน Nonstructural Protein 5 ของไวรัสเดงกี่ ต่อ Cytokine Production และ Inflammatory Response ในโรคไข้เลือดออก”

โดย

รองศาสตราจารย์ ดร.นพ.ถาวรชัย ลิ้มจินดาพร

มิถุนายน 2556

สัญญาเลขที่ RMU538007

รายงานวิจัยฉบับสมบูรณ์

โครงการ

“บทบาทของโปรตีน Nonstructural Protein 5 ของไวรัสเดงกี่ ต่อ Cytokine Production และ Inflammatory Response ในโรคไข้เลือดออก”

	คณะผู้วิจัย	สังกัด
หัวหน้าโครงการ	รศ.ดร.นพ.ถาวรชัย ลิ้มจินดาพร	มหาวิทยาลัยมหิดล
ผู้ร่วมโครงการ	ศ.ดร.เพทาย เย็นจิตโสมนัส	มหาวิทยาลัยมหิดล
	ดร.ศันสนีย น้อยสคราญ	ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ

สนับสนุนโดย
สำนักงานคณะกรรมการอุดมศึกษาและสำนักงานกองทุนสนับสนุนการวิจัย

Abstract

Project code: RMU538007

Project title: The Role of Dengue Virus Nonstructural Protein 5 in Cytokine Production and Inflammatory Response of Dengue Virus Infection

Investigator: Associate Professor Thawornchai Limjindaporn, MD, PhD

E-mail address: thawornchai.lim@mahidol.ac.th

Project period: July 2010-June 2013

Optimal levels of cytokines have anti-viral effects during dengue (DENV) infection; however, the massive cytokine secretion following severe DENV infection may contribute to the development of the disease rather than protection. Increased levels of cytokines–the so-called 'cytokine storm' relates to the pathogenesis of severe DENV infection. In this study, the expression of cytokine and inflammatory genes was compared between DENV-infected hepatic cell line, HepG2 and mock-infected HepG2 cell line using a real-time PCR array. The highly up-regulated cytokines including CXCL-10, TNF-α, and RANTES, were subsequently verified. In addition, molecular mechanism how DENV nonstructural protein 5 (DENV NS5) regulated cytokine secretion was characterized. A yeast two-hybrid assay was firstly performed to identify human proteins interacting with DENV NS5 and Daxx, which is an NF-κB inhibitor, was identified. The *in vivo* relevance of this interaction was suggested by co-immunoprecipitation and nuclear co-localization of these two proteins in HEK293 cells expressing DENV NS5. HEK293 cells expressing DENV NS5-K/A, which were mutated at the nuclear localization signals (NLS), were created in this study. In the absence of NLS, DENV NS5 could neither translocate into the nucleus nor interact with Daxx to increase RANTES production. As binding of DENV NS5 and Daxx, which is NF-κB inhibitor, may influence binding of NF-κB and Daxx, luciferase reporter and ChIP assays were performed and the result showed that interaction of DENV NS5 and Daxx might influence interaction binding between NF-κB and Daxx thereby liberating NF-κB to activate RANTES promoter in HEK293 cells expressing DENV NS5.

Keywords: Dengue Virus Infection, Nonstructural Protein 5, Cytokine Production, Inflammatory Response, NF-κB, RANTES

บทคัดย่อ

รหัสโครงการ: RMU538007

ชื่อโครงการ: บทบาทของโปรตีน nonstructural protein 5 ของไวรัสเดงกี่ต่อ cytokine production และ inflammatory response ในโรคไข้เลือดออก

ชื่อนักวิจัย: รศ.ดร.นพ.ถาวรชัย ลิ้มจินดาพร

E-mail address: thawornchai.lim@mahidol.ac.th

ระยะเวลาโครงการ: กรกฎาคม 2553-มิถุนายน 2556

การมีระดับไซโตคายน์ที่เหมาะสมเป็นปฏิกริยาของร่างกายในการจำกัดไวรัสเดงกี่ อย่างไรก็ตามหากมีการหลั่งไซโตคายน์มากเกินไปอาจส่งผลเสียในการทำลายอวัยวะต่างๆ เช่น ตับและปอด ซึ่งปัจจุบันการทำลายอวัยวะเป็นเกณฑ์อย่างหนึ่งในการวินิจฉัยโรคเดงกี่ชนิดร้ายแรง การศึกษานี้ได้เปรียบเทียบการแสดงออกของยีนที่เกี่ยวข้องกับการผลิตไซโตคายน์จำนวนมากในเซลล์ตับที่ติดเชื้อไวรัสเดงกี่กับเซลล์ตับปรกติพบว่ามีการเพิ่มขึ้นจำนวนมากของไซโตคายน์หลายชนิดเช่น CXCL-10, TNF-α, และ RANTES กลไกการเพิ่มขึ้นของไซโตคายน์ RANTES ถูกรายงานในการวิจัยนี้ว่ามีความเกี่ยวข้องกับโปรตีน nonstructural protein 5 ของไวรัสเดงกี่ โดยพบว่าโปรตีน nonstructural protein 5 ของไวรัสเดงกี่จับกับโปรตีน Daxx ของมนุษย์ซึ่งปรกติทำหน้าที่เกี่ยวข้องกับการยับยั้งการทำงานของโปรตีนควบคุมที่ชื่อว่า NF-ĸB ซึ่งมีผลต่อการผลิตไซโตคายน์ในเซลล์ การเปลี่ยนแปลงกรดอะมิโนในโปรตีน nonstructural protein 5 ของไวรัสเดงกี่ส่งผลต่อการเข้านิวเคียส การจับกับโปรตีน Daxx และการหลั่งไซโตคายน์ RANTES ในเซลล์ นอกจากนี้ยังพบว่ากลไกหนึ่งคือการจับกันระหว่างโปรตีน nonstructural protein 5 ของไวรัสเดงกี่กับโปรตีน Daxx ทำให้มีการลดการจับกันระหว่างโปรตีน Daxx กับโปรตีนควบคุม NF-ĸB และส่งผลให้โปรตีนควบคุม NF-ĸB อิสระมีมากขึ้น สามารถกระตุ้นโปรโมเตอร์ของ RANTES ได้มากขึ้น

คำหลัก: โรคไข้เลือดออก, โปรตีน nonstructural protein 5, การสร้างไซโตคายน์, กระบวนการอักเสบ, โปรตีนควบคุม NF-ĸB, ไซโตคายน์ RANTES

Introduction

Dengue virus (DENV) infection is a mosquito-borne viral disease and is endemic in several countries (1). Clinical severity of the disease ranges from a predominantly febrile disease, dengue fever (DF), to dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (2). The patients with DHF generally present hemorrhagic tendencies, plasma leakage, thrombocytopenia, and hemoconcentration. Furthermore, hepatic dysfunction is a feature of DENV infection (3, 4). Elevation of aminotransferases and hepatic failure are observed in the patients with DHF/DSS (5).

Optimal levels of cytokines have anti-viral effects during DENV infection (6-8); however, the massive cytokine secretion following severe DENV infection may contribute to the development of the disease rather than protection (9-12). Increased levels of cytokines –the so-called 'cytokine storm', which relate to the pathogenesis of severe DENV infection, are observed both *in vitro* and *in vivo* models of DENV infection (13-20).

A complex interaction between DENV proteins and the host immune response contributes to DHF/DSS (21-27). Both inhibition of the antiviral response and stimulation of cytokine production by DENV proteins have been reported. DENV NS2B3 complex, DENV NS4B and DENV NS5 contribute to the inhibition of type I IFN response (21, 23, 25). DENV NS4B and DENV NS5 also enhance the production of DHF-associated cytokines (26, 27). DENV NS5 is a bi-functional enzyme containing three domains (28-30). The N-terminus from residues 1 to 368 contains a 2'-O-methyltransferase while the C-terminus from residues 405 to 900 contains an RNA-dependent RNA polymerase. The interdomain region contains nuclear localization sequences (NLS), which are divided into aNLS and bNLS, respectively. Mutations in both aNLS and bNLS result in the accumulation of DENV NS5 in the cytoplasm (31). DENV NS2B3 complex, DENV NS4B and DENV NS5 contribute to the inhibition of type I IFN response (21, 23, 25). DENV NS4B and DENV NS5 also enhance the production of DHF-associated cytokines (26, 27).

In this study, we firstly compared the expression of cytokine genes between mock-infected and DENV-infected HepG2 cells using a real-time PCR array. The highly up-regulated cytokines were subsequently verified at both the mRNA and protein levels. Secondly, the potential therapeutic agent to reduce massive cytokine production, namely compound A, was used to inhibit cytokine production. Thirdly, the molecular

mechanisms how DENV NS5 contributed to cytokine production was shown by comparing the expression of cytokine genes between HEK293T expressing DENV NS5 and HEK293T expressing vector control. Finally, a yeast two-hybrid assay was performed to identify host proteins interacting with DENV NS5 and a death-domain-associate protein (Daxx), which serves as transcription repressor was identified. Nuclear NS5 interacted with an NF-ΚB inhibitor, Daxx, thereby liberating NF-ΚB to activate RANTES promoter as revealed by luciferase reporter assay. Increased DNA-binding activity of NF-ΚB on RANTES promoter was also demonstrated by ChIP assay. The roles of DENV NS5 in nuclear translocation, Daxx interaction, and cytokine production were tested and DENV NS5 was demonstrated to interact with human Daxx to increase RANTES production.

Materials and Methods

Part I: Expression of cytokine genes between mock-infected and DENV-infected HepG2 cells using a real-time PCR array and the potential therapeutic agent, namely compound A, in reduction of massive cytokine production in DENV-infected cells.

1.1 Cell culture and Infection of HepG2 cells

Up to 2 x10^6 HepG2 cells were seeded in a 60 mm dish and cultured for 24 h before infection. HepG2 cells were grown in DMEM medium (Gibco-BRL), supplemented with 10% fetal bovine serum (FBS), 2mM L-glutamine, 1% non-essential amino acids, 1mM sodium pyruvate, 100U/ml of penicillin and streptomycin at 37^{o}C in a humidified atmosphere containing 5% CO_2. HepG2 cells were washed with PBS and infected with DENV serotype 2 (DENV-2) strain 16681 at a multiplicity of infection (MOI) of 5. Cells were collected at 12, 24, 36 or 48 h post-infection. DENV NS1 antibody staining analysed by flow cytometry demonstrated DENV infection (32). Cell viability was determined by the trypan blue exclusion test (33).

1.2 Real-time PCR array

Total RNA was isolated either from mock-infected HepG2 cells or from DENV-infected HepG2 cells at 24 h post-infection using the High Pure RNA isolation kit (Roche). RNAs with an OD_{260nm}/OD_{280nm} absorbance ratio of at least 2.0 were used. Total RNA was reverse-transcribed into cDNA using the RT^2 First strand Kit (SABiosciences), mixed with RT^2 qPCR mastermix containing SYBR Green (SA Biosciences), and aliquoted in equal volumes to each well of the real-time PCR arrays. The Human Cytokine RT² Profiler™ PCR Array (SA Biosciences) has 84 genes related to the cytokine pathway. The real-time PCR cycling program was run on a Roche Light Cycler 480. The threshold cycle (Ct) of each gene was determined and subsequently analysed by RT^2 Profiler PCR Array data Analysis software (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php). The highly up-regulated chemokine gene, CXCL10, and cytokine gene, TNF-α, were selected and confirmed at the mRNA level by quantitative Real-time PCR using different sets of primers, including CXCL10 F5'GAATCGAAGGCCATCAAGAA3', CXCL10 R5'AAGCAGGGTCAGAACATCCA 3' ; TNF-αF5'TGCTTGTTCCTCAGCCTCTT3',TNF-α R 5'ATGGGCTACAGGCTTGTCACT3').

1.3 ELISA

The effect of CpdA on CXCL10 and TNF-α production was determined following infection of HepG2 cells with DENV-2 at a MOI of 5 for 2 h, washing and incubation for 24 h in the presence of PBS, 5μM CpdA, 10μM CpdA , 20μM CpdA or 50μM dexamethasone (Dex) (Sigma Aldrich), respectively. The levels of cytokines were then measured from CpdA-untreated and CpdA-treated DENV-infected HepG2 cells by ELISA (R&D Systems).

1.4 Chemotaxis assay

To test the effect of CpdA on leukocyte migration, the DENV-infected HepG2 cells were treated with CpdA as described in the preceding experiment. The chemotaxis assay was performed using the protocol described previously (34). Briefly, 600 μl of cell culture supernatant collected from either CpdA-untreated or CpdA-treated DENV-infected HepG2 cells was added to the lower chambers of the transwell cassette (Corning Costar, Lowell, MA, USA). Up to $1x10^5$ monocyte THP-1 cells were added to

the upper chamber and incubated at 37ºC in 5% CO_2 for 6 h. A 500 µl aliquot of the cells migrated to the lower chamber was counted by flow cytometry in a FACCalibur acquiring events for a fixed time period of 60 sec using CellQuest software (Becton Dickinson, Basel, Switzerland).

1.5 Focus-Forming Unit (FFU)

Firstly, viability of mock-infected or DENV-infected HepG2 cells in the presence or absence of CpdA were first measured by trypan blue exclusion test (33). Secondly, DENV-infected HepG2 cells were treated with CpdA as described in the preceding experiment. Focus-forming unit (FFU) was performed using the protocol described previously (35). Briefly, titration of DENV was carried out using 96-well plates. Vero cells were seeded at 3×10^4 cells/well 24 h prior to infection. Ten-fold serially diluted culture media was added to Vero cells at room temperature (RT) for 2 h. After adsorption, the cells were overlaid with 100 µl of 1.5% gum tragacanth containing 2% FBS in MEM before further incubation at 37 °C for 3 days. The cultures were then fixed with 3.7% formaldehyde for 10 min at RT, treated with 1% Triton X-100 in PBS for another 10 min, washed three times with PBS, and incubated with anti-DENV E (4G2) for 30 min at 37 °C in a humidified chamber. Cells were washed with PBS and incubated with rabbit anti-mouse IgG-HRP at the dilution 1: 1000 for 30 min at 37 °C in a humidified chamber. After triple washing, the substrate solution was added. Stained foci were visible and the reaction was terminated by washing cells with PBS. DENV-infected foci were counted with a light microscope and viral concentration in the supernatant calculated as focus-forming units (FFU) per milliliter.

1.6 Statistical analysis

All data were obtained from three independent experiments and reported as the mean ± SEM. Statistical differences between the groups were tested with an unpaired *t*-test using StatView version 5.0 and *P* value less than 0.05 was considered significant.

Part II: Functional studies for the role of DENV NS5 in cytokine production and inflammatory response

2.1 Yeast two-hybrid screening. Two-hybrid screening was performed by the interaction mating method described by Finley and Brent(36). DENV NS5 was PCR amplified by *Pfu* DNA polymerase from a cDNA clone of DENV serotype 2 strain 16681(37) using nucleotide primers, 5'TTG ACT GTA TCG CCG GGA ACT GGC AAC ATA 3' and 5'CCG GAA TTA GCT TGG CTG CAG CCA CAG AAC TCC TCG 3'. The PCR reaction was carried out in a GeneAmp PCR System 9700 (Applied Biosystems) with an initial denaturation step of 94ºC for 5 min and followed by 35 cycles of denaturation at 94ºC for 45 sec, annealing at 55ºC for 30 sec, extension at 72ºC for 3 min, and one cycle of final extension at 72ºC for 10 min. Subsequently**,** the amplified DNA was cloned by yeast recombination (38) into the yeast expression vector pEG-NRT(39), which was derived from pEG202(40), and contains a *HIS3* selectable marker. The constitutive ADH promoter is used to express DENV NS5 with the DNA binding domain protein LexA. Nuclear localization signal (NLS), 5'recombination tag (5'RT), multiple cloning site (MCS) and 3'recombination tag (3'RT) were inserted between LexA and ADH terminator. The resulting bait plasmid, pEG-NS5, was verified by DNA sequencing and transformed into *S. cerevisiae* strain RFY 206 (MATa *his3A200 leu2-3 lys2A201 ura3-52 trp1A::hisG*) containing a Lexop-*lac*Z reporter plasmid, pSH18-34 (36). A galactose inducible HeLa cell cDNA prey library was constructed in plasmid pJZ 4-5 containing a *TRP1* selectable marker and transformed into strain RFY 231 (MATα *his3 leu2::3Lexop-LEU2 ura3 trp1 LYS2*)(36). The bait strain then was mated with the library strains and plated on galactose drop-out medium lacking histidine, tryptophan, uracil and leucine (gal/raf -u, -h, -w, -l) to select for diploids. The production of a DENV NS5 binding protein by a prey plasmid was expected to activate the 3Lexop-*LEU*2 reporter. Putative positive clones were patched to four indicator plates: (glu/-u, -h, -w, -l), (gal/raf -u, -h, -w, -l), (glu/X-Gal-u, -h, -w), and (gal/raf/ X-Gal-u, -h, -w). Prey plasmids were rescued from clones exhibiting a galactose-inducible Leu^+ *lac*Z^+ phenotype by transformation into a Trp^- *Escherichia coli* strain KC8(36). Putative positive library plasmids were purified and subjected to DNA sequencing and BLAST analysis.

2.2 Generation of HEK293 cells expressing DENV NS5, or DENV NS5-K/A. DENV NS5 with a C-terminal FLAG-tag was PCR amplified by *Pfx* DNA polymerase (Invitrogen) from plasmid pET-DENV-2-NS5 using nucleotide primers, 5'ACA GGA TCC ACC ATG GGA ACT GGC AAC ATA GGA GAG ACG 3' and 5' TGT CTC GAG TTA CTT GTC ATC GTC ATC CTT GTA ATC CCA CAG AAC TCC TGC TTC TTC C3'. The PCR reaction was carried out in a GeneAmp PCR System 9700 with an initial denaturation step of 94ºC for 5 min and followed by 30 cycles of denaturation at 94ºC for 30 sec, annealing at 55ºC for 30 sec, extension at 68ºC for 3 min, and one cycle of final extension at 68ºC for 10 min. The PCR product was sub-cloned into plasmid pcDNA3.1/Hygro (Invitrogen) and the fidelity of the insert in the resulting plasmid, pcDNA3.1/ DENV NS5, was verified by DNA sequencing.

Site-directed mutagenesis was employed to generate plasmid pcDNA3.1/DENV NS5 (K371A, K372A, K387A, K388A, K389A), namely DENV NS5-K/A. Firstly, DENV NS5 (K387A, K388A, K389A) was amplified by PCR using plasmid pcDNA3.1/DENV NS5 as a template and nucleotide primers, 5'GGA AAG AAT TAG GGG CGG CAG CGA CAC CCA GGA TGT G 3' and 5'CAC ATC CTG GGT GTC GCT GCC GCC CCT AAT TCT TTC C 3'. The PCR reaction was carried out in a GeneAmp PCR System 9700 with an initial denaturation step of 94ºC for 2 min and followed by 18 cycles of denaturation at 94ºC for 30 sec, annealing at 55ºC for 1 min, extension at 68ºC for 18 min. The PCR product was digested with *Dpn*I to eliminate methylated parental DNA template and transformed into competent *E.coli* strain DH5. The correct mutant clone with K387A, K388A, K389A was verified by digestion with *Fnu4*HI and DNA sequencing. Secondly, DENV NS5 (K371A, K372A, K387A, K388A, K389A), was amplified by PCR using plasmid pcDNA3.1/DENV NS5(K387A, K388A, K389A) as a template and nucleotide primers, 5'CCG AAA GAA GGC ACG GCG GCA CTA ATG AAA ATA AC 3' and 5'GTT ATT TTC ATT AGT GCC GCC GTG CCT TCT TTC GG3'. The PCR reaction and transformation is similar to those described previously. The correct mutant clone with K371A, K372A, K387A, K388A, K389A, namely DENV NS5-K/A, was verified by digestion with *Fnu4*HI and DNA sequencing.

HEK 293 cells were transiently transfected with pcDNA3.1 or pcDNA3.1/DENV NS5 or pcDNA3.1/DENV NS5-K/A by Lipofectamine® 2000 transfection (Invitrogen). Two days after transfection, the cells were collected, fixed and permeabilized with 0.1% Triton X-100 in PBS. Re-suspended cells were blocked with DMEM containing 1% BSA before incubation with mouse anti-FLAG (Sigma) antibody for 1 h. After incubation, the

cells were washed twice with chilled DMEM containing 1% BSA. Then, goat anti-mouse antibody conjugated with FITC (Molecular Probes) was used as secondary antibody for 30 min. The cells were washed again and analyzed by using FACSort™ flow cytometer (Becton–Dickinson). HEK293 cells expressing either DENV NS5, or DENV NS5-K/A were also tested for the presence of DENV NS5 by Western blot analysis using anti-FLAG (Sigma).

2.3 Co-immunoprecipitation. Forty-eight h post-transfection, HEK293 cells expressing either DENV NS5 or DENV NS5-K/A were lysed in RIPA buffer containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 20 mM Tris–HCl; pH 7.4, 5 mM EDTA, and protease inhibitor cocktail. Five μg of goat anti-Daxx antibody (Santa Cruz Biotechnology) or 5 μg of mouse anti-FLAG antibody were added to lysates. The mixture was incubated with gentle rotation at 4°C for 6 h. The incubation was continued 24 h after addition of Protein G Sepharose (Amersham Pharmacia Biosciences). Subsequently, Protein G Sepharose was collected by centrifugation at 13,000 x g for 5 min and washed twice with 0.1% Triton X-100, 150 mM NaCl, 20 mM Tris–HCl; pH 7.4, 5 mM EDTA. The bound proteins were eluted by boiling in SDS-PAGE loading buffer, separated by SDS-PAGE, and transferred to nitrocellulose membrane. After blocking with 5% skim milk, the membranes were incubated with either a mouse anti-FLAG antibody or a rabbit anti-Daxx antibody followed by probing with either a rabbit anti-mouse antibody conjugated-horseradish peroxidase (HRP) or a swine anti-rabbit antibody conjugated-HRP (DakoCytomation) respectively. Chemiluminescent signals generated by SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) were detected using a G:BOX chemiluminescence imaging system (Syngene).

2.4 Co-localization. HEK293 cells were grown on coverslips and then tranfected with plasmid expressing either DENV NS5 or DENV NS5-K/A for 48 h. The transfected cells were fixed with 0.1% formaldehyde in PBS, rinsed, permeabilized with 0.1% Triton X-100 in PBS and blocked with 1% BSA. The cells were stained at RT for 1 h with both a mouse anti-FLAG and a rabbit anti-Daxx primary antibodies, washed and incubated at RT for 1 h with both an Alexa 488-conjugated rabbit anti-mouse Ig antibody (Molecular Probes) and a Cy3-conjugated donkey anti-rabbit Ig antibody (Jackson Immunoresearch Laboratories) as secondary antibodies. Fluorescent images were captured with a confocal microscope (model LSM 510, Carl Zeiss).

2.5 Real-time RT-PCR. Total RNA from HEK293 cells expressing either DENV NS5, or DENV NS5-K/A and cultured in the presence of 50 ng/ml TNF-α (Santa Cruz biotechnology) was isolated using Trizol reagent (Invitrogen). Cytokines were quantified by real-time RT-PCR using the following primer pairs: IL-8: 5'-TCC TGC AGA GGA TCA AGA CA-3' and 5'-GAG CAC TTG CCA CTG GTG TA-3' , CXCL9: 5'-CAG ATT CAG CAG ATG TGA AGG A-3' and 5'-GAA ATT CAA CTG GTG GGT GGT-3', RANTES: 5'-CAA GGA AAA CTG GGT GCA GA-3'and 5'-TCT CCC GTG CAA TAT CTA GGA A-3', respectively. The assay was performed using LightCycler® 480 SYBR Green I Master Mix (Roche) and a LightCycler® 480 Instrument equipped with a 96-well thermal cycler (Roche). Briefly, RNA samples were reverse-transcribed using the SuperScript III First-Strand Synthesis System (Invitrogen). Then cDNA templates were subjected to a 10-min initial denaturation at 95°C prior to 50 cycles of PCR (95°C for 10 sec, 60°C for 10 sec, and 72°C for 20 sec) in the presence of *Taq* DNA polymerase and the gene-specific primer pairs. The mRNA level was normalized with human beta-actin mRNA using the $\Delta\Delta$Ct method(41).

2.6 ELISA. Production of RANTES in HEK293 cells expressing either DENV NS5 or DENV NS5-K/A in the presence or absence of TNF-α, was measured by Instant ELISA® (eBioscience) according to the manufacturer's instruction.

2.7 Luciferase reporter gene assay. This technique is widely used to study eukaryotic gene expression regulation, receptor activity, transcription factors, and intracellular signaling. Dual-Luciferase® Reporter Assay System was chosen to be a tool for this study. The term of "dual reporter" refers to "experimental reporter", which correlates with the effect of specific experimental conditions and "control reporter", which provides an internal control and serves as the baseline response. These 2 reporters are simultaneous co-transfected, expressed and measured within single sample. The experimental reporter activity is normalized to control reporter activity to minimize experimental variability caused by differences in cell viability or transfection efficiency. The experimental reporter activity is due to the activity of firefly (*Photinus pyralis*) luciferase whist control reporter activity is due to the activity of *Renilla* (*Renilla reniformis)* luciferase. In this system, fiefly luciferase and *Renilla* luciferase are engineered in pGL3-basic plasmid and pRL-SV40 plasmid, respectively. All steps of luciferase reporter gene assay were performed according to the manufacturer's

instruction. Briefly, cells were lysed with sufficient volume of Passive Lysis Buffer (PLB) by direct adding on the cells in cultured plate. Then, cultured plate was placed on the rocking platform with gently rocking and incubated at room temperature for 15 min. To ensure the complete lysis, cells were gently scraped and homogenized by pipetting cells up and down before transferring to the new sterile tube. The luciferase activities were measured sequentially in which fiefly luciferase was firstly measured by adding its substrate (LAR II reagent). After quantifying the firefly luminescence, this reaction was quenched, and the *Renilla* luciferase reaction was simultaneously initiated by adding Stop & Glo® Reagent to the same tube. The Stop & Glo® Reagent stopped the activity of fiefly luciferase and was a substrate for *Renilla* luciferase. The experimental reporter activity was calculated as the formula shown below and the activity was presented as a relative light unit (RLU).

2.8 Chromatin immunoprecipitation (ChIP) assay. To study the interaction between protein and DNA, ChIP assay is a powerful and versatile technique used for studying protein-DNA interactions within the natural chromatin context of the cells (42). In this study, SimpleChIP® Emzymatic Chromatin IP Kit was applied for study the interaction between p65 subunit of NF-ΚB and RANTES promoter. Steps of ChIP assay were followed according to the manufacturer's instruction.

2.9 Real-time PCR array

Total RNA was isolated either from HEK293T expressing DENV NS5 and HEK293T expressing vector control. at 48 h posttransfection using the High Pure RNA isolation kit (Roche). RNAs with an OD_{260nm}/OD_{280nm} absorbance ratio of at least 2.0 were used. Total RNA was reverse-transcribed into cDNA using the RT^2 First strand Kit (SABiosciences), mixed with RT^2 qPCR mastermix containing SYBR Green (SA Biosciences), and aliquoted in equal volumes to each well of the real-time PCR arrays. The Human Cytokine RT² Profiler™ PCR Array (SA Biosciences) has 84 genes related to the cytokine pathway. The real-time PCR cycling program was run on a Roche Light Cycler 480. The threshold cycle (Ct) of each gene was determined and subsequently analysed by RT^2 Profiler PCR Array data Analysis software (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php).

Results and Discussion

Part I: Expression of cytokine genes between mock-infected and DENV-infected HepG2 cells using a real-time PCR array and the potential therapeutic agent, namely compound A, in reduction of massive cytokine production in DENV-infected cells.

1.1 Cytokine expression profile in DENV-infected HepG2 cells

The average percent of HepG2 cells infected with MOI of 5 at 24 h, 36 h and 48 h were 80, 92, and 90, respectively (Fig.1A, 1B). We selected DENV-infected HepG2 cells at 24 h post-infection for accessing changes in expression of cytokine genes as DENV-infected HepG2 cells underwent cell death at 36 h and 48h post infection (Fig.1C).

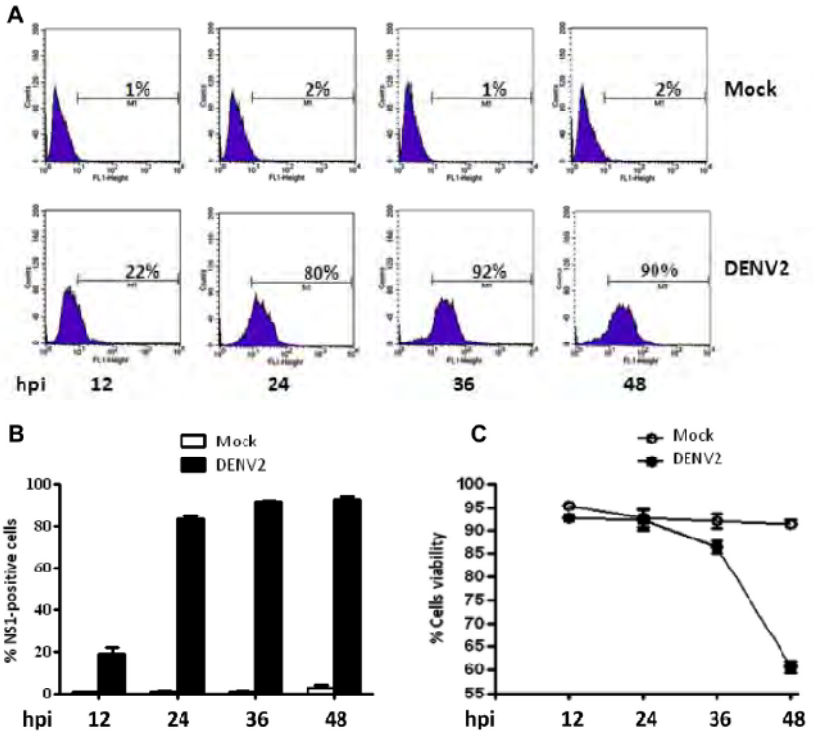

Figure 1

Total RNA was prepared from mock-infected HepG2 cells or DENV-infected HepG2 cells and used to probe microarrays. Table 1 and Fig.2A demonstrated the genes exhibiting a change in cytokine gene expression more than 2 fold.

Table 1

Gene No.	Fold change	Gene name	Gene description
1	660.77	CXCL10	Chemokine (C-X-C motif) ligand 10
2	191.08	CCL5	Chemokine (C-C motif) ligand 5
3	168.66	TNF-α	Tumor necrosis factor (TNF superfamily, member2)
4	71.41	CCL4	Chemokine (C-C motif) ligand 4
5	44.57	CXCL3	Chemokine (C-X-C motif) ligand 3
6	24.39	CXCL2	Chemokine (C-X-C motif) ligand 2
7	22.91	LTB	Lymphotoxin beta (TNF superfamily, member3)
8	22.75	IL8	Interleukin 8
9	17.85	CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)
10	14.01	CXCL5	Chemokine (C-X-C motif) ligand 5
11	11.78	FOS	V-fos FBJ murine osteosarcoma viral oncogene homolog
12	11.46	LTA	Lymphotoxin alpha (TNF superfamily, member1)
13	6.76	IL23A	Interleukin 23, alpha subunit p19
14	4.02	CEBPB	CCAAT/enhancer binding protein (C/EBP), beta
15	3.48	CCL3	Chemokine (C-C motif) ligand 3
16	3.38	CXCL9	Chemokine (C-X-C motif) ligand 9
17	2.84	NOS2A	Nitric oxide synthase 2A (inducible, hepatocytes)
18	2.77	TLR3	Toll-like receptor 3
19	2.46	IL9	Interleukin 9
20	2.23	IL6	Interleukin 6 (interferon beta2)
21	2.16	CCR3	Chemokine (C-C motif) receptor 3
22	2.10	CCL2	Chemokine (C-C motif) ligand 2
23	2.07	TNFSF14	Tumor necrosis factor (ligand) superfamily, member14
24	2.05	IL1F10	Interleukin 1 family, member 10 (theta)

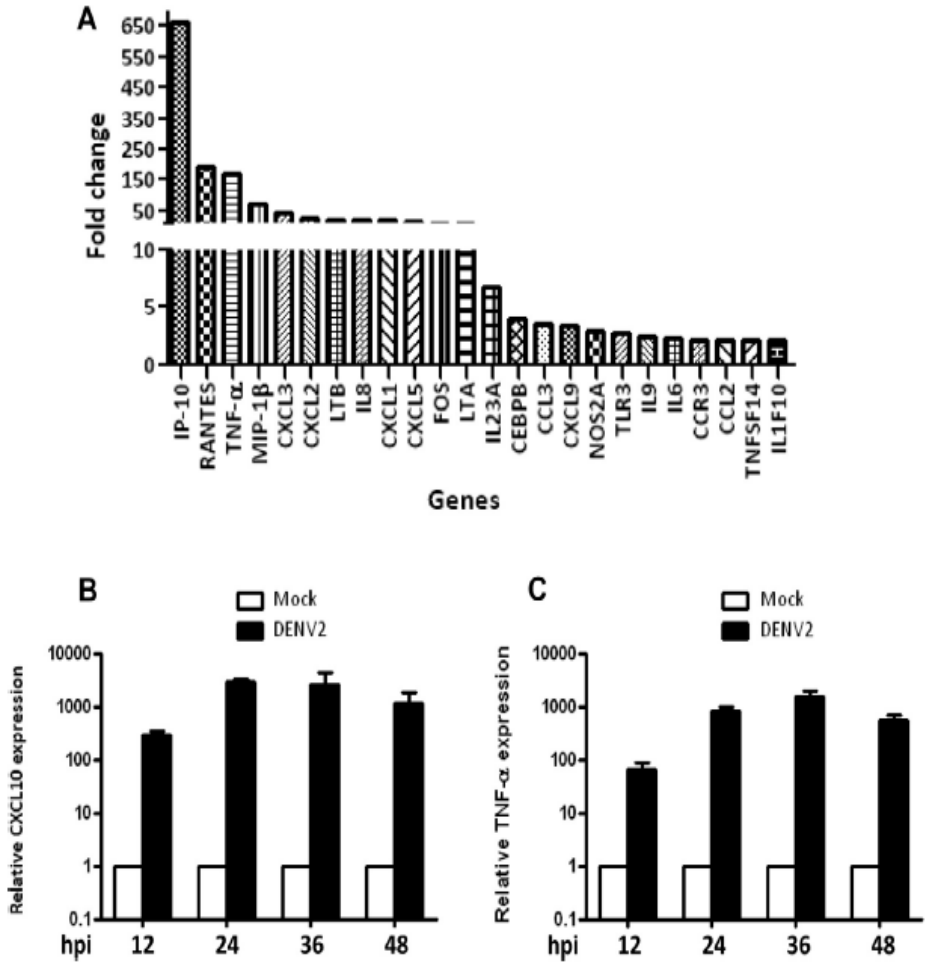

Figure 2

CXCL10 and TNF-α were selected as representatives for DENV-induced cytokines in further analyses, as CXCL10 and TNF-α are the highly up-regulated cytokines in patients with DHF (11, 13, 19, 43, 44). CXCL10 is a potent chemokine elevated during DENV infection. The early induction of CXCL10 during DENV infection plays a role in innate immunity through the recruitment and activation of NK cells (8). However, intrahepatic infiltrating NK and T cells cause liver cell death in DENV infection (45). Our results demonstrated that CXCL10 mRNA and protein expression in DENV-infected HepG2 cells are highly increased post DENV infection, respectively (Fig.2B, 3A, 3C). Also, TNF-α mRNA and protein expression increased significantly post DENV infection, respectively, in DENV-infected HepG2 cells (Fig.2C, 3B, 3D) supporting the massive cytokine secretion followed after severe DENV infection (9-12).

1.2 CpdA reduced DENV-induced CXCL10, and TNF-α secretion in HepG2 cells

Proinflammatory cytokines in DENV infection may increase vascular permeability, damage organs, and lead to hypovolumic shock (46). Glucocorticoids (GCs) are among the most effective anti-inflammatory drugs. Dexamathasone was used to inhibit TNF-α secretion in DENV-infected monocyte cell line (47). However, the side effects of steroidal ligands may deteriorate the patients in clinical setting. We therefore asked whether the dissociated glucocorticoid receptor modulator, CpdA, which has no metabolic side effects as compared to steroidal ligands, would inhibit DENV-induced CXCL10 and TNF-α secretion in HepG2 cells, an approach that might alleviate the DENV-induced cytokine storm. An animal model of DENV-induced liver injury showed the up-regulation of CXCL10. Neutralization of CXCL10 abrogated NK cell recruitment to the liver and diminished liver cell death(45). In addition, intrahepatic CD8+ cells are cytotoxic against DENV-infected cells (45). *In vitro* and *in vivo* models implicate TNF-α in DENV-induced tissue damage (12, 48, 49). CXCL10 and TNF-α secretion form mock or DENV-infected HepG2 cells were measured in the presence or absence of CpdA. The results showed that CpdA significantly reduced DENV-induced CXCL10 and TNF-α secretion at both mRNA and protein levels in a dose-dependent manner (Fig.3A-D). The decreased production of CXCL10 by CpdA may decrease immune cell migration, thereby decreasing the cytokine secretion in the DENV-infected liver.

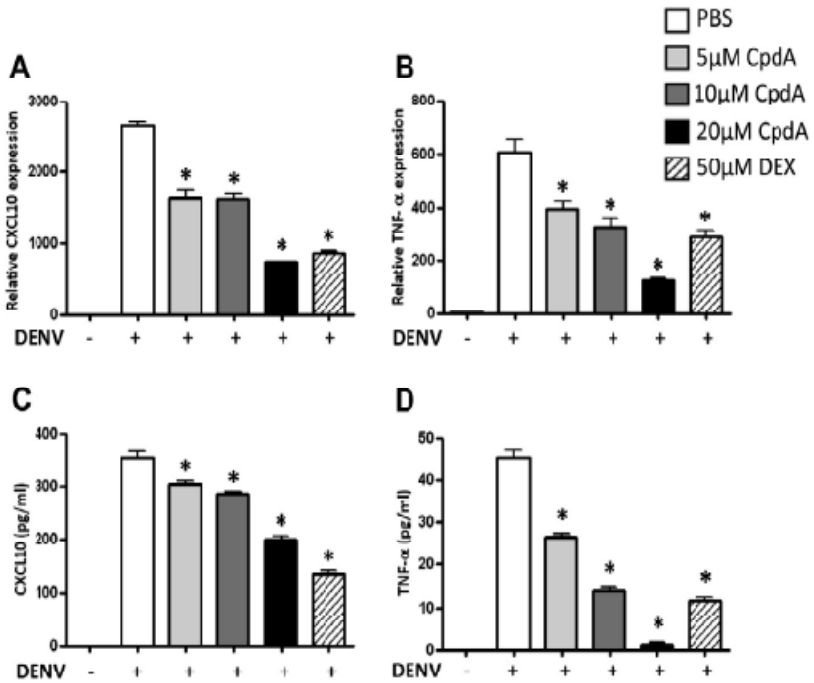

Figure 3

We then asked whether CpdA would inhibit DENV-induced leukocyte migration. As expected, CpdA reduced DENV-induced leukocyte migration about 3-fold in a dose-dependent manner (Fig. 4A). Therefore, CpdA may have a therapeutic potential in decreasing massive CXCL10 and TNF-α during DENV infection.

1.3 CpdA reduced DENV production in HepG2 cells

The candidate for a therapeutic agent against DENV should not only decrease massive cytokine production in DENV-infected cells, but should also decrease DENV production in the DENV-infected cells. DENV replication in HepG2 cells activates NF-κB concomitantly with viral protein synthesis, before the appearance of apoptotic cells (50). We therefore asked whether CpdA, which interferes with NF-κB-driven expression, would inhibit DENV production in HepG2 cells, an approach that might decrease the production of DENV in infected hepatocytes. The results showed that 20 μM of CpdA significantly decreased yield of viral progeny a minimum of 30% and 50 μM of CpdA significantly decreased yield of viral progeny a minimum of 70% demonstrating for the first time that CpdA inhibited virus production in HepG2 cells (Fig. 4B). The decreased DENV production was not from the decreased viable cells as the number of mock-infected and DENV-infected HepG2 cells was similar at the 24 h post infection (Fig. 4C). Similar to our study, addition of reduced glutathione (GSH) to the medium of DENV-infected HepG2 cells was also shown to inhibit activity of NF-κB thereby decreasing DENV production(51). It is interesting to ask and further investigate why inhibition of NF-kB decreases DENV production. This study revealed the up-regulation of several cytokine genes, including CXCL10 and TNF-α, during DENV infection in HepG2 cells. CpdA reduced massive DENV-induced secretion of CXCL10 and TNF-α cytokines, decreased leukocyte migration and decreased DENV production in HepG2 cells. The molecular mechanisms how CpdA inhibits NF-κB signaling in DENV infection merits further investigation.

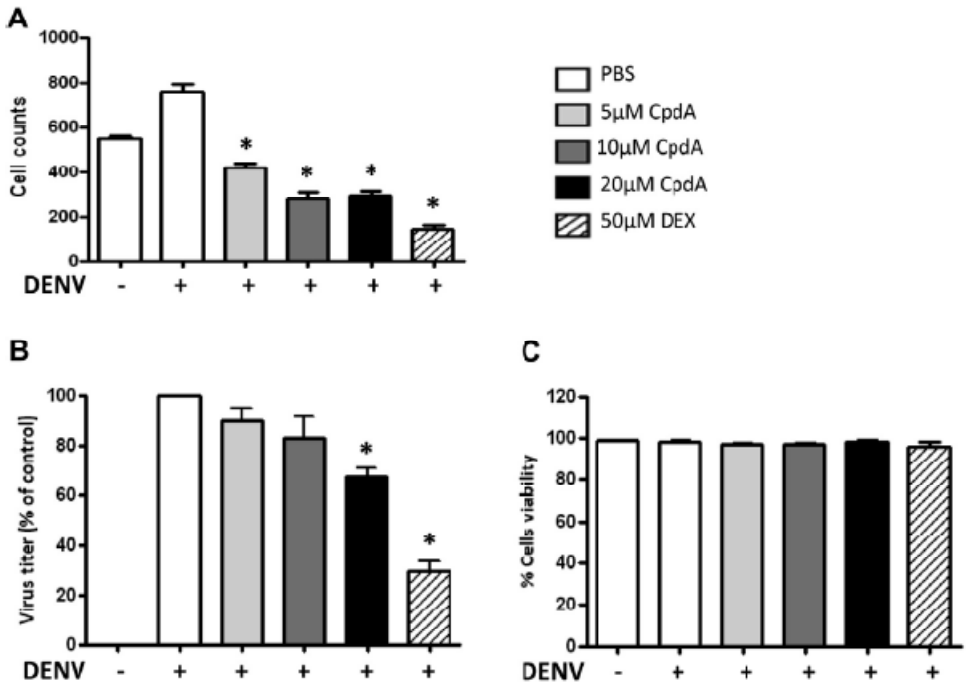

Figure 4

Part II: Functional studies for the role of DENV NS5 in cytokine production and inflammatory response

2.1 Identification of DENV NS5 interacting proteins by a yeast two-hybrid screen

To identify human proteins that interacted with DENV NS5, we screened over 10^7 clones from a HeLa cDNA library using DENV NS5 as bait. Thirty putative positive clones were obtained. To verify the interaction, recovered prey plasmids were introduced into yeast strain RFY 231 along with the *lac*Z plasmid and bait plasmid and again tested on the indicator plates. Twelve library plasmids were purified and subjected to DNA sequencing. BLAST analysis revealed that the twelve clones corresponded to the proteins Daxx, Fas-associated factor 1 (FAF1), Calpain 2, Protein phosphatase 1 (PP-1), Splicing factor (SF3a), and Double-strand break repair protein (Mre11A).

The specificity of the interaction between DENV NS5 and Daxx, is shown in Fig. 5A wherein cells containing the DENV NS5 and Daxx, exhibited galactose-dependent leucine prototrophy and *lacZ* expression. Daxx has a strong transcriptional repression activity and can bind to several transcription factors in the nucleus (52, 53). In addition, the majority of Daxx is present in the nucleus similar to that of DENV NS5 (54, 55). Daxx is a 740 amino acid protein that contains amino-terminal amphipathic helices

(PAH1, PAH2), a coiled-coiled domain (CC), an acidic domain (D/E), and a carboxyl-terminal serine/proline/threonine rich domain (S/P/T). Among them, the S/P/T domain can mediate the interaction of Daxx with numerous proteins. The region of Daxx that interacted with DEN NS5 was verified in the prey plasmid by DNA sequencing and BLAST analysis and the 211 carboxyl-terminal residues, which cover the S/P/T domain than can bind to multiple proteins including DENV capsid protein in the nucleus of DENV-infected cells, were identified (56) (Fig. 5B).

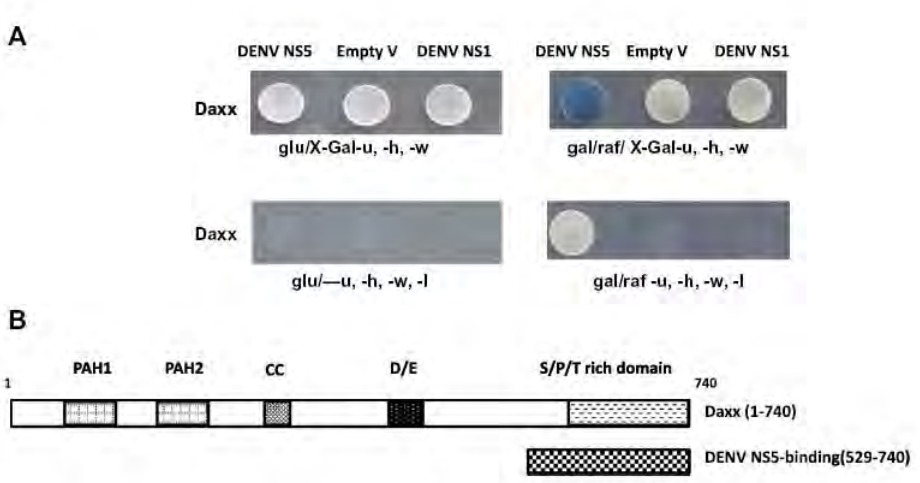

Figure 5

2.2 Interaction of DENV NS5 and Daxx in the nucleus of HEK293 cells expressing DENV NS5

The interaction of DENV NS5 and Daxx was confirmed in mammalian HEK293 cells. Co-immunoprecipitation of DENV NS5 and Daxx was observed in HEK293 cells expressing DENV NS5. Anti-flag antibody co-immunoprecipitated Daxx protein and, conversely, anti-Daxx antibody co-precipitated DENV NS5 (Fig. 6A, B). Co-localization of DENV NS5 and Daxx was evident in the nucleus of HEK293 cells expressing DENV NS5 (Fig. 6C). These data supported the proposed interaction of DENV NS5 and Daxx and further suggested that association occurs mainly in the nucleus of HEK293 cells expressing DENV NS5.

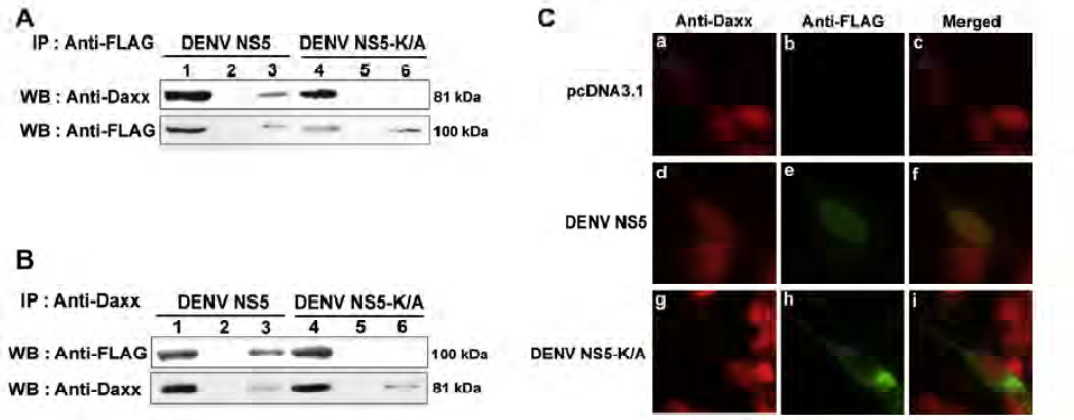

Figure 6

2.3 Interaction DENV NS5 and Daxx modulates RANTES production

DENV NS5 was previously shown to enhance the production of IL-8 (27). In addition, enhancement of TNF-α-stimulated NF-κB activation by DENV NS5 was reported (48). In the present study, the level of DHF-associated cytokines including IL-8, CXCL-9 and RANTES was measured in HEK293 cells expressing DENV NS5 in the presence of TNF-α. The mRNA expression of RANTES was significantly up-regulated in HEK293 cells expressing DENV NS5 compared to empty vector-transfected HEK293 cells (Fig. 7A). RANTES is a member of the C-C chemokine family and its expression was increased both in DENV-infected cell lines and DENV-infected patients (17) and activation of RANTES production by other viral infections was reported to be regulated by NF-κB (57, 58). Therefore, enhancement of TNF-α-stimulated NF-κB activation of RANTES production may modulated by DENV NS5.

Interaction between DENV NS5 and Daxx, which normally interacts with NF-κβ (59), may modulate RANTES production. Therefore, HEK293 cells expressing either DENV NS5 or DENV NS5-K/A were created to assess in the role of NS5 nuclear translocation in Daxx interaction and RANTES production. Site-directed mutagenesis was employed to generate mutant DENV NS5 (K371A, K372A, K387A, K388A, K389A), namely DENV NS5-K/A, Whilst DENV NS5 was present in the nuclei of HEK293 cells expressing DENV NS5, HEK293 cells expressing DENV NS5-K/A had no detectable DENV NS5 protein in the nucleus (Fig. 6C). Secondly, co-immunoprecipitation and co-localization of DENV NS5-K/A and Daxx was examined. Co-immunoprecipitation of DENV NS5 and Daxx was observed only in HEK293 cells expressing DENV NS5, but not in HEK293 cells expressing DENV NS5-K/A. Anti-flag antibody co-

immunoprecipitates Daxx protein and, conversely, anti-Daxx antibody co-precipitated DENV NS5 in HEK293 cells expressing DENV NS5 but not in HEK293 cells expressing DENV NS5-K/A. (Fig. 6A, B). Furthermore, co-localization of DENV NS5 and Daxx was evident in the nucleus of HEK293 cells expressing DENV NS5 but not in HEK293 cells expressing DENV NS5-K/A. (Fig. 6C). Finally, the RANTES production in either HEK293 cells expressing DENV NS5 or DENV NS5-K/A. was measured by real-time RT-PCR and ELISA, respectively. As expected, both mRNA expression and RANTES production were significantly higher in HEK293 cells expressing DENV NS5 than those in HEK293 cells expressing DENV NS5-K/A (Fig. 7B, C). This difference was not due to differences in transfection efficiency or protein expression since DENV NS5 and DENV NS5-K/A were expressed at similar levels as determined by immunofluorescence staining (Fig. 7D). Thus, in the absence of a functional NLS, DENV NS5 could neither translocate into the nucleus nor interact with Daxx to increase RANTES secretion.

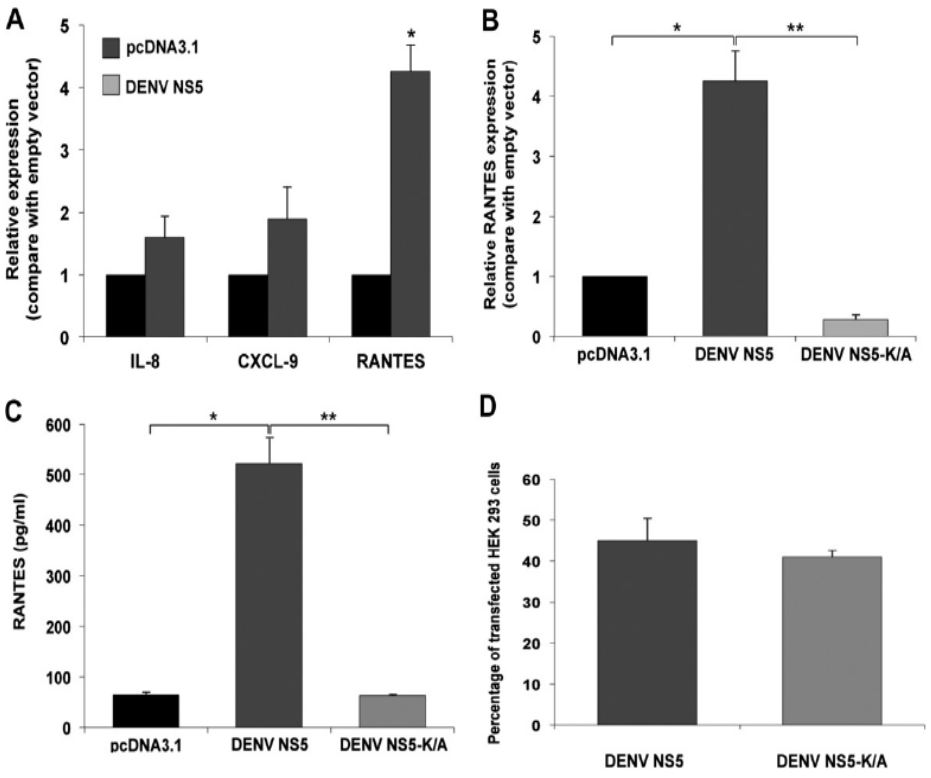

Figure 7

As TNF-α signaling activates NF-κB and RANTES production during DENV infection (13, 14) and NF-κB is known to activate the RANTES promoter (58), DENV NS5 may increase the amount of NF-κB available to activate RANTES expression through its interaction with Daxx, which normally interacts with NF-κB (59) (Fig.8).

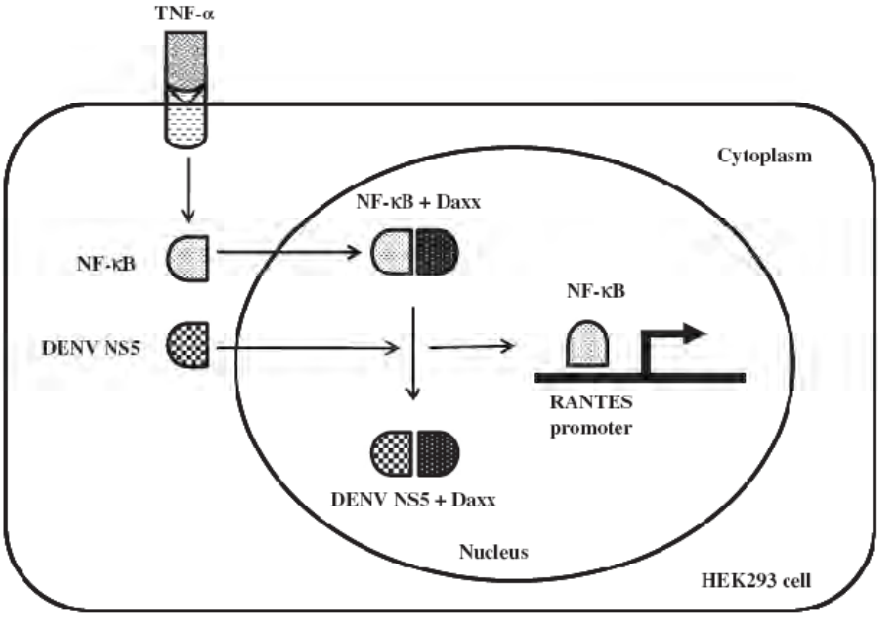

Figure 8

2.4 DENV NS5 increased the amount of NF-κB available to activate RANTES expression

To examine whether NF-κB is a major transcription factor for DENV NS5-activated RANTES promoter, 2 binding sites of NF-κB on RANTES promoter were mutated using PCR-based site-directed mutagenesis as described previously. DENV NS5 plasmid was then transfected in HEK293 cells either with plasmid pGL3-basic or with reporter plasmid contains RANTES promoter or with reporter plasmid contains RANTES promoter with mutated NF-κB binding sites. After 48 h post transfection, RANTES promoter activity was present in term of RLU normalized with DENV NS5 co-transfected with empty pGL3-basic (1). Results shown are typical of three independent experiments. The significant difference was tested with an unpaired *t-test* using GraphPad Prism 5. Figure 9 showed that when both binding sites of NF-κB on

RANTES promoter were mutated, the ability of NS5 to induce the luciferase activity decreased approximately 12 fold compared to that of WT-RANTES promoter.

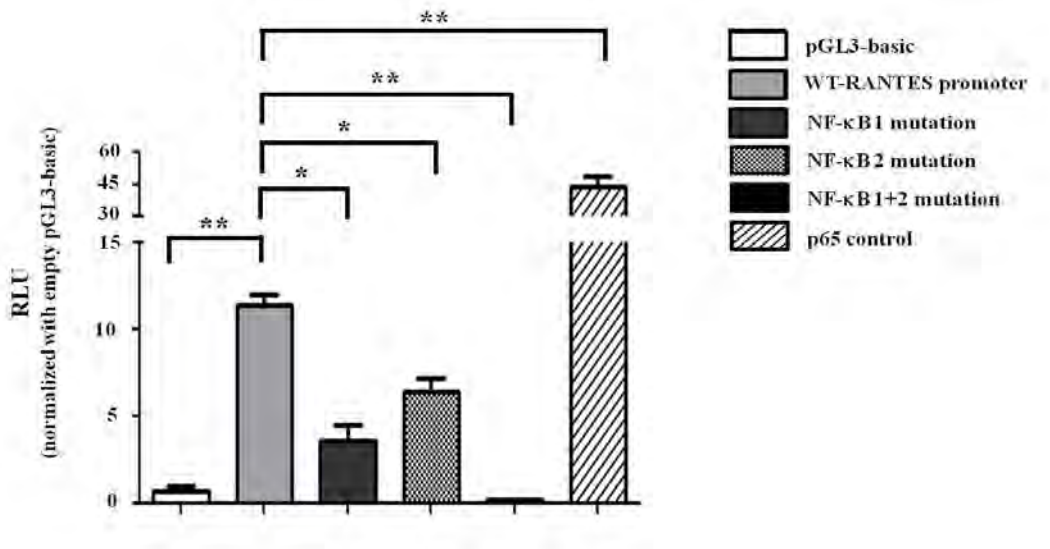

Figure 9

In this study, SimpleChIP® Emzymatic Chromatin IP Kit was applied for study the interaction between p65 subunit of NF-ΚB and RANTES promoter in the presence of DENV NS5. HEK 293 cells were transfected with DENV NS5 plasmid. After 48 h post transfction, cells were subjected of DNA-protein cross-linking, chromatin digestion, and cell sonication. Anti-p65 antibody was used to immunoprecipitate the NF-ΚB-RANTES promoter complex. Then, RANTES promoter fragments were purified and subjected to PCR using primers, which were specific for NF-ΚB binding sites on RANTES promoter. Fig.10 A, and 10B showed the increased band intensity of NF-ΚB binding site on RANTES promoter from HEK 293 cells expressing DENV NS5 immunoprecipitated by anti-p65 (Lane 5) when compared to that from HEK 293 cells expressing empty vector (Lane2). HEK 293 cells expressing DENV NS5 or empty vector immunoprecipited by isotype control were shown in lane 6 and lane 3, respectively.

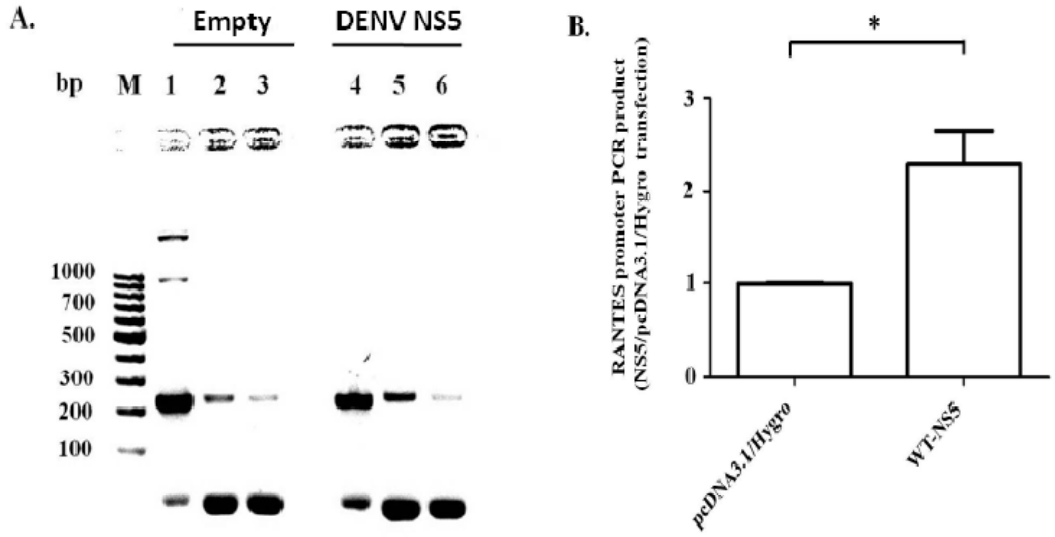

Figure 10

2.5 DENV NS5 increased the expression of other NF-κB-regulated cytokines

We next asked whether DENV NS5 increased the expression of other NF-κB-regulated cytokines beside RANTES. Total RNA was isolated either from HEK293T expressing DENV NS5 and HEK293T expressing vector control at 48 h posttransfection using the High Pure RNA isolation kit (Roche). RNAs with an OD_{260nm}/OD_{280nm} absorbance ratio of at least 2.0 were used. Total RNA was reverse-transcribed into cDNA using the RT^2 First strand Kit (SABiosciences), mixed with RT^2 qPCR mastermix containing SYBR Green (SA Biosciences), and aliquoted in equal volumes to each well of the real-time PCR arrays. The Human Cytokine RT² Profiler™ PCR Array (SA Biosciences) has 84 genes related to the cytokine pathway. The real-time PCR cycling program was run on a Roche Light Cycler 480. The threshold cycle (Ct) of each gene was determined and subsequently analysed by RT^2 Profiler PCR Array data Analysis software (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php). The result showed in Fig.11 that expression of other NF-κB-regulated cytokines including IL-10 and lymphotoxin-α was also up-regulated. These cytokines were also shown to correlated with dengue severity(44, 60).

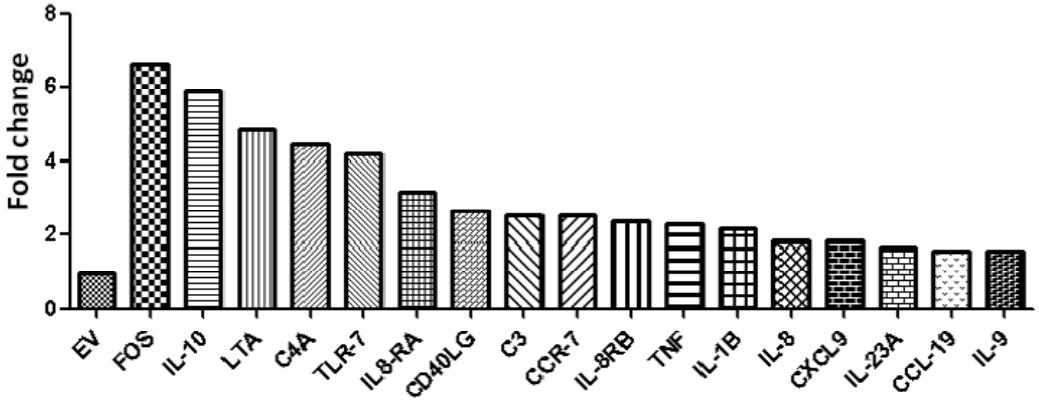

Figure 11

In conclusion, the expression of cytokine genes between mock-infected and DENV-infected HepG2 cells was compared using a real-time PCR array. The highly up-regulated cytokines including CXCL-10, and TNF-α were subsequently verified at both the mRNA and protein levels. The potential therapeutic agent, namely compound A, was used in this study to reduce massive CXCL-10, and TNF-α production. The molecular mechanisms how DENV NS5 contributed to cytokine production were characterized by several methods. Firstly, a yeast two-hybrid assay was performed to identify host proteins interacting with DENV NS5 and a death-domain-associate protein (Daxx), which serves as transcription repressor was identified. Nuclear NS5 interacted with an NF-κB inhibitor, Daxx, thereby liberating NF-κB to activate RANTES promoter as revealed by luciferase reporter assay and ChIP assay. The roles of DENV NS5 in nuclear translocation, Daxx interaction, and RANTES production were tested and DENV NS5 was demonstrated to interact with human Daxx to increase RANTES production. In addition, a real-time PCR array was used and identified several putative NF-κB-regulated cytokines beside RANTES governed by DENV NS5.

References

1. Halstead SB. Dengue. Lancet. 2007 Nov 10;370(9599):1644-52.

2. Halstead SB. Pathogenesis of dengue: challenges to molecular biology. Science. 1988 Jan 29;239(4839):476-81.

3. Kuo CH, Tai DI, Chang-Chien CS, Lan CK, Chiou SS, Liaw YF. Liver biochemical tests and dengue fever. Am J Trop Med Hyg. 1992 Sep;47(3):265-70.

4. Souza LJ, Alves JG, Nogueira RM, Gicovate Neto C, Bastos DA, Siqueira EW, et al. Aminotransferase changes and acute hepatitis in patients with dengue fever: analysis of 1,585 cases. Braz J Infect Dis. 2004 Apr;8(2):156-63.

5. Khongphatthanayothin A, Lertsapcharoen P, Supachokchaiwattana P, Satupan P, Thongchaiprasit K, Poovorawan Y, et al. Hepatosplanchnic circulatory dysfunction in acute hepatic infection: the case of dengue hemorrhagic fever. Shock. 2005 Nov;24(5):407-11.

6. Hsieh MF, Lai SL, Chen JP, Sung JM, Lin YL, Wu-Hsieh BA, et al. Both CXCR3 and CXCL10/IFN-inducible protein 10 are required for resistance to primary infection by dengue virus. J Immunol. 2006 Aug 1;177(3):1855-63.

7. Gil L, Lopez C, Blanco A, Lazo L, Martin J, Valdes I, et al. The cellular immune response plays an important role in protecting against dengue virus in the mouse encephalitis model. Viral Immunol. 2009 Feb;22(1):23-30.

8. Chen JP, Lu HL, Lai SL, Campanella GS, Sung JM, Lu MY, et al. Dengue virus induces expression of CXC chemokine ligand 10/IFN-gamma-inducible protein 10, which competitively inhibits viral binding to cell surface heparan sulfate. J Immunol. 2006 Sep 1;177(5):3185-92.

9. Atrasheuskaya A, Petzelbauer P, Fredeking TM, Ignatyev G. Anti-TNF antibody treatment reduces mortality in experimental dengue virus infection. FEMS Immunol Med Microbiol. 2003 Jan 21;35(1):33-42.

10. Guabiraba R, Marques RE, Besnard AG, Fagundes CT, Souza DG, Ryffel B, et al. Role of the chemokine receptors CCR1, CCR2 and CCR4 in the pathogenesis of experimental dengue infection in mice. PLoS One. 2010;5(12):e15680.

11. Rathakrishnan A, Wang SM, Hu Y, Khan AM, Ponnampalavanar S, Lum LC, et al. Cytokine expression profile of dengue patients at different phases of illness. PLoS One. 2012;7(12):e52215.

12. Yen YT, Chen HC, Lin YD, Shieh CC, Wu-Hsieh BA. Enhancement by tumor necrosis factor alpha of dengue virus-induced endothelial cell production of reactive

nitrogen and oxygen species is key to hemorrhage development. J Virol. 2008 Dec;82(24):12312-24.

13. Hober D, Poli L, Roblin B, Gestas P, Chungue E, Granic G, et al. Serum levels of tumor necrosis factor-alpha (TNF-alpha), interleukin-6 (IL-6), and interleukin-1 beta (IL-1 beta) in dengue-infected patients. Am J Trop Med Hyg. 1993 Mar;48(3):324-31.

14. Avirutnan P, Malasit P, Seliger B, Bhakdi S, Husmann M. Dengue virus infection of human endothelial cells leads to chemokine production, complement activation, and apoptosis. J Immunol. 1998 Dec 1;161(11):6338-46.

15. Simmons CP, Popper S, Dolocek C, Chau TN, Griffiths M, Dung NT, et al. Patterns of host genome-wide gene transcript abundance in the peripheral blood of patients with acute dengue hemorrhagic fever. J Infect Dis. 2007 Apr 15;195(8):1097-107.

16. Libraty DH, Endy TP, Houng HS, Green S, Kalayanarooj S, Suntayakorn S, et al. Differing influences of virus burden and immune activation on disease severity in secondary dengue-3 virus infections. J Infect Dis. 2002 May 1;185(9):1213-21.

17. Lin YL, Liu CC, Chuang JI, Lei HY, Yeh TM, Lin YS, et al. Involvement of oxidative stress, NF-IL-6, and RANTES expression in dengue-2-virus-infected human liver cells. Virology. 2000 Oct 10;276(1):114-26.

18. Suksanpaisan L, Cabrera-Hernandez A, Smith DR. Infection of human primary hepatocytes with dengue virus serotype 2. J Med Virol. 2007 Mar;79(3):300-7.

19. Fink J, Gu F, Ling L, Tolfvenstam T, Olfat F, Chin KC, et al. Host gene expression profiling of dengue virus infection in cell lines and patients. PLoS Negl Trop Dis. 2007;1(2):e86.

20. Conceicao TM, El-Bacha T, Villas-Boas CS, Coello G, Ramirez J, Montero-Lomeli M, et al. Gene expression analysis during dengue virus infection in HepG2 cells reveals virus control of innate immune response. J Infect. 2009 Jan;60(1):65-75.

21. Ashour J, Laurent-Rolle M, Shi PY, Garcia-Sastre A. NS5 of dengue virus mediates STAT2 binding and degradation. J Virol. 2009 Jun;83(11):5408-18.

22. Ashour J, Morrison J, Laurent-Rolle M, Belicha-Villanueva A, Plumlee CR, Bernal-Rubio D, et al. Mouse STAT2 restricts early dengue virus replication. Cell Host Microbe. 2010 Nov 18;8(5):410-21.

23. Rodriguez-Madoz JR, Belicha-Villanueva A, Bernal-Rubio D, Ashour J, Ayllon J, Fernandez-Sesma A. Inhibition of the type I interferon response in human dendritic cells

by dengue virus infection requires a catalytically active NS2B3 complex. J Virol. 2010 Oct;84(19):9760-74.

24. Mazzon M, Jones M, Davidson A, Chain B, Jacobs M. Dengue virus NS5 inhibits interferon-alpha signaling by blocking signal transducer and activator of transcription 2 phosphorylation. J Infect Dis. 2009 Oct 15;200(8):1261-70.

25. Munoz-Jordan JL, Laurent-Rolle M, Ashour J, Martinez-Sobrido L, Ashok M, Lipkin WI, et al. Inhibition of alpha/beta interferon signaling by the NS4B protein of flaviviruses. J Virol. 2005 Jul;79(13):8004-13.

26. Kelley JF, Kaufusi PH, Volper EM, Nerurkar VR. Maturation of dengue virus nonstructural protein 4B in monocytes enhances production of dengue hemorrhagic fever-associated chemokines and cytokines. Virology. 2011 Sep 15;418(1):27-39.

27. Medin CL, Fitzgerald KA, Rothman AL. Dengue virus nonstructural protein NS5 induces interleukin-8 transcription and secretion. J Virol. 2005 Sep;79(17):11053-61.

28. Egloff MP, Benarroch D, Selisko B, Romette JL, Canard B. An RNA cap (nucleoside-2'-O-)-methyltransferase in the flavivirus RNA polymerase NS5: crystal structure and functional characterization. EMBO J. 2002 Jun 3;21(11):2757-68.

29. Forwood JK, Brooks A, Briggs LJ, Xiao CY, Jans DA, Vasudevan SG. The 37-amino-acid interdomain of dengue virus NS5 protein contains a functional NLS and inhibitory CK2 site. Biochem Biophys Res Commun. 1999 Apr 21;257(3):731-7.

30. Yap TL, Xu T, Chen YL, Malet H, Egloff MP, Canard B, et al. Crystal structure of the dengue virus RNA-dependent RNA polymerase catalytic domain at 1.85-angstrom resolution. J Virol. 2007 May;81(9):4753-65.

31. Pryor MJ, Rawlinson SM, Butcher RE, Barton CL, Waterhouse TA, Vasudevan SG, et al. Nuclear localization of dengue virus nonstructural protein 5 through its importin alpha/beta-recognized nuclear localization sequences is integral to viral infection. Traffic. 2007 Jul;8(7):795-807.

32. Noisakran S, Sengsai S, Thongboonkerd V, Kanlaya R, Sinchaikul S, Chen ST, et al. Identification of human hnRNP C1/C2 as a dengue virus NS1-interacting protein. Biochem Biophys Res Commun. 2008 Jul 18;372(1):67-72.

33. Strober W. Trypan blue exclusion test of cell viability. Curr Protoc Immunol. 2001 May;Appendix 3:Appendix 3B.

34. Scandella E, Men Y, Legler DF, Gillessen S, Prikler L, Ludewig B, et al. CCL19/CCL21-triggered signal transduction and migration of dendritic cells requires prostaglandin E2. Blood. 2004 Mar 1;103(5):1595-601.

35. Jirakanjanakit N, Sanohsomneing T, Yoksan S, Bhamarapravati N. The micro-focus reduction neutralization test for determining dengue and Japanese encephalitis neutralizing antibodies in volunteers vaccinated against dengue. Trans R Soc Trop Med Hyg. 1997 Sep-Oct;91(5):614-7.

36. Brent R, Finley RL, Jr. Understanding gene and allele function with two-hybrid methods. Annu Rev Genet. 1997;31:663-704.

37. Sriburi R, Keelapang P, Duangchinda T, Pruksakorn S, Maneekarn N, Malasit P, et al. Construction of infectious dengue 2 virus cDNA clones using high copy number plasmid. J Virol Methods. 2001 Mar;92(1):71-82.

38. Orr-Weaver TL, Szostak JW. Yeast recombination: the association between double-strand gap repair and crossing-over. Proc Natl Acad Sci U S A. 1983 Jul;80(14):4417-21.

39. Limjindaporn T, Mairiang D, Khunchai S, Wongwiwat W. A novel yeast two hybrid vector, pEG-NRT, for protein protein interaction studies. Siriraj Med J. 2007;59((suppl 2)):175-7.

40. Gyuris J, Golemis E, Chertkov H, Brent R. Cdi1, a human G1 and S phase protein phosphatase that associates with Cdk2. Cell. 1993 Nov 19;75(4):791-803.

41. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001 Dec;25(4):402-8.

42. Orlando V. Mapping chromosomal proteins in vivo by formaldehyde-crosslinked-chromatin immunoprecipitation. Trends in biochemical sciences. [Research Support, Non-U.S. Gov't
Review]. 2000 Mar;25(3):99-104.

43. Dejnirattisai W, Duangchinda T, Lin CL, Vasanawathana S, Jones M, Jacobs M, et al. A complex interplay among virus, dendritic cells, T cells, and cytokines in dengue virus infections. J Immunol. 2008 Nov 1;181(9):5865-74.

44. Malavige GN, Huang LC, Salimi M, Gomes L, Jayaratne SD, Ogg GS. Cellular and cytokine correlates of severe dengue infection. PLoS One. 2012;7(11):e50387.

45. Sung JM, Lee CK, Wu-Hsieh BA. Intrahepatic infiltrating NK and CD8 T cells cause liver cell death in different phases of dengue virus infection. PLoS One. 2012;7(9):e46292.

46. Green S, Rothman A. Immunopathological mechanisms in dengue and dengue hemorrhagic fever. Curr Opin Infect Dis. 2006 Oct;19(5):429-36.

47. Reis SR, Sampaio AL, Henriques M, Gandini M, Azeredo EL, Kubelka CF. An in vitro model for dengue virus infection that exhibits human monocyte infection, multiple cytokine production and dexamethasone immunomodulation. Mem Inst Oswaldo Cruz. 2007 Dec;102(8):983-90.

48. Wati S, Rawlinson SM, Ivanov RA, Dorstyn L, Beard MR, Jans DA, et al. Tumour necrosis factor alpha (TNF-{alpha}) stimulation of cells with established dengue virus type 2 infection induces cell death that is accompanied by a reduced ability of TNF-{alpha} to activate nuclear factor {kappa}B and reduced sphingosine kinase-1 activity. J Gen Virol. 2011 Apr;92(Pt 4):807-18.

49. Nagila A, Netsawang J, Suttitheptumrong A, Morchang A, Khunchai S, Srisawat C, et al. Inhibition of p38MAPK and CD137 signaling reduce dengue virus-induced TNF-alpha secretion and apoptosis. Virol J. 2013;10(1):105.

50. Marianneau P, Cardona A, Edelman L, Deubel V, Despres P. Dengue virus replication in human hepatoma cells activates NF-kappaB which in turn induces apoptotic cell death. J Virol. 1997 Apr;71(4):3244-9.

51. Tian Y, Jiang W, Gao N, Zhang J, Chen W, Fan D, et al. Inhibitory effects of glutathione on dengue virus production. Biochem Biophys Res Commun. 2010 Jul 2;397(3):420-4.

52. Michaelson JS, Bader D, Kuo F, Kozak C, Leder P. Loss of Daxx, a promiscuously interacting protein, results in extensive apoptosis in early mouse development. Genes Dev. 1999 Aug 1;13(15):1918-23.

53. Torii S, Egan DA, Evans RA, Reed JC. Human Daxx regulates Fas-induced apoptosis from nuclear PML oncogenic domains (PODs). EMBO J. 1999 Nov 1;18(21):6037-49.

54. Yang X, Khosravi-Far R, Chang HY, Baltimore D. Daxx, a novel Fas-binding protein that activates JNK and apoptosis. Cell. 1997 Jun 27;89(7):1067-76.

55. Chang HY, Nishitoh H, Yang X, Ichijo H, Baltimore D. Activation of apoptosis signal-regulating kinase 1 (ASK1) by the adapter protein Daxx. Science. 1998 Sep 18;281(5384):1860-3.

56. Limjindaporn T, Netsawang J, Noisakran S, Thiemmeca S, Wongwiwat W, Sudsaward S, et al. Sensitization to Fas-mediated apoptosis by dengue virus capsid protein. Biochem Biophys Res Commun. 2007 Oct 19;362(2):334-9.

57. Genin P, Algarte M, Roof P, Lin R, Hiscott J. Regulation of RANTES chemokine gene expression requires cooperativity between NF-kappa B and IFN-regulatory factor transcription factors. J Immunol. 2000 May 15;164(10):5352-61.

58. Moriuchi H, Moriuchi M, Fauci AS. Nuclear factor-kappa B potently up-regulates the promoter activity of RANTES, a chemokine that blocks HIV infection. J Immunol. 1997 Apr 1;158(7):3483-91.

59. Park J, Lee JH, La M, Jang MJ, Chae GW, Kim SB, et al. Inhibition of NF-kappaB acetylation and its transcriptional activity by Daxx. J Mol Biol. 2007 Apr 27;368(2):388-97.

60. Malavige GN, Gomes L, Alles L, Chang T, Salimi M, Fernando S, et al. Serum IL-10 as a marker of severe dengue infection. BMC Infect Dis. 2013;13(1):341.

ผลงาน (Outputs)

1. ผลงานตีพิมพ์ (Publications)

1.1 Role of cathepsin B in dengue virus-mediated apoptosis. Morchang A, Panaampon J, Suttitheptumrong A, Yasamut U, Noisakran S, Yenchitsomanus PT, Limjindaporn T. Biochem Biophys Res Commun. 2013 Aug 16;438(1):20-5.

1.2 Compound A, a dissociated glucocorticoid receptor modulator, reduces dengue virus-induced cytokine secretion and dengue virus production. Suttitheptumrong A, Khunchai S, Panaampon J, Yasamut U, Morchang A, Puttikhunt C, Noisakran S, Haegeman G, Yenchitsomanus PT, Limjindaporn T. Biochem Biophys Res Commun. 2013 Jun 28;436(2):283-8.

1.3 Inhibition of p38MAPK and CD137 signaling reduce dengue virus-induced TNF-α secretion and apoptosis. Nagila A, Netsawang J, Suttitheptumrong A, Morchang A, Khunchai S, Srisawat C, Puttikhunt C, Noisakran S, Yenchitsomanus PT, Limjindaporn T. Virol J. 2013 Apr 4;10:105.

1.4 Identification of new protein interactions between dengue fever virus and its hosts, human and mosquito. Mairiang D, Zhang H, Sodja A, Murali T, Suriyaphol P, Malasit P, Limjindaporn T, Finley RL Jr. PLoS One. 2013;8(1):e53535.

1.5 Interaction of dengue virus nonstructural protein 5 with Daxx modulates RANTES production. Khunchai S, Junking M, Suttitheptumrong A, Yasamut U, Sawasdee N, Netsawang J, Morchang A, Chaowalit P, Noisakran S, Yenchitsomanus PT, Limjindaporn T. Biochem Biophys Res Commun. 2012 Jun 29;423(2):398-403.

1.6 Role of CD137 signaling in dengue virus-mediated apoptosis. Nagila A, Netsawang J, Srisawat C, Noisakran S, Morchang A, Yasamut U, Puttikhunt C, Kasinrerk W, Malasit P, Yenchitsomanus PT, Limjindaporn T. Biochem Biophys Res Commun. 2011 Jul 8;410(3):428-33.

2. การเสนอผลงานวิชาการ (Presentations)

2.1 การเสนอผลงานวิชาการแบบบรรยายเรื่อง “Viral protein interaction with molecular chaperone to regulate infection “ การประชุมวิชาการHuman Genomics and Molecular Biology 2010. 13 ธันวาคม พ.ศ. 2554

2.2 การเสนอผลงานวิชาการแบบบรรยายเรื่อง “Cell death gene expression profile: role of RIPK2 in dengue virus-mediated apoptosis“ การประชุมวิชาการ Human Genomics and Molecular Biology 2011. 13 ธันวาคม พ.ศ. 2554

2.3 การนำเสนอผลงานทางวิชาการแบบบรรยายเรื่อง “CD137 Signaling Induces Cytokines to Aggravate Dengue Virus-Induced Apoptosis” ในงานประชุมวิชาการระดับนานาชาติ Singapore International Conference on Dengue and Emerging Infection ระหว่างวันที่ 21-23 พฤศจิกายน 2555

2.4 การบรรยายเกี่ยวกับโรคไข้เลือดออกในหัวข้อเรื่อง “The Role of Dengue Virus Capsid Protein in Apoptosis “ ในวันที่ 20 พฤศจิกายน 2555 ตามคำเชิญของภาควิชาจุลชีววิทยา มหาวิทยาลัยแห่งชาติสิงคโปร์ (National University of Singapore)

2.5 การเสนอผลงานวิชาการแบบบรรยายเรื่อง “Interaction of dengue virus nonstructural protein 5 with Daxx modulates RANTES production“ การประชุมวิชาการนักวิจัยรุ่นใหม่พบเมธีวิจัยอาวุโส สกว. ครั้งที่ 12, 10-12 ตุลาคม พ.ศ. 2555

2.6 การเสนอผลงานวิชาการแบบบรรยายเรื่อง “Role of p38MAPK in dengue virus-mediated apoptosis“ การประชุมวิชาการนานาชาติ International Conference in Medicine and Public Health to Commemorate 150th Anniversary of the Birth of Queen Sri Savarindira, Faculty of Medicine Siriraj Hospital, Mahidol University. 17-21 กันยายน พ.ศ. 2555

2.7 การเสนอผลงานวิชาการแบบบรรยายเรื่อง “2-(4-acetoxyphenyl)-2-chloro-*N*-methyl-ethylammonium chloride reduces production of MIP-1β in dengue virus-infected HepG2 cells ” การประชุมวิชาการ 28th National Graduate Research Conference, Assumption University of Thailand, 28-29 มิถุนายน 2556

2.8 การเสนอผลงานวิชาการแบบโปสเตอร์เรื่อง “Cytokine gene expression profie in DENV-infected HepG2 cells“การประชุมวิชาการนานาชาติ International Conference in Medicine and Public Health to Commemorate 150th Anniversary of the Birth of Queen Sri Savarindira, Faculty of Medicine Siriraj Hospital, Mahidol University. 17-21 กันยายน พ.ศ. 2555

2.9 การเสนอผลงานวิชาการแบบโปสเตอร์เรื่อง “ Compound A inhibits cytokine and chemokine secretion of DENV-infected HepG2 cells” ในงานประชุมวิชาการระดับนานาชาติ Molecular Medicine Conference 2012 ระหว่างวันที่ 19-22 ธันวาคม 2555

2.10 การเสนอผลงานวิชาการแบบโปสเตอร์เรื่อง “ Curcumin attenuated DENV-mediated apoptosis“ การประชุมวิชาการนานาชาติ International Anatomical Sciences and Cell Biology Conference, Chiang Mai, 6-8 ธันวาคม พ.ศ. 2555

2.11 การเสนอผลงานวิชาการแบบโปสเตอร์เรื่อง “ The role of cathepsin in dengue virus-induced apoptosis “ การประชุมวิชาการโครงการปริญญาเอกกาญจนาภิเษกครั้งที่ 14, 5-7 เมษายน 2556

2.12 การเสนอผลงานวิชาการแบบโปสเตอร์เรื่อง “ Depletion of adaptor protein complex-1μ subunit alters localization of envelope protein and reduces dengue virus production“ การประชุมวิชาการโครงการปริญญาเอกกาญจนาภิเษกครั้งที่ 14, 5-7 เมษายน 2556

2.13 การเสนอผลงานทางวิชาการแบบโปสเตอร์เรื่อง “The Effect of Methyl Gallate in Dengue Virus-Infected HepG2 cells” การประชุมวิชาการ 28th National Graduate Research Conference, Assumption University of Thailand, 28-29 มิถุนายน 2556

2.14 การเสนอผลงานทางวิชาการแบบโปสเตอร์เรื่อง “The Effect of tyrosin kinase inhibitors in deugne virus-infected lung cell line”การประชุมวิชาการ 28th National Graduate Research Conference, Assumption University of Thailand, 28-29 มิถุนายน 2556

3. การผลิตนักศึกษาบัณฑิตศึกษา (Ph.D. and M.S.)

3.1 Amar Nagila, Ph.D. in Biochemistry, Faculty of Medicine Siriraj Hospital, Mahidol University.

3.2 Attaphan Morchang, M.S. in Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University.

3.3 Umpa Yasamut, M.S. in Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University.

3.4 Aroonroong Suttitheptumrong, M.S. in Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University.

3.5 Sasiprapa Khunchai, M.S. in Anatomy, Faculty of Medicine Siriraj Hospital, Mahidol University.

3.6 Chayadol Tantasit, M.S. in Anatomy, Faculty of Medicine Siriraj Hospital, Mahidol University

Biochemical and Biophysical Research Communications 436 (2013) 283–288

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Compound A, a dissociated glucocorticoid receptor modulator, reduces dengue virus-induced cytokine secretion and dengue virus production

Aroonroong Suttitheptumrong [a,b], Sasiprapa Khunchai [a,b], Jutatip Panaampon [a], Umpa Yasamut [a,b], Atthapan Morchang [a,b], Chunya Puttikhunt [d], Sansanee Noisakran [d], Guy Haegeman [a], Pa-thai Yenchitsomanus [a], Thawornchai Limjindaporn [a,c,*]

[a] Division of Molecular Medicine, Department of Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand
[b] Graduate Program in Immunology, Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand
[c] Department of Anatomy, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand
[d] Medical Biotechnology Unit, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Thailand

ARTICLE INFO

Article history:
Received 8 May 2013
Available online 4 June 2013

Keywords:
Dengue virus
Dissociated glucocorticoid receptor modulator
Compound A
Dengue virus production
Cytokine secretion

ABSTRACT

Dengue Virus (DENV) infection is an important mosquito-borne viral disease and its clinical symptoms range from a predominantly febrile disease, dengue fever (DF), to dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Increased levels of cytokines – the so-called 'cytokine storm', contribute to the pathogenesis of DHF/DSS. In this study, we compared the expression of cytokine genes between mock-infected and DENV-infected HepG2 cells using a real-time PCR array and revealed several up-regulated chemokines and cytokines, including CXCL10 and TNF-α. Compound A (CpdA), a plant-derived phenyl aziridine precursor containing anti-inflammatory action and acting as a dissociated nonsteroidal glucocorticoid receptor modulator, was selected as a candidate agent to modulate secretion of DENV-induced cytokines. CpdA is not a glucocorticoid but has an anti-inflammatory effect with no metabolic side effects as steroidal ligands. CpdA significantly reduced DENV-induced CXCL10 and TNF-α secretion and decreased leukocyte migration indicating for the first time the therapeutic potential of CpdA in decreasing massive immune activation during DENV infection.

1. Introduction

Dengue virus (DENV) infection is a mosquito-borne viral disease and is endemic in several countries [1]. Clinical severity of the disease ranges from a predominantly febrile disease, dengue fever (DF), to dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [2]. The patients with DHF generally present hemorrhagic tendencies, plasma leakage, thrombocytopenia, and hemoconcentration. Furthermore, hepatic dysfunction is a feature of DENV infection [3,4]. Elevation of aminotransferases and hepatic failure are observed in the patients with DHF/DSS [5].

Optimal levels of cytokines have anti-viral effects during DENV infection [6–8]; however, the massive cytokine secretion following severe DENV infection may contribute to the development of the disease rather than protection [9–12]. Increased levels of cytokines – the so-called 'cytokine storm', which relate to the pathogenesis of severe DENV infection, are observed both *in vitro* and *in vivo* models of DENV infection [13–20].

Compound A (CpdA) or 2-(4-acetoxyphenyl)-2-chloro-*N*-methyl-ethylammonium chloride, from the Namibian shrub *Salsola tuberculatiformis*, is a dissociated glucocorticoid receptor (GR) ligand, which has an anti-inflammatory effect as steroidal ligands but has no metabolic side effects [21–23]. CpdA favors GR monomer formation over GR dimer formation and interferes with NF-κB-driven expression of inflammatory cytokines [21–23]. However, CpdA cannot stimulate glucocorticoid-responsive enhancer elements (GREs) in the target gene promoters. Therefore, the metabolic side effects of steroidal ligands do not occur. CpdA was previously shown to reduce inflammation with less side effects in both mouse models of asthma and autoimmune neuritis [22,24].

In this study, we firstly compared the expression of cytokine genes between mock-infected and DENV-infected HepG2 cells using a real-time PCR array. The highly up-regulated cytokines, including CXCL10 and TNF-α, were subsequently verified at both the mRNA and protein levels. As expected, CpdA significantly reduced DENV-induced CXCL10 and TNF-α secretion indicating for the first time that modulating the immune responses by CpdA

* Corresponding author. Address: Department of Anatomy, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand.
E-mail address: thawornchai.lim@mahidol.ac.th (T. Limjindaporn).

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.05.094

may have a therapeutic potential in decreasing massive cytokine production during DENV infection.

2. Materials and methods

2.1. Cell culture and infection of HepG2 cells

Up to 2 $\times 10^6$ HepG2 cells were seeded in a 60 mm dish and cultured for 24 h before infection. HepG2 cells were grown in DMEM medium (Gibco-BRL), supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% non-essential amino acids, 1 mM sodium pyruvate, 100 U/ml of penicillin and streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. HepG2 cells were washed with PBS and infected with DENV serotype 2 (DENV-2) strain 16881 at a multiplicity of infection (MOI) of 5. Cells were collected at 12, 24, 36 or 48 h post-infection. DENV NS1 antibody staining analysed by flow cytometry demonstrated DENV infection [25]. Cell viability was determined by the trypan blue exclusion test [26].

2.2. Real-time PCR array

Total RNA was isolated either from mock-infected HepG2 cells or from DENV-infected HepG2 cells at 24 h post-infection using the High Pure RNA isolation kit (Roche). RNAs with an OD_{260nm}/OD_{280nm} absorbance ratio of at least 2.0 were used. Total RNA was reverse-transcribed into cDNA using the RT^2 First strand Kit (SA Biosciences), mixed with RT^2 qPCR mastermix containing SYBR Green (SA Biosciences), and aliquoted in equal volumes to each well of the real-time PCR arrays. The Human Cytokine RT^2 Profiler™ PCR Array (SA Biosciences) has 84 genes related to the cytokine pathway. The real-time PCR cycling program was run on a Roche Light Cycler 480. The threshold cycle (Ct) of each gene was determined and subsequently analysed by RT^2 Profiler PCR Array data Analysis software (http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php). The highly up-regulated chemokine gene, CXCL10, and cytokine gene, TNF-α, were selected and confirmed at the mRNA level by quantitative Real-time PCR using different sets of primers, including CXCL10 F5′ GAATCGAAGGCCATCAAGAA 3′, CXCL10 R5′ AAGCAGGGTCAGAACATCCA 3′; TNF-αF5′ TGCTTGTTCCTCAGCCTCTT 3′, TNF-α R 5′ ATGGGCTACAGGCTTGTCACT 3′).

2.3. Elisa

The effect of CpdA on CXCL10 and TNF-α production was determined following infection of HepG2 cells with DENV-2 at a MOI of 5 for 2 h, washing and incubation for 24 h in the presence of PBS, 5 μM CpdA, 10 μM CpdA, 20 μM CpdA or 50 μM dexamethasone (Dex) (Sigma Aldrich), respectively. The levels of cytokines were then measured from CpdA-untreated and CpdA-treated DENV-infected HepG2 cells by ELISA (R&D Systems).

2.4. Chemotaxis assay

To test the effect of CpdA on leukocyte migration, the DENV-infected HepG2 cells were treated with CpdA as described in the preceding experiment. The chemotaxis assay was performed using the protocol described previously [27]. Briefly, 600 μl of cell culture supernatant collected from either CpdA-untreated or CpdA-treated DENV-infected HepG2 cells was added to the lower chambers of the transwell cassette (Corning Costar, Lowell, MA, USA). Up to 1 $\times 10^5$ monocyte THP-1 cells were added to the upper chamber and incubated at 37 °C in 5% CO_2 for 6 h. A 500 μl aliquot of the cells migrated to the lower chamber was counted by flow cytometry in a FACCalibur acquiring events for a fixed time period of 60 s using CellQuest software (Becton Dickinson, Basel, Switzerland).

2.5. Focus-forming unit (FFU)

Firstly, viability of mock-infected or DENV-infected HepG2 cells in the presence or absence of CpdA were first measured by trypan blue exclusion test [26]. Secondly, DENV-infected HepG2 cells were treated with CpdA as described in the preceding experiment. Focus-forming unit (FFU) was performed using the protocol described previously [28]. Briefly, titration of DENV was carried out using 96-well plates. Vero cells were seeded at 3 $\times 10^4$ cells/well 24 h prior to infection. Ten-fold serially diluted culture media was added to Vero cells at room temperature (RT) for 2 h. After adsorption, the cells were overlaid with 100 μl of 1.5% gum tragacanth containing 2% FBS in MEM before further incubation at 37 °C for 3 days. The cultures were then fixed with 3.7% formaldehyde for 10 min at RT, treated with 1% Triton X-100 in PBS for another 10 min, washed three times with PBS, and incubated with anti-DENV E (4G2) for 30 min at 37 °C in a humidified chamber. Cells were washed with PBS and incubated with rabbit anti-mouseIgG-HRP at the dilution 1: 1000 for 30 min at 37 °C in a humidified chamber. After triple washing, the substrate solution was added. Stained foci were visible and the reaction was terminated by washing cells with PBS. DENV-infected foci were counted with a light microscope and viral concentration in the supernatant calculated as focus-forming units (FFU) per milliliter.

2.6. Statistical analysis

All data were obtained from three independent experiments and reported as the mean ± SEM. Statistical differences between the groups were tested with an unpaired *t*-test using StatView version 5.0 and *P* value less than 0.05 was considered significant.

3. Results and discussion

3.1. Cytokine expression profile in DENV-infected HepG2 cells

The average percent of HepG2 cells infected with MOI of 5 at 24 h, 36 h and 48 h were 80, 92, and 90, respectively (Fig. 1A). We selected DENV-infected HepG2 cells at 24 h post-infection for accessing changes in expression of cytokine genes as DENV-infected HepG2 cells underwent cell death at 36 h and 48 h post infection (Fig. 1C). Total RNA was prepared from mock-infected HepG2 cells or DENV-infected HepG2 cells and used to probe microarrays. Table 1 and Fig. 2A demonstrated the genes exhibiting a change in cytokine gene expression more than 2-fold. CXCL10 and TNF-α were selected as representatives for DENV-induced cytokines in further analyses, as CXCL10 and TNF-α are the highly up-regulated cytokines in patients with DHF [11,13,19,29,30]. CXCL10 is a potent chemokine elevated during DENV infection. The early induction of CXCL10 during DENV infection plays a role in innate immunity through the recruitment and activation of NK cells [8]. However, intrahepatic infiltrating NK and T cells cause liver cell death in DENV infection [31]. Our results demonstrated that CXCL10 mRNA and protein expression in DENV-infected HepG2 cells are highly increased post DENV infection, respectively (Figs. 2B and 3A and C). Also, TNF-α mRNA and protein expression increased significantly post DENV infection, respectively, in DENV-infected HepG2 cells (Figs. 2C and 3B and D) supporting the massive cytokine secretion followed after severe DENV infection [9–12].

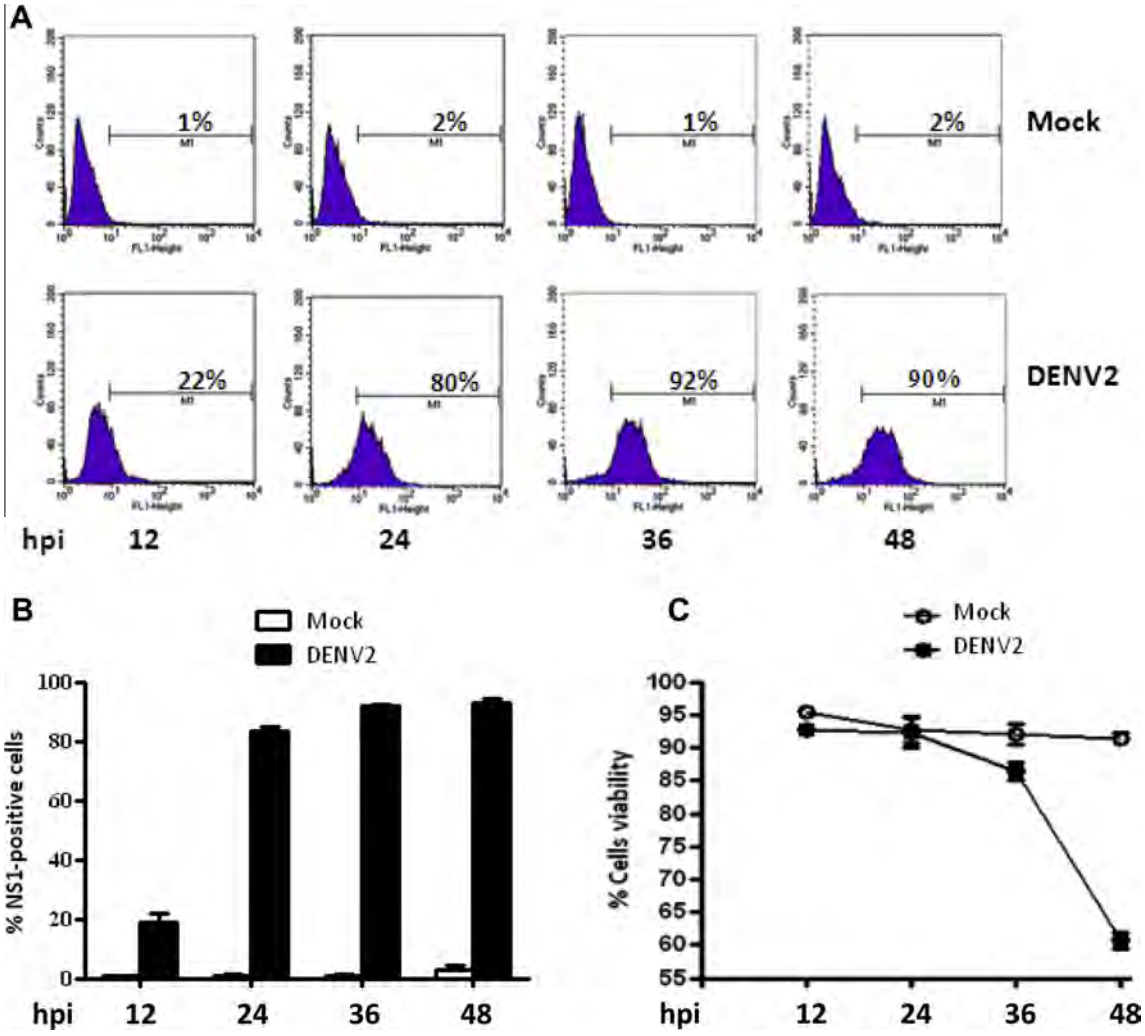

Fig. 1. DENV infection of HepG2 cells. HepG2 cells were infected with DENV serotype 2 at a MOI of 5. DENV NS1 antibody staining was performed at 12, 24, 36 and 48 h post infection and measured by flow cytometry to show DENV infection (A and B). Cell viabilities of mock and DENV-infected HepG2 cells at the indicated times were also determined by trypan blue exclusion test (C). The results are the average of three independent experiments.

3.2. CpdA reduced DENV-induced CXCL10, and TNF-α secretion in HepG2 cells

Proinflammatory cytokines in DENV infection may increase vascular permeability, damage organs, and lead to hypovolumic shock [32]. Glucocorticoids (GCs) are among the most effective anti-inflammatory drugs. Dexamethasone was used to inhibit TNF-α secretion in DENV-infected monocyte cells [33]. However, the side effects of steroidal ligands may deteriorate the patients in clinical setting. We therefore asked whether the dissociated glucocorticoid receptor modulator, CpdA, which has no metabolic side effects as compared to steroidal ligands, would inhibit DENV-induced CXCL10 and TNF-α secretion in HepG2 cells, an approach that might alleviate the DENV-induced cytokine storm. An animal model of DENV-induced liver injury showed the up-regulation of CXCL10. Neutralization of CXCL10 abrogated NK cell recruitment to the liver and diminished liver cell death [31]. In addition, intrahepatic CD8+ cells are cytotoxic against DENV-infected cells [31]. *In vitro* and *in vivo* models implicate TNF-α in DENV-induced tissue damage [12,34,35]. CXCL10 and TNF-α secretion form mock or DENV-infected HepG2 cells were measured in the presence or absence of CpdA. The results showed that CpdA significantly reduced DENV-induced CXCL10 and TNF-α secretion at both mRNA and protein levels in a dose-dependent manner (Fig. 3A–D). The decreased production of CXCL10 by CpdA may decrease immune cell migration, thereby decreasing the cytokine secretion in the DENV-infected liver. We then asked whether CpdA would inhibit DENV-induced leukocyte migration. As expected, CpdA reduced DENV-induced leukocyte migration about 3-fold in a dose-dependent manner (Fig. 4A). Therefore, CpdA may have a therapeutic potential in decreasing massive CXCL10 and TNF-α during DENV infection.

3.3. CpdA reduced DENV production in HepG2 cells

The candidate for a therapeutic agent against DENV should not only decrease massive cytokine production in DENV-infected cells, but should also decrease DENV production in the DENV-infected cells. DENV replication in HepG2 cells activates NF-κB concomitantly with viral protein synthesis, before the appearance of apoptotic cells [36]. We therefore asked whether CpdA, which interferes with NF-κB-driven expression, would inhibit DENV production in HepG2 cells, an approach that might decrease the production of DENV in infected hepatocytes. The results showed that 20 μM of CpdA significantly decreased yield of viral progeny a minimum of 30% and 50 μM of Dex significantly decreased yield of viral progeny a minimum of 70% demonstrating for the first time that CpdA inhibited virus production in HepG2 cells (Fig. 4B). The decreased

Table 1

Gene No.	Fold change	Gene name	Gene description
1	660.77	CXCL10	Chemokine (C-X-C motif) ligand 10
2	191.08	CCL5	Chemokine (C-C motif) ligand 5
3	168.66	TNF-α	Tumor necrosis factor (TNF superfamily, member2)
4	71.41	CCL4	Chemokine (C-C motif) ligand 4
5	44.57	CXCL3	Chemokine (C-X-C motif) ligand 3
6	24.39	CXCL2	Chemokine (C-X-C motif) ligand 2
7	22.91	LTB	Lymphotoxin beta (TNF superfamily, member3)
8	22.75	IL8	Interleukin 8
9	17.85	CXCL1	Chemokine (C-X-C motif) ligand 1 (melanoma growth stimulating activity, alpha)
10	14.01	CXCL5	Chemokine (C-X-C motif) ligand 5
11	11.78	FOS	V-fos FBJ murine osteosarcoma viral oncogene homolog
12	11.46	LTA	Lymphotoxin alpha (TNF superfamily, member1)
13	6.76	IL23A	Interleukin 23, alpha subunit p19
14	4.02	CEBPB	CCAAT/enhancer binding protein (C/EBP), beta
15	3.48	CCL3	Chemokine (C-C motif) ligand 3
16	3.38	CXCL9	Chemokine (C-X-C motif) ligand 9
17	2.84	NOS2A	Nitric oxide synthase 2A (inducible, hepatocytes)
18	2.77	TLR3	Toll-like receptor 3
19	2.46	IL9	Interleukin 9
20	2.23	IL6	Interleukin 6 (interferon beta2)
21	2.16	CCR3	Chemokine (C-C motif) receptor 3
22	2.10	CCL2	Chemokine (C-C motif) ligand 2
23	2.07	TNFSF14	Tumor necrosis factor (ligand) superfamily, member14
24	2.05	IL1F10	Interleukin 1 family, member 10 (theta)

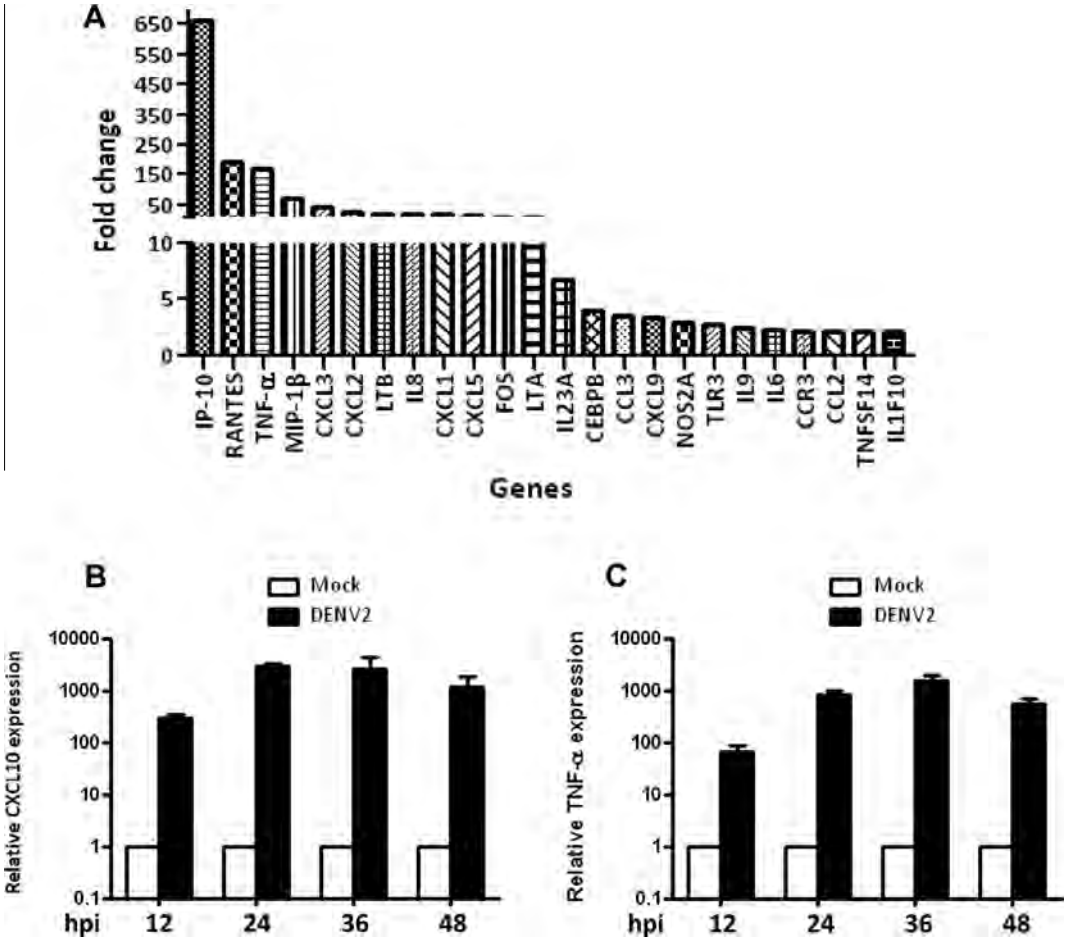

Fig. 2. CXCL10 and TNF-α were among the highly up-regulated cytokines in DENV-infected HepG2 cells. The genes exhibiting a change in cytokine gene expression more than 2-fold was shown in (A). CXCL10 (B) and TNF-α (C) were confirmed at the transcriptional level using different set of primers in DENV-infected HepG2 cells at a MOI of 5 at using real-time PCR at different time points. The results are the average of three independent experiments.

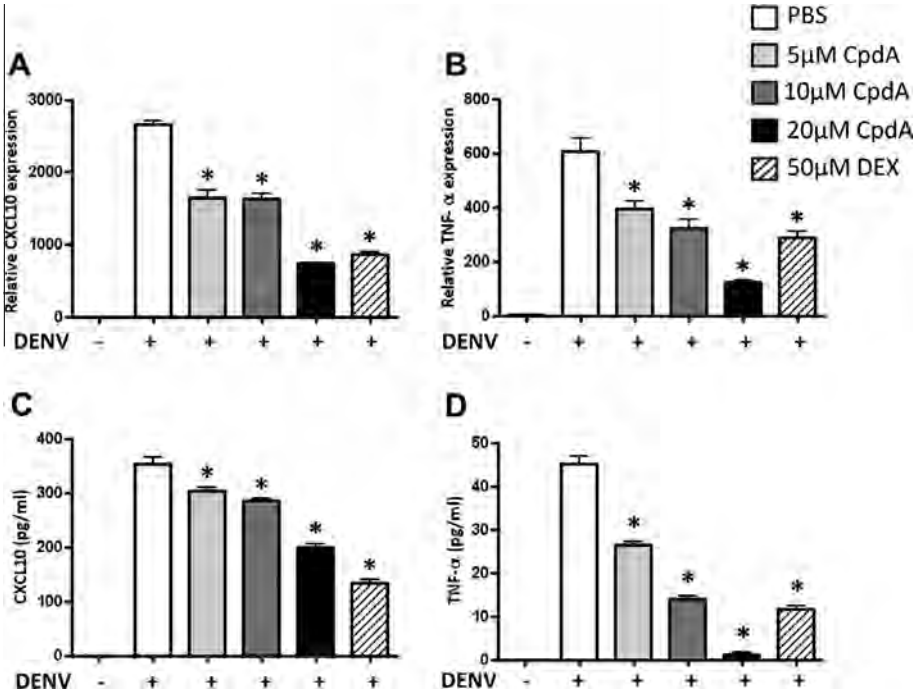

Fig. 3. CpdA reduced DENV-induced CXCL10, and TNF-α secretion. HepG2 cells were infected with DENV at a MOI of 5 and incubated for 24 h in the presence of PBS, 5 μM CpdA, 10 μM CpdA, 20 μM CpdA or 50 μM Dex. The expression levels of CXCL10 and TNF-α were then measured from CpdA-untreated and CpdA-treated DENV-infected HepG2 cells by Real-time PCR (A and B) and ELISA (C and D), respectively. The results are the average of three independent experiments. The asterisks indicate statistically significant differences between samples from CpdA-untreated and CpdA-treated DENV-infected HepG2 cells (*$p < 0.05$).

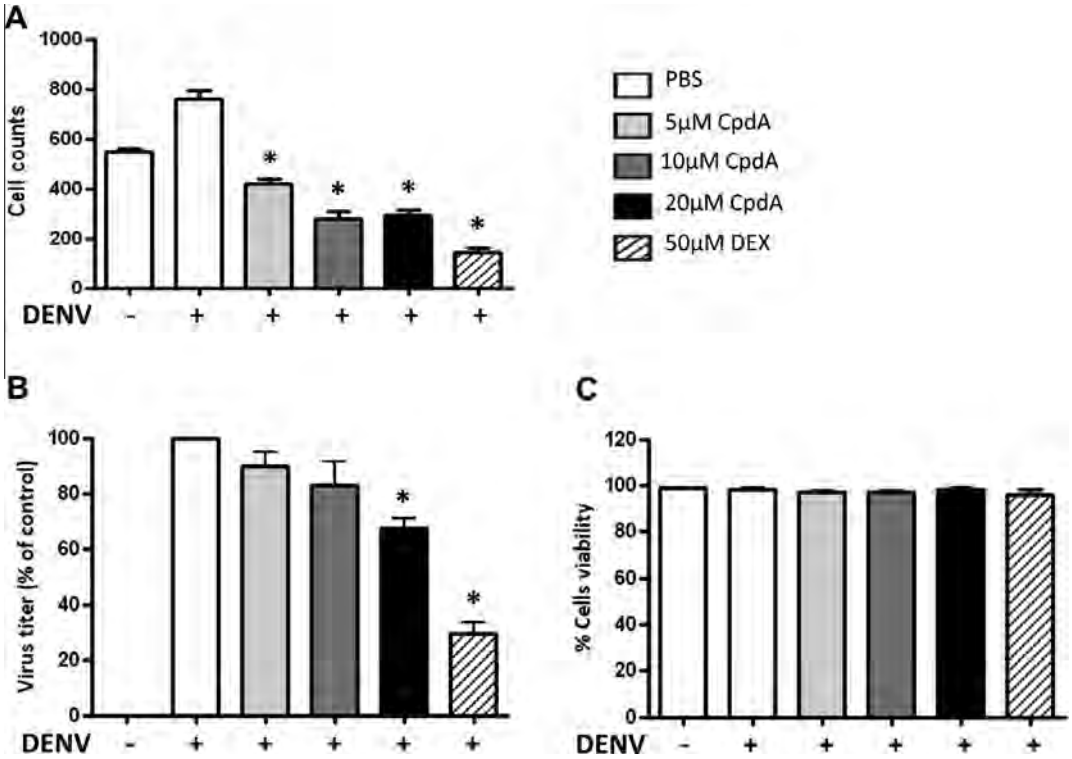

Fig. 4. CpdA reduced the yield of infectious DENV production. HepG2 cells were infected with DENV at a MOI of 5 and incubated for 24 h in the presence of PBS, 5 μM CpdA, 10 μM CpdA, 20 μM CpdA or 50 μM Dex Leukocyte migration was performed using the chemotaxis assay (A). Supernatant was sampled and analyzed for infectious DENV by FFU assay (B). Viability of mock-infected or DENV-infected HepG2 cells in the presence or absence of CpdA was measured by trypan blue exclusion test (C). The results are the average of three independent experiments. The asterisks indicate statistically significant differences between samples from CpdA-untreated and CpdA-treated DENV-infected HepG2 cells (*$p < 0.05$).

DENV production was not from the decreased viable cells as the number of mock-infected and DENV-infected HepG2 cells was similar at the 24 h post infection (Fig. 4C). Similar to our study, addition of reduced glutathione (GSH) to the medium of DENV-infected HepG2 cells was also shown to inhibit activity of NF-κB thereby decreasing DENV production [37]. It is interesting to ask and further investigate why inhibition of NF-kB decreases DENV production.

In summary, this study revealed the up-regulation of several cytokine genes, including CXCL10 and TNF-α, during DENV infection in HepG2 cells. CpdA reduced massive DENV-induced secretion of CXCL10 and TNF-α cytokines, decreased leukocyte migration and decreased DENV production in HepG2 cells. The molecular mechanisms how CpdA inhibits NF-κB signaling in DENV infection merits further investigation.

Acknowledgments

TL is supported by Mid-Career Thailand Research Fund–Commission of Higher Education (TRF-CHE) Research Grant, Siriraj Research Developmental Fund, and Mahidol University Grant. We would like to thank Siriraj Developmental Scholarship for supporting to AS, CHE Joint Ph.D. Program under Strategic Scholarships for Frontier Research Network for supporting to SK, and TRF-Royal Golden Jubilee (TRF-RGJ) Ph.D. Program for supporting to UY and AM. PY is a TRF-Senior Research Scholar.

References

[1] S.B. Halstead, Dengue, Lancet 370 (2007) 1644–1652.
[2] S.B. Halstead, Pathogenesis of dengue: challenges to molecular biology, Science 239 (1988) 476–481.
[3] C.H. Kuo, D.I. Tai, C.S. Chang-Chien, C.K. Lan, S.S. Chiou, Y.F. Liaw, Liver biochemical tests and dengue fever, Am. J. Trop. Med. Hyg. 47 (1992) 265–270.
[4] L.J. Souza, J.G. Alves, R.M. Nogueira, C. Gicovate Neto, D.A. Bastos, E.W. Siqueira, J.T. Souto Filho, A. Cezario Tde, C.E. Soares, C. Carneiro Rda, Aminotransferase changes and acute hepatitis in patients with dengue fever: analysis of 1585 cases, Braz. J. Infect. Dis. 8 (2004) 156–163.
[5] A. Khongphatthanayothin, P. Lertsapcharoen, P. Supachokchaiwattana, P. Satupan, K. Thongchaiprasit, Y. Poovorawan, C. Thisyakorn, Hepatosplanchnic circulatory dysfunction in acute hepatic infection: the case of dengue hemorrhagic fever, Shock 24 (2005) 407–411.
[6] M.F. Hsieh, S.L. Lai, J.P. Chen, J.M. Sung, Y.L. Lin, B.A. Wu-Hsieh, C. Gerard, A. Luster, F. Liao, Both CXCR3 and CXCL10/IFN-inducible protein 10 are required for resistance to primary infection by dengue virus, J. Immunol. 177 (2006) 1855–1863.
[7] L. Gil, C. Lopez, A. Blanco, L. Lazo, J. Martin, I. Valdes, Y. Romero, Y. Figueroa, G. Guillen, L. Hermida, The cellular immune response plays an important role in protecting against dengue virus in the mouse encephalitis model, Viral Immunol. 22 (2009) 23–30.
[8] J.P. Chen, H.L. Lu, S.L. Lai, G.S. Campanella, J.M. Sung, M.Y. Lu, B.A. Wu-Hsieh, Y.L. Lin, T.E. Lane, A.D. Luster, F. Liao, Dengue virus induces expression of CXC chemokine ligand 10/IFN-gamma-inducible protein 10, which competitively inhibits viral binding to cell surface heparan sulfate, J. Immunol. 177 (2006) 3185–3192.
[9] A. Atrasheuskaya, P. Petzelbauer, T.M. Fredeking, G. Ignatyev, Anti-TNF antibody treatment reduces mortality in experimental dengue virus infection, FEMS Immunol. Med. Microbiol. 35 (2003) 33–42.
[10] R. Guabiraba, R.E. Marques, A.G. Besnard, C.T. Fagundes, D.G. Souza, B. Ryffel, M.M. Teixeira, Role of the chemokine receptors CCR1, CCR2 and CCR4 in the pathogenesis of experimental dengue infection in mice, PLoS One 5 (2010) e15680.
[11] A. Rathakrishnan, S.M. Wang, Y. Hu, A.M. Khan, S. Ponnampalavanar, L.C. Lum, R. Manikam, S.D. Sekaran, Cytokine expression profile of dengue patients at different phases of illness, PLoS One 7 (2012) e52215.
[12] Y.T. Yen, H.C. Chen, Y.D. Lin, C.C. Shieh, B.A. Wu-Hsieh, Enhancement by tumor necrosis factor alpha of dengue virus-induced endothelial cell production of reactive nitrogen and oxygen species is key to hemorrhage development, J. Virol. 82 (2008) 12312–12324.
[13] D. Hober, L. Poli, B. Roblin, P. Gestas, E. Chungue, G. Granic, P. Imbert, J.L. Pecarere, R. Vergez-Pascal, P. Wattre, et al., Serum levels of tumor necrosis factor-alpha (TNF-alpha), interleukin-6 (IL-6), and interleukin-1 beta (IL-1 beta) in dengue-infected patients, Am. J. Trop. Med. Hyg. 48 (1993) 324–331.
[14] P. Avirutnan, P. Malasit, B. Seliger, S. Bhakdi, M. Husmann, Dengue virus infection of human endothelial cells leads to chemokine production, complement activation, and apoptosis, J. Immunol. 161 (1998) 6338–6346.
[15] C.P. Simmons, S. Popper, C. Dolocek, T.N. Chau, M. Griffiths, N.T. Dung, T.H. Long, D.M. Hoang, N.V. Chau, T.T. Thao le, T.T. Hien, D.A. Relman, J. Farrar, Patterns of host genome-wide gene transcript abundance in the peripheral blood of patients with acute dengue hemorrhagic fever, J. Infect. Dis. 195 (2007) 1097–1107.
[16] D.H. Libraty, T.P. Endy, H.S. Houng, S. Green, S. Kalayanarooj, S. Suntayakorn, W. Chansiriwongs, D.W. Vaughn, A. Nisalak, F.A. Ennis, A.L. Rothman, Differing influences of virus burden and immune activation on disease severity in secondary dengue-3 virus infections, J. Infect. Dis. 185 (2002) 1213–1221.
[17] Y.L. Lin, C.C. Liu, J.I. Chuang, H.Y. Lei, T.M. Yeh, Y.S. Lin, Y.H. Huang, H.S. Liu, Involvement of oxidative stress, NF-IL-6, and RANTES expression in dengue-2-virus-infected human liver cells, Virology 276 (2000) 114–126.
[18] L. Suksanpaisan, A. Cabrera-Hernandez, D.R. Smith, Infection of human primary hepatocytes with dengue virus serotype 2, J. Med. Virol. 79 (2007) 300–307.
[19] J. Fink, F. Gu, L. Ling, T. Tolfvenstam, F. Olfat, K.C. Chin, P. Aw, J. George, V.A. Kuznetsov, M. Schreiber, S.G. Vasudevan, M.L. Hibberd, Host gene expression profiling of dengue virus infection in cell lines and patients, PLoS Negl. Trop. Dis. 1 (2007) e86.
[20] T.M. Conceicao, T. El-Bacha, C.S. Villas-Boas, G. Coello, J. Ramirez, M. Montero-Lomeli, A.T. Da Poian, Gene expression analysis during dengue virus infection in HepG2 cells reveals virus control of innate immune response, J. Infect. 60 (2009) 65–75.
[21] K. De Bosscher, W. Vanden Berghe, I.M. Beck, W. Van Molle, N. Hennuyer, J. Hapgood, C. Libert, B. Staels, A. Louw, G. Haegeman, A fully dissociated compound of plant origin for inflammatory gene repression, Proc. Natl. Acad. Sci. USA 102 (2005) 15827–15832.
[22] L.L. Reber, F. Daubeuf, M. Plantinga, L. De Cauwer, S. Gerlo, W. Waelput, S. Van Calenbergh, J. Tavernier, G. Haegeman, B.N. Lambrecht, N. Frossard, K. De Bosscher, A dissociated glucocorticoid receptor modulator reduces airway hyperresponsiveness and inflammation in a mouse model of asthma, J. Immunol. 188 (2012) 3478–3487.
[23] P. Dewint, V. Gossye, K. De Bosscher, W. Vanden Berghe, K. Van Beneden, D. Deforce, S. Van Calenbergh, U. Muller-Ladner, B. Vander Cruyssen, G. Verbruggen, G. Haegeman, D. Elewaut, A plant-derived ligand favoring monomeric glucocorticoid receptor conformation with impaired transactivation potential attenuates collagen-induced arthritis, J. Immunol. 180 (2008) 2608–2615.
[24] Z. Zhang, Z.Y. Zhang, H.J. Schluesener, Compound A, a plant origin ligand of glucocorticoid receptors, increases regulatory T cells and M2 macrophages to attenuate experimental autoimmune neuritis with reduced side effects, J. Immunol. 183 (2009) 3081–3091.
[25] S. Noisakran, S. Sengsai, V. Thongboonkerd, R. Kanlaya, S. Sinchaikul, S.T. Chen, C. Puttikhunt, W. Kasinrerk, T. Limjindaporn, W. Wongwiwat, P. Malasit, P.T. Yenchitsomanus, Identification of human hnRNP C1/C2 as a dengue virus NS1-interacting protein, Biochem. Biophys. Res. Commun. 372 (2008) 67–72.
[26] W. Strober, Trypan blue exclusion test of cell viability. Curr. Protoc. Immunol. Appendix 3 (2001) Appendix 3B.
[27] E. Scandella, Y. Men, D.F. Legler, S. Gillessen, L. Prikler, B. Ludewig, M. Groettrup, CCL19/CCL21-triggered signal transduction and migration of dendritic cells requires prostaglandin E2, Blood 103 (2004) 1595–1601.
[28] N. Jirakanjanakit, T. Sanohsomneing, S. Yoksan, N. Bhamarapravati, The micro-focus reduction neutralization test for determining dengue and Japanese encephalitis neutralizing antibodies in volunteers vaccinated against dengue, Trans. R. Soc. Trop. Med. Hyg. 91 (1997) 614–617.
[29] W. Dejnirattisai, T. Duangchinda, C.L. Lin, S. Vasanawathana, M. Jones, M. Jacobs, P. Malasit, X.N. Xu, G. Screaton, J. Mongkolsapaya, A complex interplay among virus, dendritic cells, T cells, and cytokines in dengue virus infections, J. Immunol. 181 (2008) 5865–5874.
[30] G.N. Malavige, L.C. Huang, M. Salimi, L. Gomes, S.D. Jayaratne, G.S. Ogg, Cellular and cytokine correlates of severe dengue infection, PLoS One 7 (2012) e50387.
[31] J.M. Sung, C.K. Lee, B.A. Wu-Hsieh, Intrahepatic infiltrating NK and CD8 T cells cause liver cell death in different phases of dengue virus infection, PLoS One 7 (2012) e46292.
[32] S. Green, A. Rothman, Immunopathological mechanisms in dengue and dengue hemorrhagic fever, Curr. Opin. Infect. Dis. 19 (2006) 429–436.
[33] S.R. Reis, A.L. Sampaio, M. Henriques, M. Gandini, E.L. Azeredo, C.F. Kubelka, An in vitro model for dengue virus infection that exhibits human monocyte infection, multiple cytokine production and dexamethasone immunomodulation, Mem. Inst. Oswaldo Cruz 102 (2007) 983–990.
[34] S. Wati, S.M. Rawlinson, R.A. Ivanov, L. Dorstyn, M.R. Beard, D.A. Jans, S.M. Pitson, C.J. Burrell, P. Li, J.M. Carr, Tumour necrosis factor alpha (TNF-{alpha}) stimulation of cells with established dengue virus type 2 infection induces cell death that is accompanied by a reduced ability of TNF-{alpha} to activate nuclear factor {kappa}B and reduced sphingosine kinase-1 activity, J. Gen. Virol. 92 (2011) 807–818.
[35] A. Nagila, J. Netsawang, A. Suttitheptumrong, A. Morchang, S. Khunchai, C. Srisawat, C. Puttikhunt, S. Noisakran, P.T. Yenchitsomanus, T. Limjindaporn, Inhibition of p38MAPK and CD137 signaling reduce dengue virus-induced TNF-alpha secretion and apoptosis, Virol. J. 10 (2013) 105.
[36] P. Marianneau, A. Cardona, L. Edelman, V. Deubel, P. Despres, Dengue virus replication in human hepatoma cells activates NF-kappaB which in turn induces apoptotic cell death, J. Virol. 71 (1997) 3244–3249.
[37] Y. Tian, W. Jiang, N. Gao, J. Zhang, W. Chen, D. Fan, D. Zhou, J. An, Inhibitory effects of glutathione on dengue virus production, Biochem. Biophys. Res. Commun. 397 (2010) 420–424.

Biochemical and Biophysical Research Communications 423 (2012) 398–403

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Interaction of dengue virus nonstructural protein 5 with Daxx modulates RANTES production

Sasiprapa Khunchai [a,b,1], Mutita Junking [a,1], Aroonroong Suttitheptumrong [a,b], Umpa Yasamut [a,b], Nunghathai Sawasdee [a], Janjuree Netsawang [d], Atthapan Morchang [a,b], Prapaipit Chaowalit [a], Sansanee Noisakran [e], Pa-thai Yenchitsomanus [a,*], Thawornchai Limjindaporn [a,b,c,*]

[a] Division of Molecular Medicine, Department of Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand
[b] Graduate Program in Immunology, Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand
[c] Department of Anatomy, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand
[d] Faculty of Medical Technology, Rangsit University, Bangkok, Thailand
[e] Medical Biotechnology Research Unit, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Bangkok, Thailand

ARTICLE INFO

Article history:
Received 8 May 2012
Available online 1 June 2012

Keywords:
Dengue virus
Nonstructural protein 5
NS5
Daxx
RANTES
CCL5

ABSTRACT

Dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS), caused by dengue virus (DENV) infection, are important public health problems in the tropical and subtropical regions. Abnormal hemostasis and plasma leakage are the main patho-physiological changes in DHF/DSS. A remarkably increased production of cytokines, the so called 'cytokine storm', is observed in the patients with DHF/DSS. A complex interaction between DENV proteins and the host immune response contributes to cytokine production. However, the molecular mechanism(s) by which DENV nonstructural protein 5 (NS5) mediates these responses has not been fully elucidated. In the present study, yeast two-hybrid assay was performed to identify host proteins interacting with DENV NS5 and a death-domain-associate protein (Daxx) was identified. The *in vivo* relevance of this interaction was suggested by co-immunoprecipitation and nuclear co-localization of these two proteins in HEK293 cells expressing DENV NS5. HEK293 cells expressing DENV NS5-K/A, which were mutated at the nuclear localization sequences (NLS), were created to assess its functional roles in nuclear translocation, Daxx interaction, and cytokine production. In the absence of NLS, DENV NS5 could neither translocate into the nucleus nor interact with Daxx to increase the DHF-associated cytokine, RANTES (CCL5) production. This work demonstrates the interaction between DENV NS5 and Daxx and the role of the interaction on the modulation of RANTES production.

1. Introduction

Dengue virus (DENV) belongs to the *Flaviviridae* family, which contain a single positive-stranded RNA genome encoding a single polypeptide precursor. Host and viral proteases cleave this polypeptide into three structural proteins (capsid, membrane, and envelope) and seven nonstructural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5), respectively. Clinical symptoms of DENV infection range from a predominantly febrile disease, dengue fever (DF), to dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), which typically occurs in cases of reinfection with a different serotype of DENV. Patients with DHF present with hemorrhagic tendencies, plasma leakage, thrombocytopenia, and hemoconcentration [1]. In addition, increased levels of cytokines – so called 'cytokine storm', which relate to the pathogenesis of severe DENV infection, are observed in the patients with DHF/DSS [2].

A complex interaction between DENV proteins and the host immune response contributes to DHF/DSS [3–9]. Both inhibition of the antiviral response and stimulation of cytokine production by DENV proteins have been reported. For example, DENV NS2B3 complex, DENV NS4B and DENV NS5 contribute to the inhibition of type I IFN response [3,5,7]. DENV NS4B and DENV NS5 also enhance the production of DHF-associated cytokines [8,9].

DENV NS5 is a bi-functional enzyme containing three domains [10–12]. The N-terminus from residues 1 to 368 contains a 2′-*O*-methyltransferase while the C-terminus from residues 405 to 900 contains an RNA-dependent RNA polymerase. The interdomain region contains nuclear localization sequences (NLS), which are divided into aNLS and bNLS, respectively. Mutations in both aNLS and bNLS result in the accumulation of DENV NS5 in the cytoplasm [13].

* Corresponding authors at: Department of Anatomy, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand (T. Limjindaporn).
E-mail addresses: grpye@mahidol.ac.th (P.-t. Yenchitsomanus), limjindaporn@yahoo.com (T. Limjindaporn).
[1] The first two authors contributed equally to this work.

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.05.137

In the present study, a yeast two-hybrid assay was performed to identify host proteins interacting with DENV NS5 and a death-domain-associate protein (Daxx) which serves as transcription repressor was identified. The roles of DENV NS5 in nuclear translocation, Daxx interaction, and cytokine production were tested and DENV NS5 was demonstrated to interact with human Daxx to increase RANTES production.

2. Materials and methods

2.1. Yeast two-hybrid screening

Two-hybrid screening was performed by the interaction mating method described by Finley and Brent [14]. DENV NS5 was PCR amplified by *Pfu* DNA polymerase from a cDNA clone of DENV serotype 2 strain 16681 [15] using nucleotide primers, 5′TTG ACT GTA TCG CCG GGA ACT GGC AAC ATA3′ and 5′CCG GAA TTA GCT TGG CTG CAG CCA CAG AAC TCC TCG3′. The PCR reaction was carried out in a GeneAmp PCR System 9700 (Applied Biosystems) with an initial denaturation step of 94 °C for 5 min and followed by 35 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 30 s, extension at 72 °C for 3 min, and one cycle of final extension at 72 °C for 10 min. Subsequently, the amplified DNA was cloned by yeast recombination [16] into the yeast expression vector pEG-NRT [17], which was derived from pEG202 [18], and contains a *HIS3* selectable marker. The constitutive ADH promoter is used to express DENV NS5 with the DNA binding domain protein LexA. Nuclear localization sequences, 5′recombination tag (5′RT), multiple cloning site (MCS) and 3′recombination tag (3′RT) were inserted between LexA and ADH terminator. The resulting bait plasmid, pEG-NS5, was verified by DNA sequencing and transformed into *Saccharomyces cerevisiae* strain RFY 206 (MATa *his3A200 leu2-3 lys2A201 ura3-52 trp1A::hisG*) containing a Lexop-*lac*Z reporter plasmid, pSH18–34 [14]. A galactose inducible HeLa cell cDNA prey library was constructed in plasmid pJZ 4–5 containing a *TRP1* selectable marker and transformed into strain RFY 231 (MATα *his3 leu2::3Lexop-LEU2 ura3 trp1 LYS2*) [14]. The bait strain then was mated with the library strains and plated on galactose drop-out medium lacking histidine, tryptophan, uracil and leucine (gal/raf -u, -h, -w, -l) to select for diploids. The production of a DENV NS5 binding protein by a prey plasmid was expected to activate the 3Lexop-*LEU*2 reporter. Putative positive clones were patched to four indicator plates: (glu/-u, -h, -w, -l), (gal/raf -u, -h, -w, -l), (glu/X-Gal-u, -h, -w), and (gal/raf/ X-Gal-u, -h, -w). Prey plasmids were rescued from clones exhibiting a galactose-inducible Leu^+ $lacZ^+$ phenotype by transformation into a Trp^- *Escherichia coli* strain KC8 [14]. Putative positive library plasmids were purified and subjected to DNA sequencing and BLAST analysis.

2.2. Generation of HEK293 cells expressing DENV NS5, or DENV NS5-K/A

DENV NS5 with a C-terminal FLAG-tag was PCR amplified by *Pfx* DNA polymerase (Invitrogen) from plasmid pET-DENV-2-NS5 using nucleotide primers, 5′ACA GGA TCC ACC ATG GGA ACT GGC AAC ATA GGA GAG ACG3′ and 5′TGT CTC GAG TTA CTT GTC ATC GTC ATC CTT GTA ATC CCA CAG AAC TCC TGC TTC TTC C3′. The PCR reaction was carried out in a GeneAmp PCR System 9700 with an initial denaturation step of 94 °C for 5 min and followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, extension at 68 °C for 3 min, and one cycle of final extension at 68 °C for 10 min. The PCR product was sub-cloned into plasmid pcDNA3.1/Hygro (Invitrogen) and the fidelity of the insert in the resulting plasmid, pcDNA3.1/DENV NS5, was verified by DNA sequencing.

Site-directed mutagenesis was employed to generate plasmid pcDNA3.1/DENV NS5 (K371A, K372A, K387A, K388A, K389A), namely DENV NS5-K/A. Firstly, DENV NS5 (K387A, K388A, K389A) was amplified by PCR using plasmid pcDNA3.1/DENV NS5 as a template and nucleotide primers, 5′GGA AAG AAT TAG GGG CGG CAG CGA CAC CCA GGA TGT G3′ and 5′CAC ATC CTG GGT GTC GCT GCC GCC CCT AAT TCT TTC C3′. The PCR reaction was carried out in a GeneAmp PCR System 9700 with an initial denaturation step of 94 °C for 2 min and followed by 18 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 1 min, extension at 68 °C for 18 min. The PCR product was digested with *Dpn*I to eliminate methylated parental DNA template and transformed into competent *E.coli* strain DH5. The correct mutant clone with K387A, K388A, K389A was verified by digestion with *Fnu4*HI and DNA sequencing. Secondly, DENV NS5 (K371A, K372A, K387A, K388A, K389A), was amplified by PCR using plasmid pcDNA3.1/DENV NS5(K387A, K388A, K389A) as a template and nucleotide primers, 5′CCG AAA GAA GGC ACG GCG GCA CTA ATG AAA ATA AC3′ and 5′GTT ATT TTC ATT AGT GCC GCC GTG CCT TCT TTC GG3′. The PCR reaction and transformation is similar to those described previously. The correct mutant clone with K371A, K372A, K387A, K388A, K389A, namely DENV NS5-K/A, was verified by digestion with *Fnu4*HI and DNA sequencing.

HEK 293 cells were transiently transfected with pcDNA3.1 or pcDNA3.1/DENV NS5 or pcDNA3.1/DENV NS5-K/A by Lipofectamine® 2000 transfection (Invitrogen). Two days after transfection, the cells were collected, fixed and permeabilized with 0.1% Triton X-100 in PBS. Re-suspended cells were blocked with DMEM containing 1% BSA before incubation with mouse anti-FLAG (Sigma) antibody for 1 h. After incubation, the cells were washed twice with chilled DMEM containing 1% BSA. Then, goat anti-mouse antibody conjugated with FITC (Molecular Probes) was used as secondary antibody for 30 min. The cells were washed again and analyzed by using FACSort™ flow cytometer (Becton–Dickinson). HEK293 cells expressing either DENV NS5, or DENV NS5-K/A were also tested for the presence of DENV NS5 by Western blot analysis using anti-FLAG (Sigma).

2.3. Co-immunoprecipitation

Forty-eight h post-transfection, HEK293 cells expressing either DENV NS5 or DENV NS5-K/A were lysed in RIPA buffer containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 20 mM Tris–HCl; pH 7.4, 5 mM EDTA, and protease inhibitor cocktail. Five micrograms of goat anti-Daxx antibody (Santa Cruz Biotechnology) or 5 μg of mouse anti-FLAG antibody were added to lysates. The mixture was incubated with gentle rotation at 4 °C for 6 h. The incubation was continued 24 h after addition of Protein G Sepharose (Amersham Pharmacia Biosciences). Subsequently, Protein G Sepharose was collected by centrifugation at 13,000*g* for 5 min and washed twice with 0.1% Triton X-100, 150 mM NaCl, 20 mM Tris–HCl; pH 7.4, 5 mM EDTA. The bound proteins were eluted by boiling in SDS–PAGE loading buffer, separated by SDS–PAGE, and transferred to nitrocellulose membrane. After blocking with 5% skim milk, the membranes were incubated with either a mouse anti-FLAG antibody or a rabbit anti-Daxx antibody followed by probing with either a rabbit anti-mouse antibody conjugated-horseradish peroxidase (HRP) or a swine anti-rabbit antibody conjugated-HRP (DakoCytomation), respectively. Chemiluminescent signals generated by SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific) were detected using a G:BOX chemiluminescence imaging system (Syngene).

2.4. Co-localization

HEK293 cells were grown on coverslips and then transfected with plasmid expressing either DENV NS5 or DENV NS5-K/A for 48 h. The transfected cells were fixed with 0.1% formaldehyde in

PBS, rinsed, permeabilized with 0.1% Triton X-100 in PBS and blocked with 1% BSA. The cells were stained at RT for 1 h with both a mouse anti-FLAG and a rabbit anti-Daxx primary antibodies, washed and incubated at RT for 1 h with both an Alexa 488-conjugated rabbit anti-mouse Ig antibody (Molecular Probes) and a Cy3-conjugated donkey anti-rabbit Ig antibody (Jackson Immunoresearch Laboratories) as secondary antibodies. Fluorescent images were captured with a confocal microscope (model LSM 510, Carl Zeiss).

2.5. Real-time RT-PCR

Total RNA from HEK293 cells expressing either DENV NS5, or DENV NS5-K/A and cultured in the presence of 50 ng/ml TNF-α (Santa Cruz biotechnology) was isolated using Trizol reagent (Invitrogen). Cytokines were quantified by real-time RT-PCR using the following primer pairs: IL-8: 5′-TCC TGC AGA GGA TCA AGA CA-3′ and 5′-GAG CAC TTG CCA CTG GTG TA-3′, CXCL9: 5′-CAG ATT CAG CAG ATG TGA AGG A-3′ and 5′-GAA ATT CAA CTG GTG GGT GGT-3′, RANTES: 5′-CAA GGA AAA CTG GGT GCA GA-3′and 5′-TCT CCC GTG CAA TAT CTA GGA A-3′, respectively. The assay was performed using LightCycler® 480 SYBR Green I Master Mix (Roche) and a LightCycler® 480 Instrument equipped with a 96-well thermal cycler (Roche). Briefly, RNA samples were reverse-transcribed using the SuperScript^{III} First-Strand Synthesis System (Invitrogen). Then cDNA templates were subjected to a 10-min initial denaturation at 95 °C prior to 50 cycles of PCR (95 °C for 10 s, 60 °C for 10 s, and 72 °C for 20 s) in the presence of *Taq* DNA polymerase and the gene-specific primer pairs. The mRNA level was normalized with human beta-actin mRNA using the ΔΔCt method [19].

2.6. Enzyme-linked immunosorbent assay (ELISA)

Production of RANTES in HEK293 cells expressing either DENV NS5 or DENV NS5-K/A in the presence of TNF-α, was measured by Instant ELISA® (eBioscience) according to the manufacturer's instruction.

3. Results and discussion

3.1. Identification of DENV NS5 interacting proteins by a yeast two-hybrid screen

To identify human proteins that interacted with DENV NS5, we screened over 10^7 clones from a HeLa cDNA library using DENV NS5 as bait. Thirty putative positive clones were obtained. To verify the interaction, recovered prey plasmids were introduced into yeast strain RFY 231 along with the *lacZ* plasmid and bait plasmid and again tested on the indicator plates. Twelve library plasmids were purified and subjected to DNA sequencing. BLAST analysis revealed that the twelve clones corresponded to the proteins Daxx, Fas-associated factor 1 (FAF1), Calpain 2, Protein phosphatase 1 (PP-1), Splicing factor (SF3a), and Double-strand break repair protein (Mre11A).

The specificity of the interaction between DENV NS5 and Daxx, is shown in Fig. 1A wherein cells containing the DENV NS5 and Daxx, exhibited galactose-dependent leucine prototrophy and *lacZ* expression. Daxx has a strong transcriptional repression activity and can bind to several transcription factors in the nucleus [20,21]. In addition, the majority of Daxx is present in the nucleus similar to that of DENV NS5 [22,23]. Daxx is a 740 amino acid protein that contains amino-terminal amphipathic helices (PAH1, PAH2), a coiled–coiled domain (CC), an acidic domain (D/E), and a carboxyl-terminal serine/proline/threonine rich domain (S/P/T). Among them, the S/P/T domain can mediate the interaction of Daxx with numerous proteins. The region of Daxx that interacted with DEN NS5 was verified in the prey plasmid by DNA sequencing and BLAST analysis and the 211 carboxyl-terminal residues, which cover the S/P/T domain than can bind to multiple proteins including DENV capsid protein in the nucleus of DENV-infected cells, were identified [24] (Fig. 1B).

3.2. Interaction of DENV NS5 and Daxx in the nucleus of HEK293 cells expressing DENV NS5

The interaction of DENV NS5 and Daxx was confirmed in mammalian HEK293 cells. Co-immunoprecipitation of DENV NS5 and

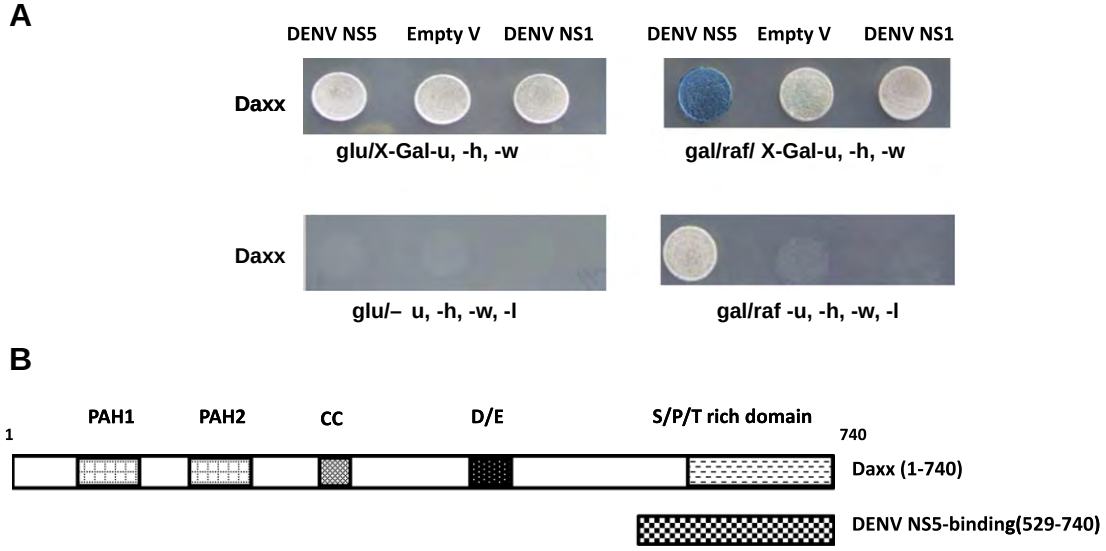

Fig. 1. DENV NS5-human Daxx interaction in a yeast two-hybrid system. (A) Yeast strain RFY231 was co-transformed with a bait plasmid, a Daxx prey plasmid and *lacZ* reporter plasmid. The bait plasmids used were pEG-NS5, expressing the lexA-DEN NS5 fusion protein, the empty bait plasmid pEG202, or an unrelated bait plasmid pEG-NS1 (DENV NS1). A specific interaction was indicated by galactose-dependent β-galactosidase expression, as evidenced by blue colonies on the galactose containing X-Gal plate and white colonies on the glucose containing X-Gal plate, and by galactose-dependent growth on the leucine deficient plate. (B) The region of Daxx that interacted with DEN NS5 was the 211 carboxyl-terminal residues, a region which binds multiple cellular proteins.

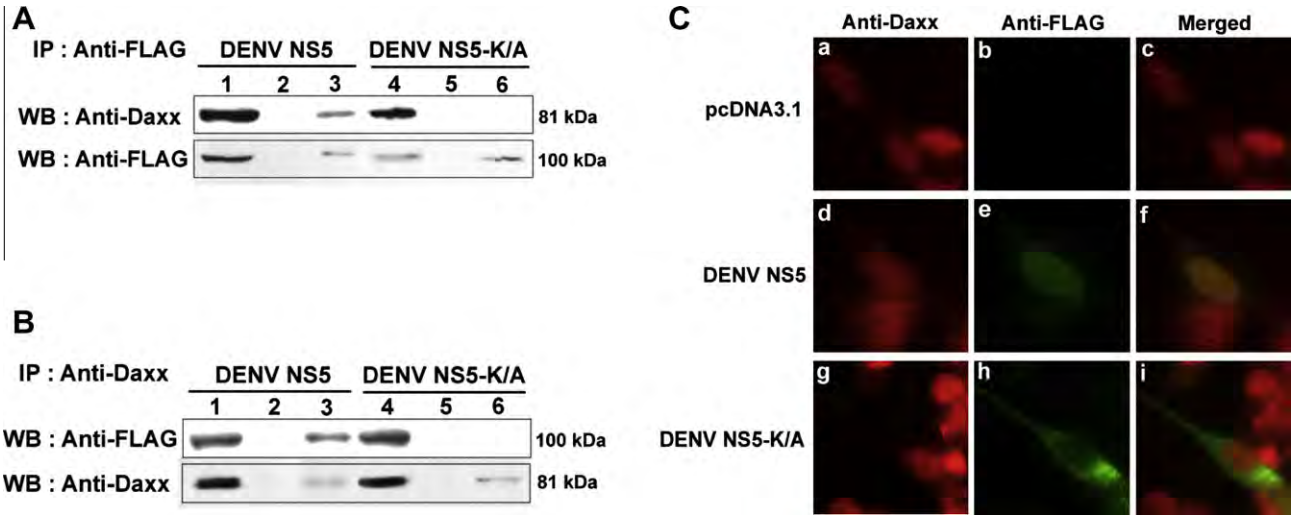

Fig. 2. Interaction between DENV NS5 and human Daxx. (A) Lysates of HEK293 cells expressing DENV NS5 or DENV NS5-K/A were immunoprecipitated with a mouse anti-FLAG antibody. Immune complexes were detected by Western blot analysis using either a mouse anti-FLAG or a rabbit anti-Daxx antibodies. Lane 1, and 4: input; Lane 2, and 5: IP with no antibody; Lane 3 and 6: IP with anti-FLAG antibody. (B) Lysates of HEK293 cells expressing DENV NS5 or DENV NS5-K/A were immunoprecipitated with a rabbit anti-Daxx antibody. Immune complexes were detected by Western blot analysis using either a mouse anti-FLAG antibody or a rabbit anti-Daxx antibody. Lane 1, and 4: input; Lane 2, and 5: IP with no antibody; Lane 3 and 6: IP with anti-Daxx antibody. (C) HEK293 cells expressing DENV NS5 or DENV NS5-K/A were fixed and immunostained with rabbit anti-Daxx antibody (panel a, d, and g) and mouse anti-FLAG antibody (panel b, e, and h). The merged image (panel c, f, and i) demonstrated co-localization between DENV NS5 and Daxx.

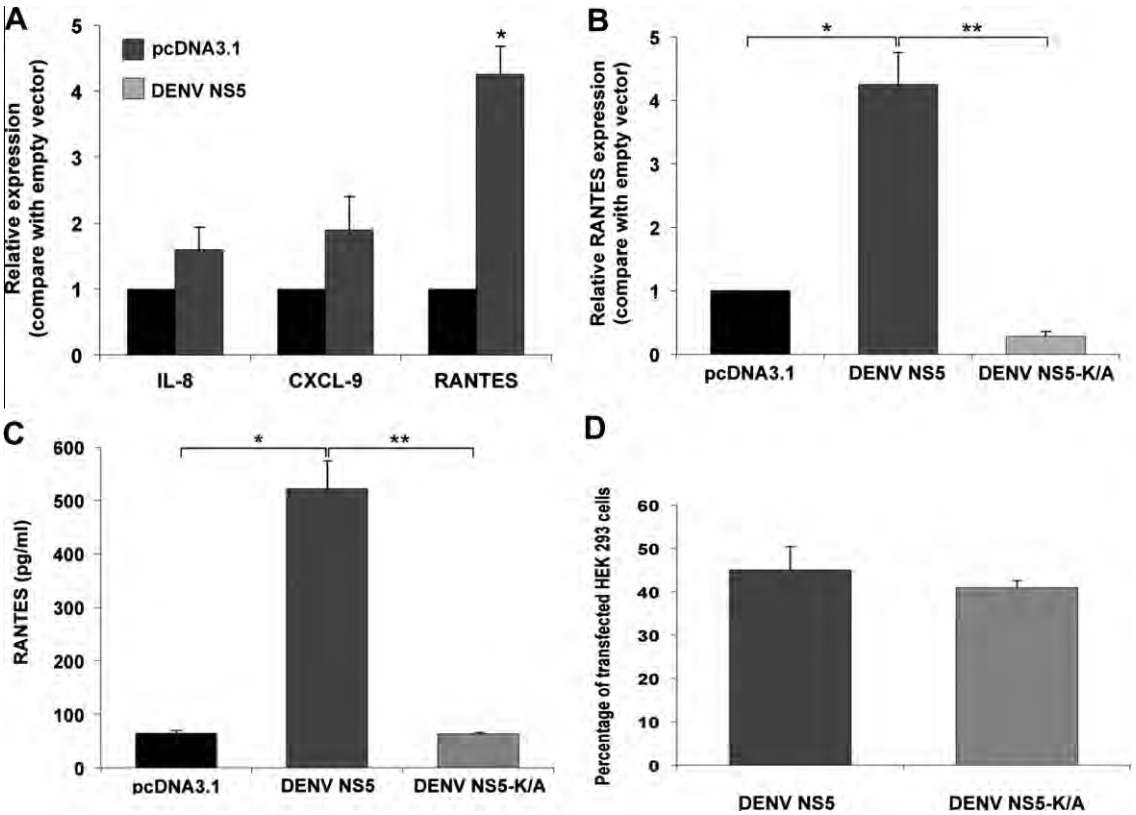

Fig. 3. Interaction between DENV NS5 and human Daxx modulates RANTES production. (A) The amount of mRNA of DHF-associated cytokines was measured in HEK293 cells expressing DENV NS5 in the presence of TNF-α. Relative gene expression (fold change) was determined by real-time PCR using primers specific to IL-8, CXCL-9 and RANTES. The results are the average of three independent experiments. The asterisks indicate statistically significant differences between HEK293 cells expressing DENV NS5 and empty vector ($^{*}p < 0.05$). (B) The mRNA expression of RANTES was measured in HEK293 cells expressing DENV NS5 in the presence of TNF-α. Relative gene expression (fold change) of RANTES was determined by real-time PCR. The results are the average of three independent experiments. The asterisks indicate statistically significant differences between HEK293 cells expressing DENV NS5 and DENV NS5-K/A ($^{*}p < 0.05$, $^{**}p < 0.01$). (C) The production of RANTES was measured by ELISA in HEK293 cells expressing DENV NS5 in the presence of TNF-α. The results are the average of three independent experiments. The asterisks indicate statistically significant differences between HEK293 cells expressing DENV NS5 and DENV NS5-K/A ($^{*}p < 0.05$, $^{**}p < 0.01$). (D) The expression of DENV NS5 or DENV NS5-K/A was determined by flow cytometry using a mouse anti-FLAG antibody.

Daxx was observed in HEK293 cells expressing DENV NS5. Anti-flag antibody co-immunoprecipitated Daxx protein and, conversely, anti-Daxx antibody co-precipitated DENV NS5 (Fig. 2A and B). Co-localization of DENV NS5 and Daxx was evident in the nucleus of HEK293 cells expressing DENV NS5 (Fig. 2C). These data supported the proposed interaction of DENV NS5 and Daxx and further suggested that association occurs mainly in the nucleus of HEK293 cells expressing DENV NS5.

3.3. Interaction DENV NS5 and Daxx modulates RANTES production

DENV NS5 was previously shown to enhance the production of IL-8 [9]. In addition, enhancement of TNF-α-stimulated NF-κB activation by DENV NS5 was reported [25]. In the present study, the level of DHF-associated cytokines including IL-8, CXCL-9 and RANTES was measured in HEK293 cells expressing DENV NS5 in the presence of TNF-α. The mRNA expression of RANTES was significantly up-regulated in HEK293 cells expressing DENV NS5 compared to empty vector-transfected HEK293 cells (Fig. 3A). RANTES is a member of the C–C chemokine family and its expression was increased both in DENV-infected cell lines and DENV-infected patients [26] and activation of RANTES production by other viral infections was reported to be regulated by NF-κB [27,28]. Therefore, enhancement of TNF-α-stimulated NF-κB activation of RANTES production may modulated by DENV NS5.

Interaction between DENV NS5 and Daxx, which normally interacts with NF-κβ [29], may modulate RANTES production. Therefore, HEK293 cells expressing either DENV NS5 or DENV NS5-K/A were created to assess in the role of NS5 nuclear translocation in Daxx interaction and RANTES production. Site-directed mutagenesis was employed to generate mutant DENV NS5 (K371A, K372A, K387A, K388A, K389A), namely DENV NS5-K/A, Whilst DENV NS5 was present in the nuclei of HEK293 cells expressing DENV NS5, HEK293 cells expressing DENV NS5-K/A had no detectable DENV NS5 protein in the nucleus (Fig. 2C). Secondly, co-immunoprecipitation and co-localization of DENV NS5-K/A and Daxx was examined. Co-immunoprecipitation of DENV NS5 and Daxx was observed only in HEK293 cells expressing DENV NS5, but not in HEK293 cells expressing DENV NS5-K/A. Anti-flag antibody co-immunoprecipitates Daxx protein and, conversely, anti-Daxx antibody co-precipitated DENV NS5 in HEK293 cells expressing DENV NS5 but not in HEK293 cells expressing DENV NS5-K/A. (Fig. 2A and B). Furthermore, co-localization of DENV NS5 and Daxx was evident in the nucleus of HEK293 cells expressing DENV NS5 but not in HEK293 cells expressing DENV NS5-K/A. (Fig. 2C). Finally, the RANTES production in either HEK293 cells expressing DENV NS5 or DENV NS5-K/A. was measured by real-time RT-PCR and ELISA, respectively. As expected, both mRNA expression and RANTES production were significantly higher in HEK293 cells expressing DENV NS5 than those in HEK293 cells expressing DENV NS5-K/A (Fig. 3B and C). This difference was not due to differences in transfection efficiency or protein expression since DENV NS5 and DENV NS5-K/A were expressed at similar levels as determined by immunofluorescence staining (Fig. 3D). Thus, in the absence of a functional NLS, DENV NS5 could neither translocate into the nucleus nor interact with Daxx to increase RANTES secretion.

As TNF-α signaling activates NF-κB and RANTES production during DENV infection [2,30] and NF-κB is known to activate the RANTES promoter [28], DENV NS5 may increase the amount of NF-κB available to activate RANTES expression through its interaction with Daxx, which normally interacts with NF-κB [29] (Fig. 4). The molecular mechanisms that control the competitive binding of DENV NS5, Daxx, and NF-κB require further investigation. However, this work is the first to demonstrate the *in vivo* interaction between DENV NS5 and Daxx and its role in modulating RANTES production.

Acknowledgments

TL is supported by Mid-Career Thailand Research Fund–Commission of Higher Education (TRF-CHE) Research Grant, Mahidol University Grant, and Siriraj Chalearmprakiat Fund, SK by CHE Ph.D. Program, MJ by TRF-Post Doctoral Research Grant, AM and UM by TRF-Royal Golden Jubilee Ph.D. Program. PY is a TRF-Senior Research Scholar. We are grateful to Dr. William A Fonzi, Georgetown University, USA, for helpful discussion and continuous support, Dr. Russell L Finley Jr., Wayne State University, USA, for

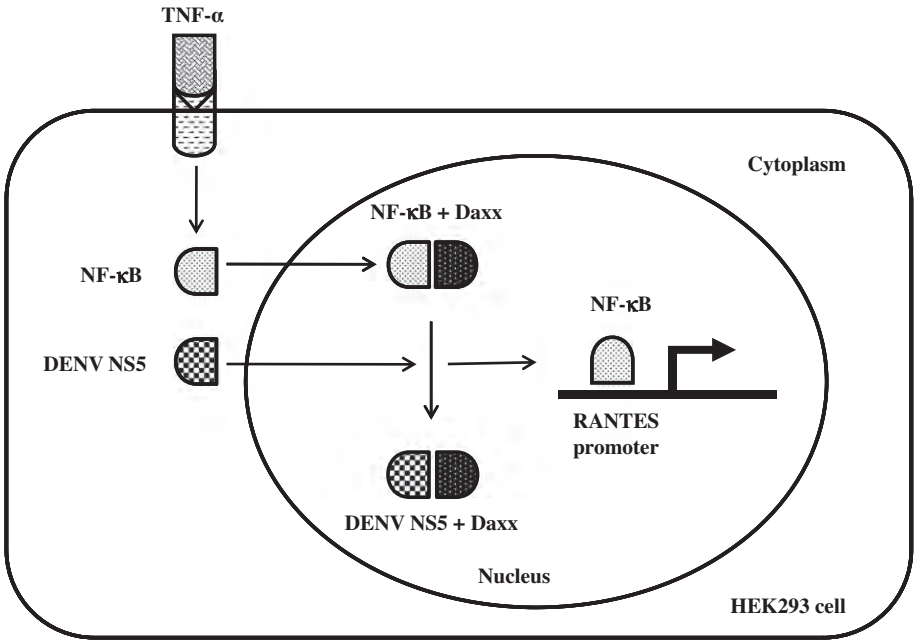

Fig. 4. Proposed model for modulation of Rantes production by DENV NS5. TNF-α signaling activates NF-κB and the competitive binding between DENV NS5 and Daxx limits the interaction between Daxx and NF-κB thereby releasing NF-κB to activate RANTES production.

reagents of yeast two-hybrid system, and Dr. Nopporn Sitthisombut, Chiang Mai University, for pBluescript II KS containing cDNA of DENV serotype 2 strain 16681.

References

[1] S.B. Halstead, Pathogenesis of dengue: challenges to molecular biology, Science 239 (1988) 476–481.
[2] P. Avirutnan, P. Malasit, B. Seliger, S. Bhakdi, M. Husmann, Dengue virus infection of human endothelial cells leads to chemokine production, complement activation, and apoptosis, J. Immunol. 161 (1998) 6338–6346.
[3] J. Ashour, M. Laurent-Rolle, P.Y. Shi, A. Garcia-Sastre, NS5 of dengue virus mediates STAT2 binding and degradation, J. Virol. 83 (2009) 5408–5418.
[4] J. Ashour, J. Morrison, M. Laurent-Rolle, A. Belicha-Villanueva, C.R. Plumlee, D. Bernal-Rubio, K.L. Williams, E. Harris, A. Fernandez-Sesma, C. Schindler, A. Garcia-Sastre, Mouse STAT2 restricts early dengue virus replication, Cell Host Microbe 8 (2010) 410–421.
[5] J.R. Rodriguez-Madoz, A. Belicha-Villanueva, D. Bernal-Rubio, J. Ashour, J. Ayllon, A. Fernandez-Sesma, Inhibition of the type I interferon response in human dendritic cells by dengue virus infection requires a catalytically active NS2B3 complex, J. Virol. 84 (2010) 9760–9774.
[6] M. Mazzon, M. Jones, A. Davidson, B. Chain, M. Jacobs, Dengue virus NS5 inhibits interferon-alpha signaling by blocking signal transducer and activator of transcription 2 phosphorylation, J. Infect. Dis. 200 (2009) 1261–1270.
[7] J.L. Munoz-Jordan, M. Laurent-Rolle, J. Ashour, L. Martinez-Sobrido, M. Ashok, W.I. Lipkin, A. Garcia-Sastre, Inhibition of alpha/beta interferon signaling by the NS4B protein of flaviviruses, J. Virol. 79 (2005) 8004–8013.
[8] J.F. Kelley, P.H. Kaufusi, E.M. Volper, V.R. Nerurkar, Maturation of dengue virus nonstructural protein 4B in monocytes enhances production of dengue hemorrhagic fever-associated chemokines and cytokines, Virology 418 (2011) 27–39.
[9] C.L. Medin, K.A. Fitzgerald, A.L. Rothman, Dengue virus nonstructural protein NS5 induces interleukin-8 transcription and secretion, J. Virol. 79 (2005) 11053–11061.
[10] M.P. Egloff, D. Benarroch, B. Selisko, J.L. Romette, B. Canard, An RNA cap (nucleoside-2′-*O*-)-methyltransferase in the flavivirus RNA polymerase NS5: crystal structure and functional characterization, EMBO J. 21 (2002) 2757–2768.
[11] J.K. Forwood, A. Brooks, L.J. Briggs, C.Y. Xiao, D.A. Jans, S.G. Vasudevan, The 37-amino-acid interdomain of dengue virus NS5 protein contains a functional NLS and inhibitory CK2 site, Biochem. Biophys. Res. Commun. 257 (1999) 731–737.
[12] T.L. Yap, T. Xu, Y.L. Chen, H. Malet, M.P. Egloff, B. Canard, S.G. Vasudevan, J. Lescar, Crystal structure of the dengue virus RNA-dependent RNA polymerase catalytic domain at 1.85-angstrom resolution, J. Virol. 81 (2007) 4753–4765.
[13] M.J. Pryor, S.M. Rawlinson, R.E. Butcher, C.L. Barton, T.A. Waterhouse, S.G. Vasudevan, P.G. Bardin, P.J. Wright, D.A. Jans, A.D. Davidson, Nuclear localization of dengue virus nonstructural protein 5 through its importin alpha/beta-recognized nuclear localization sequences is integral to viral infection, Traffic 8 (2007) 795–807.
[14] R. Brent, R.L. Finley Jr., Understanding gene and allele function with two-hybrid methods, Annu. Rev. Genet. 31 (1997) 663–704.
[15] R. Sriburi, P. Keelapang, T. Duangchinda, S. Pruksakorn, N. Maneekarn, P. Malasit, N. Sittisombut, Construction of infectious dengue 2 virus cDNA clones using high copy number plasmid, J. Virol. Methods 92 (2001) 71–82.
[16] T.L. Orr-Weaver, J.W. Szostak, Yeast recombination: the association between double-strand gap repair and crossing-over, Proc. Natl. Acad. Sci. USA 80 (1983) 4417–4421.
[17] T. Limjindaporn, D. Mairiang, S. Khunchai, W. Wongwiwat, A novel yeast two hybrid vector, pEG-NRT, for protein protein interaction studies, Siriraj Med. J. 59 (2007) 175–177.
[18] J. Gyuris, E. Golemis, H. Chertkov, R. Brent, Cdi1, a human G1 and S phase protein phosphatase that associates with Cdk2, Cell 75 (1993) 791–803.
[19] K.J. Livak, T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method, Methods 25 (2001) 402–408.
[20] J.S. Michaelson, D. Bader, F. Kuo, C. Kozak, P. Leder, Loss of Daxx, a promiscuously interacting protein, results in extensive apoptosis in early mouse development, Genes Dev. 13 (1999) 1918–1923.
[21] S. Torii, D.A. Egan, R.A. Evans, J.C. Reed, Human Daxx regulates Fas-induced apoptosis from nuclear PML oncogenic domains (PODs), EMBO J. 18 (1999) 6037–6049.
[22] X. Yang, R. Khosravi-Far, H.Y. Chang, D. Baltimore, Daxx, a novel Fas-binding protein that activates JNK and apoptosis, Cell 89 (1997) 1067–1076.
[23] H.Y. Chang, H. Nishitoh, X. Yang, H. Ichijo, D. Baltimore, Activation of apoptosis signal-regulating kinase 1 (ASK1) by the adapter protein Daxx, Science 281 (1998) 1860–1863.
[24] T. Limjindaporn, J. Netsawang, S. Noisakran, S. Thiemmeca, W. Wongwiwat, S. Sudsaward, P. Avirutnan, C. Puttikhunt, W. Kasinrerk, R. Sriburi, N. Sittisombut, P.T. Yenchitsomanus, P. Malasit, Sensitization to Fas-mediated apoptosis by dengue virus capsid protein, Biochem. Biophys. Res. Commun. 362 (2007) 334–339.
[25] S. Wati, S.M. Rawlinson, R.A. Ivanov, L. Dorstyn, M.R. Beard, D.A. Jans, S.M. Pitson, C.J. Burrell, P. Li, J.M. Carr, Tumour necrosis factor alpha (TNF-{alpha}) stimulation of cells with established dengue virus type 2 infection induces cell death that is accompanied by a reduced ability of TNF-{alpha} to activate nuclear factor {kappa}B and reduced sphingosine kinase-1 activity, J. Gen. Virol. 92 (2011) 807–818.
[26] Y.L. Lin, C.C. Liu, J.I. Chuang, H.Y. Lei, T.M. Yeh, Y.S. Lin, Y.H. Huang, H.S. Liu, Involvement of oxidative stress, NF-IL-6, and RANTES expression in dengue-2-virus-infected human liver cells, Virology 276 (2000) 114–126.
[27] P. Genin, M. Algarte, P. Roof, R. Lin, J. Hiscott, Regulation of RANTES chemokine gene expression requires cooperativity between NF-kappa B and IFN-regulatory factor transcription factors, J. Immunol. 164 (2000) 5352–5361.
[28] H. Moriuchi, M. Moriuchi, A.S. Fauci, Nuclear factor-kappa B potently up-regulates the promoter activity of RANTES, a chemokine that blocks HIV infection, J. Immunol. 158 (1997) 3483–3491.
[29] J. Park, J.H. Lee, M. La, M.J. Jang, G.W. Chae, S.B. Kim, H. Tak, Y. Jung, B. Byun, J.K. Ahn, C.O. Joe, Inhibition of NF-kappaB acetylation and its transcriptional activity by Daxx, J. Mol. Biol. 368 (2007) 388–397.
[30] D. Hober, L. Poli, B. Roblin, P. Gestas, E. Chungue, G. Granic, P. Imbert, J.L. Pecarere, R. Vergez-Pascal, P. Wattre, et al., Serum levels of tumor necrosis factor-alpha (TNF-alpha), interleukin-6 (IL-6), and interleukin-1 beta (IL-1 beta) in dengue-infected patients, Am. J. Trop. Med. Hyg. 48 (1993) 324–331.

OPEN ACCESS Freely available online

PLOS ONE

Identification of New Protein Interactions between Dengue Fever Virus and Its Hosts, Human and Mosquito

Dumrong Mairiang[1¤], Huamei Zhang[1], Ann Sodja[2], Thilakam Murali[1], Prapat Suriyaphol[3], Prida Malasit[4,5], Thawornchai Limjindaporn[6], Russell L. Finley, Jr.[1,7]*

1 Center for Molecular Medicine and Genetics, Wayne State University School of Medicine, Detroit, Michigan, United States of America, **2** Department of Biology, Wayne State University, Detroit, Michigan, United States of America, **3** Bioinformatics and Data Management for Research Unit, Faculty of Medicine Siriraj Hospital, and Center for Emerging and Neglected Infectious Diseases, Mahidol University, Bangkok, Thailand, **4** Dengue Hemorrhagic Fever Research Unit, Office for Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand, **5** Medical Biotechnology Research Unit, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Bangkok, Thailand, **6** Department of Anatomy, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand, **7** Department of Biochemistry and Molecular Biology, Wayne State University School of Medicine, Detroit, Michigan, United States of America

Abstract

The four divergent serotypes of dengue virus are the causative agents of dengue fever, dengue hemorrhagic fever and dengue shock syndrome. About two-fifths of the world's population live in areas where dengue is prevalent, and thousands of deaths are caused by the viruses every year. Dengue virus is transmitted from one person to another primarily by the yellow fever mosquito, *Aedes aegypti*. Recent studies have begun to define how the dengue viral proteins interact with host proteins to mediate viral replication and pathogenesis. A combined analysis of these studies, however, suggests that many virus-host protein interactions remain to be identified, especially for the mosquito host. In this study, we used high-throughput yeast two-hybrid screening to identify mosquito and human proteins that physically interact with dengue proteins. We tested each identified host protein against the proteins from all four serotypes of dengue to identify interactions that are conserved across serotypes. We further confirmed many of the interactions using co-affinity purification assays. As in other large-scale screens, we identified some previously detected interactions and many new ones, moving us closer to a complete host – dengue protein interactome. To help summarize and prioritize the data for further study, we combined our interactions with other published data and identified a subset of the host-dengue interactions that are now supported by multiple forms of evidence. These data should be useful for understanding the interplay between dengue and its hosts and may provide candidates for drug targets and vector control strategies.

Citation: Mairiang D, Zhang H, Sodja A, Murali T, Suriyaphol P, et al. (2013) Identification of New Protein Interactions between Dengue Fever Virus and Its Hosts, Human and Mosquito. PLoS ONE 8(1): e53535. doi:10.1371/journal.pone.0053535

Editor: George Dimopoulos, Johns Hopkins University, Bloomberg School of Public Health, United States of America

Received July 17, 2012; **Accepted** December 3, 2012; **Published** January 11, 2013

Funding: Dr. Thawornchai Limjindaporn is supported by Mahidol University and currently a Thailand Research Fund (TRF)-CHE research Scholar; Dr. Prapat Suriyaphol is supported by the Commission of Higher Education (CHE) and Mahidol University under the National Research Universities Initiative; This project was supported in part by the National Institutes of Health grant HG001536 to RLF. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: rfinley@wayne.edu

¤ Current address: Medical Biotechnology Research Unit, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Bangkok, Thailand

Introduction

Infection with one of the four serotypes of dengue virus can result in dengue fever and the life-threatening sequalae, dengue hemorrhagic fever and dengue shock syndrome [1]. Dengue infects as many as 50 million people each year, resulting in thousands of deaths, especially in children [2]. Currently, there is no effective antiviral drug available and intense efforts to develop a vaccine are still ongoing [2–4]. About 2.5 billion people around the world live in areas where there is a significant risk of dengue infection and recently the virus has expanded into areas where it previously did not exist or had been eradicated [5,6]. Dengue is an arthropod-borne virus (arbovirus) that is transmitted by mosquito vectors, primarily the urban-dwelling species *Aedes aegypti* [1]. Mosquitoes become infected by taking a blood meal from an infected human, and after an incubation period of 7–14 days, during which the virus disseminates from the midgut and proliferates in salivary glands, the mosquito is capable of infecting another person [1,7,8]. Because of the essential role of the mosquito in the virus transmission cycle, a number of efforts to combat dengue have focused on vector control strategies [1,9–13]. A better understanding of how dengue interacts with its mosquito host to allow replication and dissemination would be helpful in the development of these strategies.

Dengue belongs to the *Flaviviridae* family, which includes the West Nile and Yellow fever viruses [14]. There are four distinct serotypes of dengue virus (DENV1–4), which are as different from each other as are other separately classified viruses, such as the West Nile and Japanese Encephalitis viruses [15,16]. Here we will refer to all serotypes generically as dengue virus unless we are talking about a specific serotype. The dengue viral genomes consist of a positive single-stranded RNA encoding ten proteins; capsid

(C), membrane protein (M), envelope protein (E), and seven nonstructural proteins, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 [17]. These proteins are translated as a polyprotein (Figure 1A), which is cleaved into individual proteins during maturation by the host proteases furin and signalase on the lumenal side of the ER, and by the dengue NS2B/NS3 protease complex on the cytoplasmic side [17,18]. A mature virion contains a molecule of viral RNA encapsulated in capsid and enveloped by a membrane that contains M and E proteins [17]. NS5 is a large multifunctional protein with a C-terminal RNA-dependent-RNA polymerase domain that is required for viral replication [19] and an N-terminal methyltransferase domain required for RNA capping [20]. The functions of the remaining nonstructural proteins, NS1, NS2A, NS4A and NS4B, are less well understood. A number of functional studies, however, have shown that these proteins are involved in dengue pathogenesis and immune response in humans. Individual expression of NS2A, NS4A, or NS4B proteins, for example, can enhance replication of an interferon (IFN)-sensitive virus and down-regulate the expression of IFN-β-stimulated reporter genes, suggesting that these proteins contribute to inhibition of the IFN-mediated viral defense system [21]. NS5 also inhibits expression of IFN-stimulated genes [22]. NS1, which is a secreted protein, contributes to immune evasion at least in part by interfering with the complement system [23,24]. Although viral replication and maturation occur in the cytoplasm, endoplasmic reticulum, and Golgi apparatus [17], the capsid and NS5 proteins are also detected in the nucleus [25–27]. The potential nuclear roles of these proteins are poorly understood [28,29].

A more detailed picture of how dengue virus interacts with its hosts has begun to emerge from studies aimed at identifying interactions between dengue proteins and host proteins. Several studies have focused on specific genes or pathways that relate to dengue pathogenesis. For example, an interaction between dengue capsid and human Death-domain Associated protein (DAXX) was identified and shown to increase Fas-mediated and CD137-meditated apoptosis of liver cells [25,30,31]. In another example, NS5 was found to bind either directly or indirectly to the critical IFN signaling pathway transcription factor, Signal Transducer and Activator of Transcription 2 (STAT2) leading to STAT2 degradation [22,32]. Meanwhile, several recent large-scale studies have identified novel dengue-host protein interactions. Khadka et al., used yeast two-hybrid screens to identify 139 protein interactions involving 105 human liver proteins and fragments of eight dengue serotype 2 proteins [33]. They went on to show that RNAi-mediated knock down of a few of these human proteins inhibited a dengue replicon suggesting that these host factors may be important for the dengue life cycle. In another study, Le Breton et al., used yeast two-hybrid screens to identify interactions involving 108 human proteins and the NS3 and NS5 proteins from dengue serotype 2 and several other flaviviruses [34]. In yet another study, a bacterial two-hybrid screen was used to detect 31 dengue-interacting human proteins [35]. To date, however, no large-scale two-hybrid screens have been reported to detect dengue-mosquito protein interactions. In the largest study reported thus far for mosquito proteins, Colpitts et al., used tandem affinity purification assays in an *Aedes albopictus* cell line to identify protein interactions involving 19 mosquito proteins and four dengue proteins including capsid, envelope protein, NS2A, and NS4B [36]. Several of the mosquito proteins were histones and in a follow-up study the same group reported that dengue capsid can interfere with nucleosome assembly in human liver cells [29]. Computational approaches have also been used to predict dengue-host protein interactions. Doolittle et al., for example, predicted 4,376 human-dengue and 176 mosquito-dengue protein interactions based on structural similarity between dengue proteins and host proteins [37]. In another study a number of interactions were predicted between mosquito and dengue proteins or RNA based on potential conservation of host-flavivirus interactions [38].

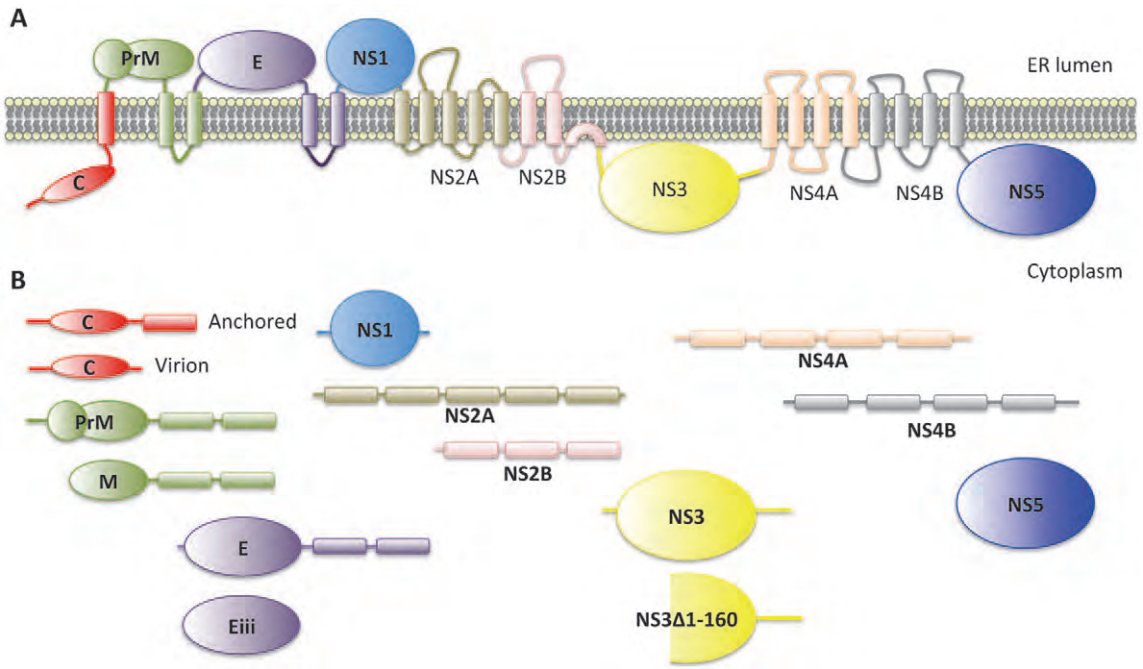

Figure 1. Dengue virus proteins. (A) The dengue polyprotein in the ER membrane prior to processing. (B) The coding regions for the fourteen dengue virus proteins and partial peptides shown were separately cloned into yeast two-hybrid plasmids.
doi:10.1371/journal.pone.0053535.g001

Despite the valuable insights that can be derived from protein interactions, the available data suggest that the dengue-host interactome is still noisy and incomplete. Protein interaction data from high throughput screens and even from literature-curated databases can include many false positive interactions that have no biological relevance [39–41]. One way to address this problem is to focus on interactions detected by multiple independent experiments because those interactions are more likely to be biologically relevant [42–50]. However, very few dengue-host protein interactions have been identified in more than one experiment. For example, the large-scale two-hybrid screens [33,34] detected only one interaction in common (between NS5 and human Matrin 3) and only one previously published interaction (between NS5 and human STAT2 [22,32]). Colpitts et al., identified only one mosquito-dengue protein interaction, between NS2A and myelin protein expression factor (AAEL003670) [36], that was also predicted by Doolittle et al [37]. The lack of overlap between different large-scale datasets is likely due in part to false positives that are detected in one screen but not another. However, even most of the interactions that have been confirmed and functionally validated were detected in only one study, suggesting that the screens also have high false negative rates. Thus, additional large-scale screens may be used to identify new protein interactions and to provide additional evidence for previously identified interactions. In this study, we used yeast two-hybrid screens to identify interactions between proteins from dengue virus and its hosts, mosquito and human. We independently tested most of the interactions by co-purification from insect cells. Furthermore, we tested interactions of host proteins against all four serotypes of dengue virus to identify either serotype-specific or serotype-independent interactions. Our data help define a more complete dengue-host interactome. When combined with the data from previous studies, the data presented here provide a higher confidence set of dengue-host protein interactions supported by multiple forms of evidence.

Results and Discussion

Intraviral protein-protein interactions

To identify interactions with dengue proteins we subcloned open reading frames (ORFs) from dengue virus serotype 2 (strain 16681) into the yeast two-hybrid bait vector for expression of the proteins with an N-terminal LexA DNA binding domain (DBD). We constructed a total of 14 baits (Figure 1B). These included baits for all ten full-length dengue proteins: nascent capsid protein (C), precursor of membrane protein (PrM), E, NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. These ten proteins are individually cleaved from the viral polypeptide during maturation [17,51,52]. We also constructed baits for mature capsid protein (CV) [53], mature membrane protein (M) [54], domain III of envelope protein (Eiii), and a fragment of NS3 lacking the N-terminal 160 amino acids (NS3Δ1–160) (Figure 1B). We subcloned the same 14 dengue ORFs into the yeast two-hybrid activation domain (AD) vector. This enabled us to test for interactions among the 14 dengue proteins. We used a two-hybrid matrix mating assay to test all 14 DBD fusion proteins against all 14 AD fusion proteins (Materials and Methods). We detected interactions between NS5 and both NS3 and NS3Δ1–160 (Figure S1). The interaction between NS5 and the C-terminal region of NS3, which contains the helicase domain, was previously demonstrated by yeast two-hybrid and co-immunoprecipitation assays [27,55] and NS3 was shown to be associated with cytoplasmic NS5 in dengue-infected cells [56]. The complex of NS3 and NS5 may be essential for viral replication since NS3 contributes the helicase to unwind a viral dsRNA intermediate allowing NS5 to synthesize a new RNA molecule. We also detected an interaction between the NS5 DBD and NS5 AD clones suggesting a homodimer. An NS5 homodimer was also observed in another yeast two-hybrid study [57]. No other novel interactions were detected. We failed to detect previously reported interactions between NS2B and NS3 [58] or between PrM and E, which were originally detected by co-purification assays and not by yeast two-hybrid [27,58,59]. It was not possible to detect the interaction corresponding to that reported between the precursor proteins NS2B-NS3 and NS4B-NS5 [60] because we did not express these fusion proteins or co-express NS2B with NS3, or NS4B with NS5 in our screen.

Dengue-mosquito protein-protein interactions

To identify mosquito proteins that interact with dengue proteins, we constructed a yeast two-hybrid AD library for *Aedes aegypti* using mRNA pooled from ten stages of development ranging from egg to adult (see Materials and Methods). We used a library mating assay to screen the mosquito library with each of 14 individual dengue bait proteins (Materials and Methods). To verify two-hybrid interactions, we subcloned the mosquito cDNAs from initial positives into new AD vectors and retested for interaction with the original dengue bait proteins. At the same time we tested for interactions with baits unrelated to the dengue baits to identify proteins that may nonspecifically interact with random proteins. In all we identified 102 interactions that were reproducible and specific by this definition (Table S1). These interactions involved eight viral bait proteins representing C, NS3, NS5, or variants of these proteins and PrM (Table 1). We did not find mosquito proteins interacting with the membrane proteins M, E, NS2A and NS2B, or with the luminal proteins Eiii and NS1. This is likely because these proteins are unable to locate or fold properly in the yeast nucleus, consistent with data from other large-scale two-hybrid screens that are generally depleted for membrane proteins [42,61,62]. None of the mosquito–dengue interactions that we identified had previously been identified.

The 102 interactions involved 93 unique mosquito proteins, 58 of which have clear human orthologs. Two of the mosquito-dengue interactions that we detected had been previously detected for the human orthologs (Table S2). These included NS5 interactions with the mosquito E3 ubiquitin ligase Seven In Absentia (AAEL009614) and the human Seven In Absentia Homolog, SIAH2 (ENSG00000181788), which was previously detected by Le Breton et al. [34]; and the interactions between NS5 and mosquito Paramyosin (AAEL010975) and human cingulin like-1 (ENSG00000128849) previously detected by Khadka et al [33]. None of the other mosquito-dengue interac-

Table 1. Number of host interactors for each dengue protein identified in this study by yeast two-hybrid screens.

Dengue Protein	Mosquito	Human
C	16	20
PrM/M	1	0
NS3	34	15
NS4A	1	0
NS4B	1	0
NS5	49	11
E, NS1, NS2A, NS2B	0	0

doi:10.1371/journal.pone.0053535.t001

tions that we detected have human-dengue counterparts found in other studies. While some of these may be genuine species-specific dengue interactions, it is also likely that the lack of overlap with previous studies is largely due to differences in the techniques and libraries used. We used library screening and directed assays (described further below), to detect 9 additional human-dengue interactions that correspond to 8 of the mosquito-dengue interactions, indicating that at least some of the mosquito-dengue interactions may be conserved (Table S1). It has been reported that some human proteins interact with proteins from a range of different viruses, perhaps because these human proteins are common viral targets or part of common cellular responses to viral infections [33,63]. We found that 15 of the mosquito proteins that we identified have human orthologs that interact with other viral proteins (Table S2). These include several that could be considered conserved interactions or interologs because they involve orthologous proteins from Hepatitis C virus (HCV). For example, we detected an interaction between dengue NS3 and mosquito titin (AAEL002565), an ortholog of human obscurin (OBSCN), which was shown to interact with HCV NS3 in a large-scale two-hybrid screen [64]. Similarly, we detected an interaction between NS3 and mosquito nucleosome assembly protein (AAEL005567), an ortholog of human Nucleosome Assembly Protein 1-Like 1 (NAP1L1), which was shown to interact with HCV NS3 [64]. The NS3 proteins from both dengue and HCV contain serine protease and RNA helicase domains and function similarly during the maturation of the viruses [17].

We looked at the expression data available for the mosquito genes that we identified. Several studies have examined the transcription response of mosquito tissues or cells to infection with dengue [65–67]. We found that genes for relatively few of the dengue interacting proteins appeared to be regulated in response to dengue infection. Among the transcripts that were modestly downregulated in the mosquito carcass in response to dengue [66], were four encoding NS3 interactors: three heat shock proteins (AAEL014845, AAEL014843, AAEL011708) and an uncharacterized ATPase (AAEL010585) that is orthologous to human valosin containing protein (VCP or p97). Interestingly, VCP, which is involved in protein processing in the endoplasmic reticulum [68,69], is also required for replication of poliovirus [70] and has been linked to the pathogenesis of hepatitis B virus [71]. None of the dengue interactors were shown to be regulated in midgut after infection [65]. Another study identified genes that are regulated in response to dengue infection in salivary glands, a key site of secondary infection in the mosquito [67]. Transcripts for two of the interactors are upregulated by dengue in the salivary gland, including one of the NS3-interacting heat shock proteins (AAEL014843) and a carboxypeptidase (AAEL010782) that interacted with capsid. Expression studies have found that genes associated with the innate immune response are regulated in response to dengue infection, including genes in the Toll-like receptor pathway and the JAK/STAT pathway [66]. We did not identify interactions involving any of these regulated genes. However, we did find that NS3 and NS5 interacted with endoplasmin (AAEL012827), a heat shock protein in the endoplasmic reticulum. Transcripts for endoplasmin are highly enriched in the salivary gland [67]. Endoplasmin, also known as gp96, is a chaperone required for proper folding of Toll-like receptors [72–74]. It is tempting to speculate that the interactions of endoplasmin with NS3 and NS5 may be a primary step in the innate immune response to dengue infection, or in the virus's attempt to circumvent the response.

In all we identified 34 NS3-interacting mosquito proteins. To explore the NS3 domains that may interact with the host proteins, we tested all of them against both full-length NS3 and NS3Δ1–160 (Table S3). As expected, all of the host proteins interacted with full-length NS3, including the three proteins that were originally isolated with NS3Δ1–160. Interestingly, most host proteins also interacted with NS3Δ1–160, indicating that they interact with the C-terminal half of NS3, which contains the helicase domain. Five host proteins were incapable of interacting with NS3Δ1–160, suggesting that they require the N-terminal protease domain of NS3 for interaction (Table S3). The NS3-interacting proteins were enriched for proteins with the gene ontology annotation "response to stress" and for proteins with the domain "heat shock protein" (Table S4), primarily because they include several heat shock proteins. Human Hsp90 and Hsp70 have been found in the dengue virus receptor complex [75], but no intracellular role for heat shock proteins during virus replication has been reported.

We identified 49 NS5-interacting mosquito proteins. The top enriched domains among these interactors were associated with myosin, found in non-muscle or smooth muscle myosin heavy chain (AAEL005656 and AAEL005733), myosin v (AAEL009357), long form paramyosin (AAEL010975), and a hypothetical protein (AAEL014104) (Table S4). Although there is no evidence linking myosin and NS5, myosin Vc was reported to be involved in the release of dengue virus from HepG2 cells [76]. Colpitts et al., detected several myosin proteins by co-affinity purification from mosquito cells; however, NS5 was not used in their study [36].

We identified 16 capsid-interacting mosquito proteins. Three of these were identified using the anchored capsid bait that contained the C-terminal membrane-spanning domain, while the others were identified using the mature capsid bait. We tested all of the capsid-interacting proteins to see if they were capable of interacting with each capsid bait protein and found that all but two proteins were capable of interacting with both baits (Table S5). The two-hybrid reporter activity was generally less with the anchored capsid compared to the mature capsid, which could explain our failure to isolate these proteins with the anchored capsid bait even though they were capable of interacting with it. This could be due to a lower expression level of the anchored capsid or to the impaired ability of the membrane domain to enter the yeast nucleus and fold properly. The capsid-interacting mosquito proteins are enriched for "nucleic acid binding" proteins and proteins with "Zn finger" domains (Table S4). Among the nucleic acid binding capsid-interacting proteins we three potentially RNA-binding proteins according to the functions of their human orthologs: a hypothetical protein (AAEL011985), putative myosin I (AAEL003676), and DEAD box ATP-dependent RNA helicase (AAEL009285). Moreover, the top protein domain enriched among the capsid interactors was the "G-patch" domain, which functions as an RNA-binding domain found in mRNA processing proteins and some retroviruses [77,78]. Since dengue capsid also directly binds to viral genomic RNA [79], it may be interesting to investigate whether interaction between capsid and G-patch proteins has any role in packaging the genome into the viral particle.

Dengue-human protein-protein interactions

While dengue-human protein interactions have been explored more extensively than dengue-mosquito protein interactions, the lack of overlap among validated interactions from different screens suggests that the dengue-human interactome is still incomplete. To complement the dengue-mosquito interactome and other dengue-human studies, we conducted two-hybrid screens using the 14 dengue protein baits (Figure 1B) and a cDNA library from human peripheral blood leukocytes (PBL). PBL contains a population of cells of the mononuclear phagocyte lineage, which are the primary

target of dengue virus infection in human [80–82]. The library screens and the reproducibility and specificity tests were conducted as in the mosquito library screens (Materials and Methods). Similar to the mosquito library screen, we did not find human proteins interacting with M, E, NS1, NS2A, NS4A and NS4B; nor did we find interactors for PrM. In total we identified 46 reproducible specific interactions between 35 human proteins and five bait proteins representing variants of dengue C, NS3, and NS5 (Table 1 and Table S6). Only six of the interactions had previously been detected or predicted (Table S6). These included two interactions (Capsid – Beta Hemoglobin (HBB) and Capsid - Ribosomal Protein L5 (RPL5)) that had been predicted based on structural similarity between dengue virus and host proteins [37] and four interactions (NS3 – Nuclear Factor of Kappa light polypeptide gene enhancer in B-cells Inhibitor, Alpha (NFKBIA), NS3 – Nuclear Receptor Binding Protein 1 (NRBP1), NS3 – Golgin B1 (GOLGB1), and NS5 – Rab Interacting Lysosomal Protein-Like 2 (RILPL2)) that were identified in separate two-hybrid screens [33,34,83].

Our library screens identified four putative conserved interactions, where both human and mosquito orthologs were identified as interacting with the same dengue proteins (Table S1). 54 out of the remaining 93 mosquito genes that we identified have human orthologs that we failed to isolate in screens of the human PBL library. Our failure to isolate human orthologs of these 54 mosquito genes could be because they are missing from the PBL cDNA library, or because the human orthologs actually do not interact with dengue proteins. To distinguish between these possibilities and to identify additional human-dengue interactions, we set out to test whether human orthologs of the mosquito proteins interact with the same dengue protein. Sequence analysis identified 96 potential human orthologs for the 54 mosquito genes (Table S1). We were able to retrieve and subclone 55 of these from a library of full-length human ORFs [84]. These 55 human genes are potential orthologs of 31 mosquito genes. We made two-hybrid AD clones for these 55 full-length ORFs and screened them against the corresponding dengue virus proteins. This resulted in identification of an additional five human-dengue interactions corresponding to four of the mosquito-dengue protein interactions (Table S1).

Combined, our human cDNA library screens and directed assays of mosquito orthologs identified 52 interactions involving 47 human proteins and three dengue proteins, capsid, NS3 and NS5 (TableS1 and Table S6). These include 46 novel interactions that were not previously detected or predicted; nine of these were detected with both human and mosquito orthologs. A global analysis of the human dengue-interacting proteins reveals no enriched GO annotations or protein domains. Similar to our finding with the mosquito proteins, a significant proportion of the dengue-interacting human proteins (19 out of 47) have been shown to interact with proteins from other viruses (Table S7) [85–91]. These include at least two interactions that could be thought of as conserved: Dengue NS3 interacted with Zinc Finger Protein 410 (ZNF410) and Calcium Binding and Coiled-Coil domain 2 (CALCOCO2), both of which have been shown to interact with HCV NS3 by a two-hybrid screen [64].

It has been shown that proteins from other viruses frequently interact with hub proteins, which are host proteins that have a large number of interactions in the host interactome [63]. To evaluate the numbers of interactions for the host proteins that we identified, we assembled a human protein interactome from several public databases (Materials and Methods). The human interactome contains 43 (92%) of the 47 dengue-interacting proteins that we identified. It also contains 144 (89%) of the 161 dengue-interacting human proteins identified exclusively in other screens [22,24,27–30,32–35,55,75,92–105], and 53 (84%) of the 63 human orthologs of mosquito proteins that we identified. For each of these gene sets, we found that the average number of interactions (or degree) per protein was significantly higher than for random samples of similar numbers of proteins. For example, the average degree of dengue-interacting proteins in our dataset was 44.0, whereas the average degree of similarly sized random samples of proteins was 22.4 (p-value = 9.3×10^{-4}) (Figure S2). The dengue interactors from mosquito were also enriched for proteins with many interactions (p-value = 2.7×10^{-3}), as were the dengue-interacting proteins identified by other studies (p-value = 6.4×10^{-7}) (Figure S2). It has been suggested that the tendency of viral proteins to interact with hub proteins may represent a feature of viral pathogenesis, since the disruption of a hub is more likely to impair the cell's protein network than the disruption of a non-hub [63,106]. While our results are consistent with this hypothesis, they could also be explained by the possibility that some proteins are particularly interactive in the protein interaction assays that have been used to detect the human interactome, including yeast two-hybrid. Thus a more thorough test of the hypothesis that dengue viral proteins tend to target hubs will require a larger set of functionally validated dengue-host interactions.

We identified a number of potentially relevant human NS3 interactors. CALCOCO2 (also known as NDP2) is a component of Nuclear Domain 10 (ND10) bodies, which play a role in the intrinsic cellular defense mechanisms against some viruses [107]. Interestingly, another major component of ND10 is DAXX, which has been shown to interact with dengue capsid [30]. Another NS3 interactor, Osteosarcoma amplified 9 (OS9), plays an important role in the unfolded protein response (UPR) [108], which is often observed in dengue-infected cells [109]. NS3 also interacted with three proteins that play roles in the Toll-like receptor-signaling pathway, which is important for the innate immune response to infection with many viruses [110,111]. This is the case also in mosquito where the homologous Toll pathway is known to be important for the immune response to dengue. It is has been shown, for example, that a negative regulator of the Toll pathway, Cactus, (AAEL000709), is downregulated in response to dengue infection, while RNAi silencing of Cactus can reduce dengue infection [66]. It is still unclear, however, how or whether dengue proteins directly interface with the Toll pathway in mosquito or with the Toll-like receptor pathway in humans. The interactions that we identified with NS3 may provide clues. One NS3 interactor that we found, as did Le Briton et al. [34], is a human ortholog of Cactus, NFKBIA, a protein that inhibits Toll-like receptor signaling by inhibiting the NF-kappaB transcription factor [112,113]. Another NS3 interactor that we identified is Coronin-1 (CORO1A), an actin-binding protein that is capable of inhibiting Toll-like receptor signaling [114]. Finally, we found that NS3 interacted with a protein named Leucine Rich Repeat (in FLII) Interacting Protein 1 (LRRFIP1). LRRFIP1 is a regulator of the Toll-like receptor-signaling pathway and has been shown to associate with dsRNA-containing endosomes/lysosomes [115], which are generated in response to viral dsRNA intermediates during replication [116]. It seems likely that at least some of these human NS3-interacting proteins may be involved in the interplay between the host defense mechanisms and the viral strategies to circumvent them. As we did for the mosquito proteins, we tested the human NS3 interacting proteins against both variants of NS3 baits (Figure 1B) and found that the N-terminal 160 amino acids of NS3 was required for only three interactions, including the previously identified interaction NS3 – NRBP1 (Table S3). The

remaining human proteins interacted with both the full-length and the C-terminal half of NS3.

The capsid interactors were isolated using the anchored capsid bait or the cytoplasmic capsid bait (Figure 1B). We tested all of the capsid interactors against both baits and found that most were able to interact with both the anchored and the cytoplasmic capsid proteins (Table S5), indicating that the C-terminal membrane spanning domain is not required for and does not dramatically interfere with most interactions. GO and protein domain enrichment analysis of the 20 capsid interactors failed to implicate any specific biological function or process (Table S8). However, the capsid interactors include a preponderance of ribosomal proteins, including RPL5, RPL6, RPL7, and RPL27, all of which are subunits of the 60S ribosome. Capsid also interacted with mosquito RPL23, and with a ribosomal RNA processing protein (RRP12) from both mosquito and human. Based in part on these ribosomal proteins and on a GTP-binding protein, GTPBP4, the capsid-interacting proteins are enriched for proteins annotated as being associated with the nucleolus (Table S8). Interestingly, dengue capsid has previously been found to accumulate in nucleoli in several cell lines [117–119], though the functional significance of this localization has not been determined. Many other viruses interact with nucleoli, and in some cases nucleoli have been shown to be essential for virus replication [120,121]. The capsid proteins from two other flaviviruses, West Nile virus and Japanese encephalitis virus, for example, each interact with specific nucleolar proteins, and in each case, these nucleolar proteins have been shown to be important for efficient viral replication [122–124]. Further studies with the proteins that we identified to interact with dengue capsid may provide insights into the role and mechanisms for accumulation of dengue capsid in the nucleolus.

Among the human NS5 interactors, "unfolded protein response (UPR)" is the top enriched GO annotation (Table S8). This enrichment is based on interactions with DERL2, an ER membrane protein involved in targeting misfolded glycoproteins for degradation [125,126], and HSPA5/Grp78/BiP, an ER protein involved in protein folding [127,128]. The UPR is known to be activated during dengue infection; however, its importance for virus replication is still undetermined [109,129–131].

Confirmation of protein interactions using additional assays

Yeast two-hybrid studies frequently detect false positive interactions that have no biological relevance. One way to gain confidence in a two-hybrid interaction is to detect it using additional assays. We used two approaches to test the confidence of interactions that we identified in the library screens. First, we reasoned that biologically relevant virus-host protein interactions are likely to be conserved across the four dengue serotypes. There is 63–68% amino acid sequence homology among the four serotypes [17]. An interaction between a host protein and the same dengue protein from multiple serotypes may imply that the interaction is more likely to have functional relevance because significant variation in the dengue protein does not interrupt the interaction. To test for conservation of interactions we repeated two-hybrid assays for all dengue-host interactions using dengue proteins from serotypes 1, 3 and 4. We found that 57 out of 102 (56.9%) dengue-mosquito protein interactions and 34 out of 46 (73.9%) dengue-human protein interactions were serotype independent; i.e., the host proteins interacted with the corresponding dengue proteins from all four serotypes (Figure 2; Tables S1 and S6). This provides additional evidence that these host proteins genuinely interact with the dengue proteins, and further points to conserved sequences or structural elements in the dengue proteins as potential interaction interfaces. A minority of the host proteins interacted with only one or a subset of the dengue serotypes (Figure 2; Tables S1 and S6). While some of these interactions may be false positives, others may be biologically relevant serotype-specific dengue-host interactions. If so, such interactions may mediate some of the serotype-specific dengue characteristics that are clinically observed [132]. Further investigation will be required to validate serotype specific interactions.

Next we employed an orthogonal assay, co-affinity purification (co-AP), to test most of the dengue-host interactions that we identified by yeast two-hybrid assays. We expressed myc-tagged versions of the mosquito and human proteins in cultured *Drosophila* cells along with NTAP-tagged dengue proteins (Materials and Methods). We purified the tagged dengue proteins and tested for co-purification of the host proteins by immunoblotting with myc antibodies (Figure 3 and Figure S3). If one of the two proteins failed to express in the cell lysate, we tried the experiment in the opposite orientation by expressing the dengue protein with a myc tag and the host protein with an NTAP tag. We were able to express and test by co-AP 136 pairs of proteins, and we detected 38 interactions (27.9%) (Table S1 and S6). This confirmation rate is similar to that reported for other large-scale tests of protein interactions by orthogonal assays [133,134], but lower than the rate reported in some individual two-hybrid studies [61,135]. One possible explanation for the discrepancy is that we define a two-hybrid positive based on reproducible activity of a highly sensitive *LEU2* reporter, and thus we may detect weaker protein-protein interactions than studies that require activation of multiple less sensitive two-hybrid reporters. The combined two-hybrid reporter activity (*LEU2* and *lacZ*) that we observed was only slightly higher for interactions that were positive by co-AP assays (average 3.4) than for interactions that were negative in co-AP assays (average 2.9), but this difference was not statistically significant. Thus, the numbers of active reporters and their levels in the two-hybrid assay was not a strong predictor of reproducibility by co-AP assay.

Figure 4 shows a summary of the dengue-host interactions that we identified. The dengue-human interaction map includes 13 proteins that have orthologs in the dengue-mosquito map and that were involved in protein-protein interactions (PPI) in both species. Three human proteins and seven mosquito proteins interacted with more than one dengue protein. The maps also show whether or not each interaction was detected with all four dengue serotypes and whether or not it was also detected by co-AP.

A snapshot of the dengue-host interactome

It is often noted that a virus such as dengue with only 10 proteins of its own should need to interact with a number of host proteins to carry out its replication cycle. Our study combined with other large-scale and small-scale studies [22,24,27–30,32–36,55,75,83,92–105] have identified 403 interactions between proteins from dengue and its hosts, not counting the more than 4,000 interactions that have been computationally predicted [37]. Since we know that most protein interaction screens and assays produce false positives, it seems likely that a number of the dengue-host PPI detected thus far are not relevant to the virus or the host's defenses against it. Among the 403 experimentally detected PPI, only seventeen PPI [23,28,33,93,94,100,103–105] have been studied further and shown to potentially have functional significance. How then can we decide which of the remainder of the interactions merit further investigation? The number of validated PPI is too small to use as a gold standard for developing a statistical scoring system to rank all PPI, as has been done for other interactomes [48,50,136]. Thus, we propose the use of two criteria for prioritizing the dengue-host PPI for further study. The

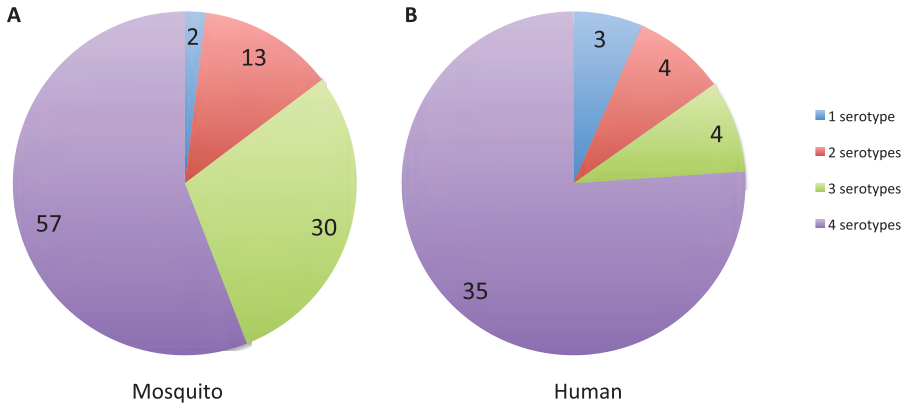

Figure 2. Pie charts showing the number of host proteins that interacted with the corresponding proteins from one, two, three, or all four dengue serotypes. (A) Mosquito proteins. (B) Human proteins.
doi:10.1371/journal.pone.0053535.g002

first criterion is based on the observation that PPI detected by multiple independent assays or studies are more likely to be biologically relevant [42–47,137]. Assuming that this is also true for the dengue-host interactions, we counted the number of assays and the number of studies that detected each of the physical interactions. Any orthogonal assay was counted as an individual piece of evidence. Two similar assays detecting the same PPI, but conducted by two independent groups, was also counted as two pieces of evidence. By this criterion, 67 of the 403 dengue PPI were detected thus far by more than one assay or study.

The second criterion proposed here is based on the fact that many biologically relevant PPI are conserved [138], and thus detection of the same interaction in two different species is tantamount to detecting the interaction more than once. Applying

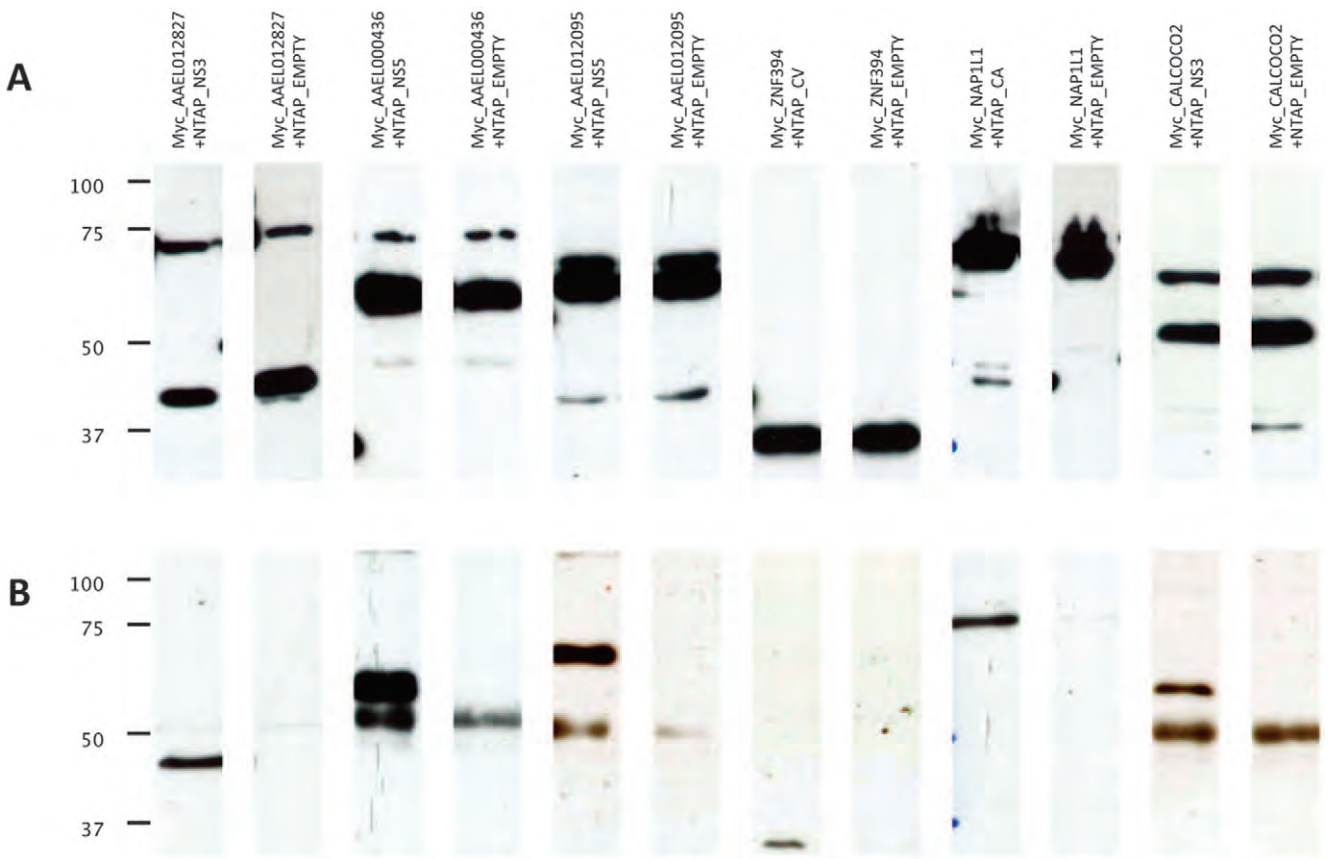

Figure 3. Examples of co-AP results. Host proteins were fused to a myc-tag while dengue proteins were fused to an NTAP-tag. The fusion proteins were expressed in S2R+ cells. NTAP-dengue proteins were purified from cell lysates, and then host proteins were detected with α-myc. (A) An α-myc immunoblot of cell lysates shows expression of mosquito and human proteins. (B) An α-myc immunoblot of NTAP-tag affinity-purified samples. Additional co-AP results are in Figure S3.
doi:10.1371/journal.pone.0053535.g003

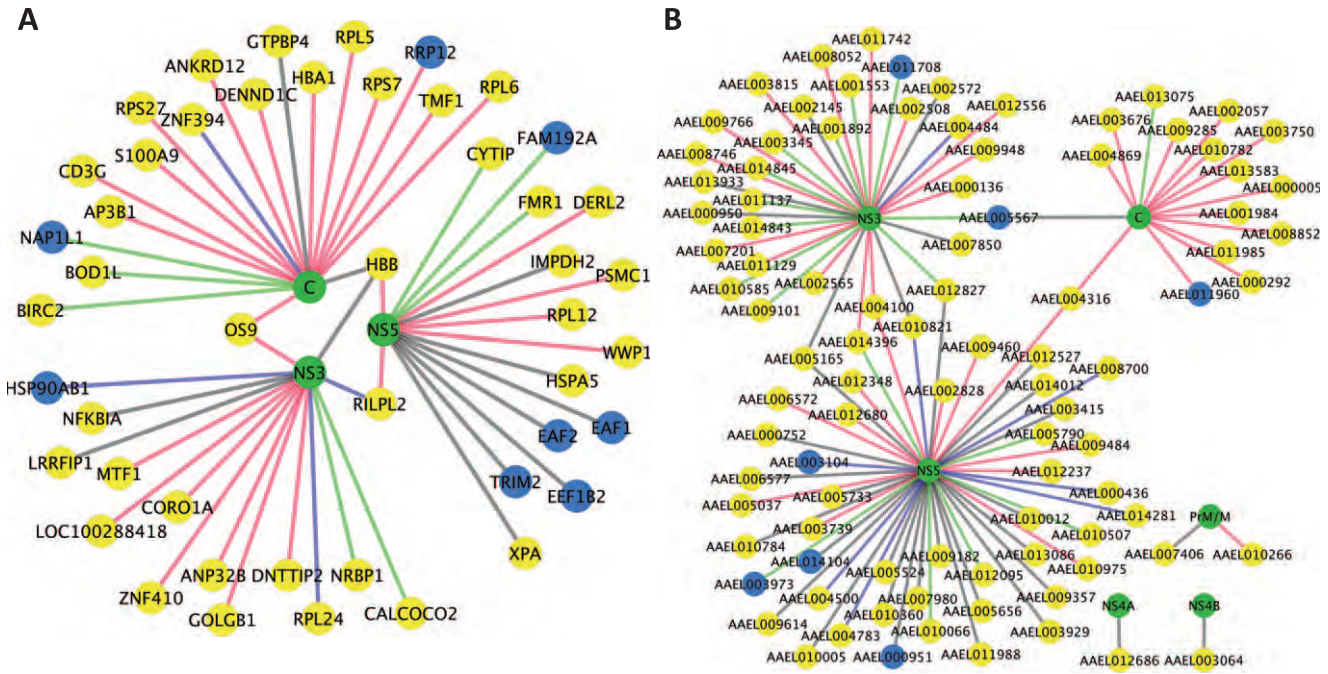

Figure 4. Dengue – host protein networks derived from two-hybrid screens and co-AP assays in this study. (A) Human-dengue interaction map. (B) Mosquito-dengue interaction map. Edges represent protein-protein interactions. Green nodes are dengue proteins, yellow nodes are host proteins, and blue nodes are host proteins found in both the human and mosquito maps. Red edges represent protein-protein interactions universally detected for all four serotypes. Blue edges represent protein-protein interactions confirmed by co-AP assays. Green edges represent the universal interactions that were confirmed by co-AP assays. Additional details are available in Cytoscape files in supplemental data (Data S1).
doi:10.1371/journal.pone.0053535.g004

this criterion to the dengue-host interactions, we counted a PPI as a potentially conserved interolog if it was found in both mosquito and human. 28 PPI (14 PPI for each species) were detected in both species. We also counted an interaction as having multiple forms of supporting evidence if it was experimentally detected and also computationally predicted [37]. Taking these criteria together, we derive a list of 35 dengue-mosquito PPI and 65 dengue-human PPI that have multiple forms of supporting evidence (Figure 5; Table S10). The list is biased against dengue and host membrane proteins, largely because the primary techniques that have been used to detect protein interactions, yeast two-hybrid and complex pull downs, are poor at detecting interactions involving membrane proteins [62]. The application of additional methods, such as protein fragment complementation [139] or co-complex purifications optimized for membrane proteins [140], will likely enable expansion of the list both by identifying new dengue-host interactions and by providing additional evidence for interactions that were thus far detected by only one method. In the meantime, using multiple forms of evidence as a guide can prioritize the existing protein interactions that merit further investigation.

For the above analysis we considered only evidence of direct protein-protein interactions. An alternative approach would be to consider other forms of evidence that may support the involvement of specific host proteins in dengue infection. Strong supporting evidence for a functionally relevant protein interaction, for example, can come from experiments showing that the host protein is required for efficient infection or propagation of the virus. Below we consider a number of protein interactions that, although detected by only one method, are supported by such functional data. Khadka et al. [33] used RNAi to test whether the expression of several human dengue-interacting proteins was required for efficient replication of a transfected dengue replicon. They showed that knockdown of six human proteins (CALR, DDX3X, ERC1, GOLGA2, TRIP11, and UBE2I) reduced the expression of a dengue reporter gene. Interactions involving two of these proteins (UBE2I and CALR) are supported by multiple forms of evidence (Figure 5A), yet the functional assay added additional support, particularly for interactions that had only been detected by the yeast two-hybrid assay. These include GOLGA2 – NS5, ERC1 – NS3, TRIP11 – NS3, TRIP11 – NS5, and the interaction of DDX3X with E, NS2A, NS4B, and NS5. Support for the functional significance of protein interactions has also come from genome-wide RNAi knockdown studies. Krishnan et al., [141] identified genes that when knocked down enhanced or inhibited infection of human cells with West Nile Virus, and then retested the hits for their effects on DENV2 infectivity. That study lends additional support to two interactions from our study (DERL2 – NS5 and ANP32B – NS3), and to another interaction (VPS11 – NS5), originally detected by two-hybrid [34]. In another study, Sessions et al., [142] identified *Drosophila* genes that were required for DENV2 propagation in *Drosophila* cells and then tested human orthologs of a subset of the hits in a similar assay. That study provided functional evidence supporting several protein interactions that had been detected only by yeast two-hybrid assays, including ENOX2 – NS3 [34], and as noted by Khadka et al. [33], SCLT1 – NS2A, ERC1 – NS5, and TTC1 – NS5. The genome-wide knockdown studies also support two interactions that we detected with mosquito genes, AAEL003750 – C and AAEL004783 – NS5. Knockdown of the human orthologs of AAEL003750, nucleoplasmin 3 (NPM3), enhanced dengue infection in human cells [141]; Guo et al., previously used this data to predict the involvement of AAEL003750 in dengue infection [38]. Knockdown of the *Drosophila* ortholog of AAEL004783, ornithine decarboxylase antizyme (Oda), inhibited propagation of

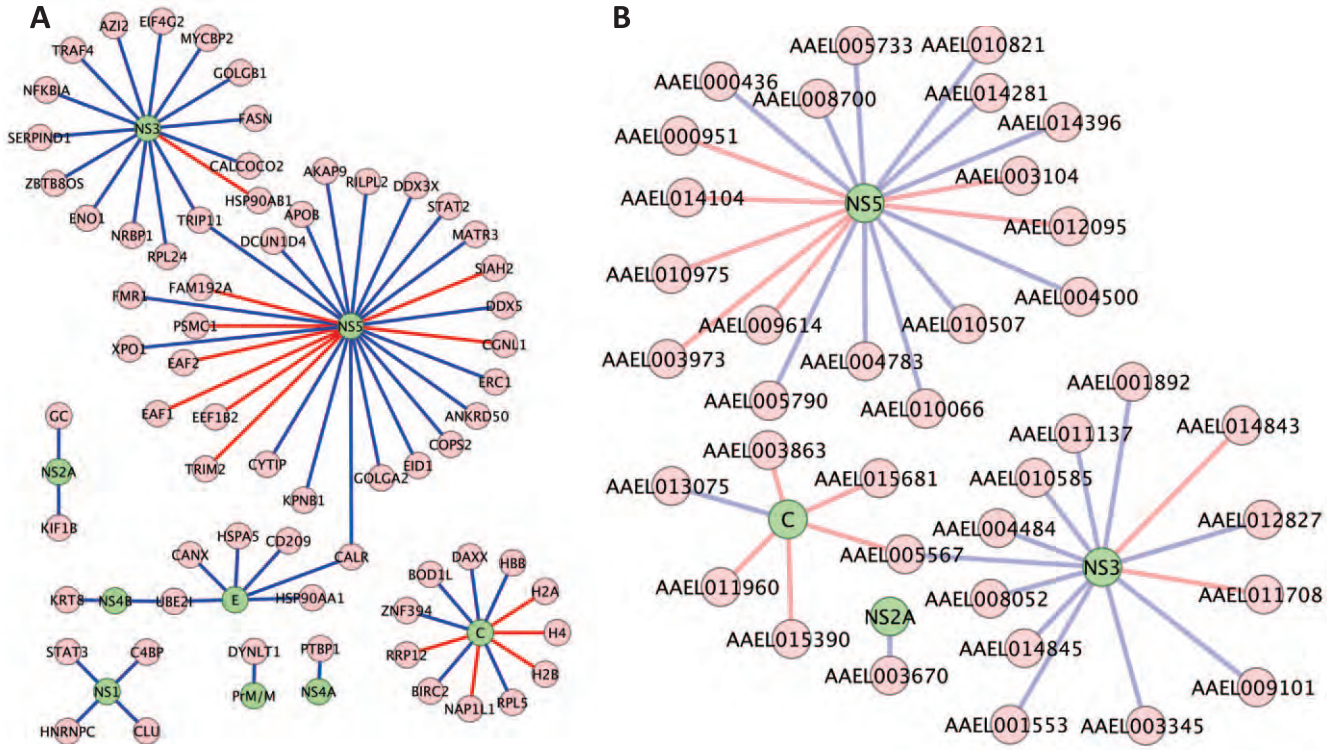

Figure 5. Dengue-host interactions supported by multiple forms of evidence. (A) Dengue-human interactome. (B) Dengue-mosquito interactome. Pink nodes represent host proteins. Green nodes represent dengue proteins. Red edges represent PPI with conserved interologs. Additional details are available in Cytoscape files in supplemental data (Data S1).
doi:10.1371/journal.pone.0053535.g005

dengue in *Drosophila* cells [142]. These examples show how integration of physical protein interaction data with functional data from high throughput studies can be used to prioritize host-dengue protein interactions for further analysis. Further investigation of these high-priority PPI and those supported by multiple forms of evidence (Figure 5) may lead to a better understanding the interplay between dengue and its hosts. Furthermore, these data should be useful for identifying host proteins or pathways that may be candidates for drug targets and vector control strategies.

Materials and Methods

Plasmids and subcloning

pHZ12 and pHZ13 are vectors containing the Gal4-responsive upstream activating sequence (UAS) driving expression of proteins with an N-terminal myc tag or TAP tag. Both vectors were made by modifying pUASattB [143], which contains the *Drosophila* marker gene mini-*white* and the phiC31 phage attachment site (attB) enabling targeted integration into specific attP sites engineered into *Drosophila* chromosomes. To construct pHZ12, a PCR fragment from pAS1 [144] was inserted downstream of the UAS in pUASattB. The PCR fragment contained a *Drosophila* translational start sequence followed by codons for 6 histidines and 3 myc tags, and recombination target sequences, 5RT (5′-TTGACTGTATCGCCG-3′) and 3RT (5′-CCGGAATTAGCTTGGCTGCAG-3′); these sequences allow insertion of open reading frames (ORFs) using recombination in *E. coli* or yeast [145]. pHZ13 was constructed by inserting a fragment containing the NTAP tag from pUAS-NTAP [146] followed by 5RT and 3RT downstream of the UAS in pUASattB. pHZ12_attR and pHZ13_attR were constructed by inserting a Gateway destination vector cassette (Invitrogen) into the cloning sites of pHZ12 and pHZ13. Briefly, the Gateway cassette was PCR amplified from pJZ4_attR with primers, DM138 and DM139 (see Table S9 for sequences) and then digested with *Xba*I and inserted into pHZ12 and pHZ13 digested with *Pme*I and *Xba*I. The ligations were used to transform *E. coli*, OmniMAXII (Invitrogen). Transformants with plasmids containing a Gateway cassette were selected on LB-*Chloramphenicol/Ampicillin* media. pJZ4_attR is a gateway destination plasmid containing an activation domain for yeast two-hybrid assays [147].

Dengue virus prototype strains for serotypes 1 (Hawaii), 2 (16681), 3(H87) and 4 (H241) were cultured in C6/36 cell lines. Viral RNA was extracted from the culture supernatant with the QIAamp Viral RNA Kit (Qiagen). cDNAs encoding each dengue virus 2 protein were generated from an infectious clone, a gift from Dr. Nopporn Sittisombut (Chiang Mai University, Chiang Mai, Thailand). cDNA synthesis was performed with primers targeting the 3′ end of each viral genome – Hawii and 16681 CACCATTCCATTTTCTGGCGTTC, H87 TGGCGTTCTGTGCCTGGAATGAT and H241 TCAACAA-CACCAATCCATCTTGCGG - using SuperScript II Reverse Transcriptase (Invitrogen) according to the product manual. Individual viral genes or gene fragments were PCR amplified from the cDNA with specific primers (Table S9). Each 5′ primer included the attB1 sequence, 5′ – GGGG-ACA-AGT-TTG-TAC-AAA-AAA-GCA-GGC-T – 3′, and each 3′ primer included the attB2 sequence, ′5 – GGGG-ACC-ACT-TTG-TAC-AAG-AAA-GCT-GGG-T– 3′. The PCR products were cloned into pDONR221 and then into destination vectors using BP Clonase II and LR Clonase (Invitrogen) according to the manufacturer's protocol. The entry clones were sequenced with M13F(-21) and M13R sequencing primers. Each viral gene or gene fragment was transferred to the following destination vectors:

pNLex_attR and pJZ4_attR for two-hybrid assays [147], and pHZ12_attR and pHZ13_attR for co-AP assays. Human open reading frames (ORFs) were similarly cloned into these destination vectors from entry vectors obtained from the human ORFeome collection [84] obtained from Open Biosystems. For co-AP assays, individual mosquito or human cDNAs were subcloned from the yeast two-hybrid library vectors into pHZ12 and pHZ13 by recombination cloning as previously described [145].

Mosquito cDNA libraries for yeast two-hybrid screens

An *Aedes aegypti* colony was established and maintained according to a published protocol [148]. Briefly, a mosquito colony was maintained at 27°C, 70–90% relative humidity in an 8-hour dark/16-hour light cycle. Newly hatched larvae were fed with ground rat food. One-day-old larvae were counted, and a group of 200–250 larvae were added in a tray containing 700–800 ml of water. Three pellets of cat food (Friskies Senior) were added to each tray. Adults were fed with either 10% sucrose or blood from a mouse. RNA was isolated separately from ten stages of development including 1) less than three-month-old embryos, 2) one-day-old larvae, 3) two-day-old larvae, 4) three-day-old larvae, 5) four-day-old larvae, 6) five-day-old larvae, 7) six-day-old larvae, 8) pupae, 9) adults and 10) adults collected three hours after a blood meal. In order to collect enough RNA, eggs from several layings were independently collected and pooled; the oldest eggs in the pool were aged less than three months. Eggs were synchronously hatched by applying a vacuum (13 to 15 inHg) for 20–40 minutes. Larvae were collected every 24 hours for six days. Pupae were collected at 120–144 hours after egg hatching. Adults were collected 3 days after emerging from pupae. The adults fed with a blood meal were collected three hours later so that the genes responding to blood ingestion were sufficiently expressed [149]. Samples were homogenized with a dounce homogenizer and RNA was isolated from tissue homogenate with an RNeasy Midi kit (Qiagen). The RNA was treated with RNase-free DNase (Qiagen) at room temperature for 15 minutes. Poly(A) RNA was enriched with the Poly(A)Purist kit (Ambion). 0.5 µg of poly(A) RNA from each of the ten stages was pooled and used for cDNA synthesis with the directional cDNA Synthesis kit (Stratagene), which uses an oligo dT 3′ primer containing an *Xho*I site, 5′-GAGAGAGAGAGAGAGAGAGAACTAGTCTCGAGTTTTT-TTTTTTTTTTTTT -3′. Next, the cDNA was ligated with an *EcoRI* adapter, 5′-OH-AATTCGGCACGAGG-3′ 3′-GCCGT-GCTCCp-5′

The cDNA was digested with *Xho*I, fractionated on Sepharose CL-2B gel filtration medium and then ligated into the yeast two-hybrid activation domain (AD) vector, pRF4-5o [150] cut with EcoRI and XhoI. Ligation reactions were ethanol precipitated, washed in 80% ethanol, resuspended in sterile distilled water, and then used to transform *E.coli*, *MegaX DH10B*^{TM} T1R Electrocomp^{TM} Cells (Invitrogen), by electroporation. 188 *E. coli* transformant colonies were randomly picked for colony PCR. 64% of the colonies had inserts with various sizes from 300 to 4,000 bp. The average size of the inserts was 1,400 bp. More than 10^7 *E. coli* colonies were collected for plasmid extraction with a QIAGEN Plasmid Giga kit.

Yeast two-hybrid screens and assays

The LexA version of the two-hybrid system from the Brent laboratory [151] was used to screen for host proteins (human or mosquito) that interact with LexA-fused dengue proteins essentially as described [152] (Table S10). A summary of the screening results is available in Table S11. The mosquito cDNA library (described above) was used to transform yeast strain RFY231 (MATα trp1::hisG his3 ura3–1 leu2::3Lexop-LEU2) using the lithium acetate method [153] and Trp+ colonies were selected. 1.1×10^8 transformant colonies were harvested and frozen in 1 ml aliquots at −80°C. Similarly, a human cDNA library (Origene Technologies) with cDNA from peripheral blood leukocytes (PBL) cloned into the AD vector pJG4–5 [154] was used to transform RFY231 and 2.2×10^8 transformant colonies were harvested and frozen in aliquots. Bait strains were created by transforming RFY206 (MATa trp1Δ::hisG his3Δ200 leu2–3 lys2Δ201 ura3-52 mal-), which also contained the *lacZ* reporter plasmid, pSH18-34(URA3+), with pNLex bait plasmids expressing dengue proteins. Each screen was carried out by mating a freshly grown aliquot of a bait strain with a thawed aliquot of yeast containing the human or mosquito libraries. About 2×10^8 colony-forming units (cfu) of the bait strain were mated with $0.5–1\times10^8$ cfu of the library strains on a YPD plate. Diploids were collected, induced in liquid galactose medium for 4 hours at 30°C, and plated on galactose medium lacking leucine. cDNA inserts from galactose-dependent Leu+ yeast were PCR amplified and re-cloned into the AD vector in fresh RFY231 yeast by gap repair [155]. The freshly transformed yeast were then used in 96-well interaction mating assays as previously described [150,156] to confirm an interaction with the original bait protein. To test for specificity, interactions were also tested between each host protein and two unrelated bait proteins, *Drosophila* Eip63E (FBgn0005640) and Cyclin J (FBgn0010317). cDNAs that passed the tests were PCR amplified and digested with *AluI* to identify unique digestion patterns, which may represent unique genes [152]. These cDNAs were then sequenced and identified. Human or mosquito proteins that were confirmed to interact with the dengue serotype 2 bait used to isolate them were also tested against the same dengue protein in all four serotypes using the 96-well interaction mating assays. cDNA from confirmed clones was PCR amplified, sequenced, and the genes were identified by nucleotide BLAST analysis.

Co-affinity purification (Co-AP) assays

D. melanogaster S2R+ cells were obtained from the *Drosophila* Genomics Resource Center at Indiana University. The cells were cultured in Schneider's media (GIBCO) supplemented with 10% FBS (Gemini) and 1%Gentamycin (GIBCO). S2R+ cells were incubated at 25°C. The cells were passaged once per week at 1:10 dilution. Co-AP assays were performed essentially as described [144]. Briefly, S2R+ cells were co-transfected with pHZ12 plasmids containing human or *Aedes aegypti* cDNA, pHZ13 plasmids containing dengue ORFs, and the expression driver plasmid pMT-GAL4 using the tranfection reagent, Effectene (Qiagen). pMT-Gal4 expresses Gal4 from the Cu^{+2}-inducible metallothionine promoter [157]. 1×10^6 cells were seeded onto each well of a 12-well cell culture plate 18–24 hours before transfection with 250 ng of each plasmid, pHZ12, pHZ13 and pMT-GAL4. 12–18 hours after transfection the media was supplemented with 1 mM $CuSO_4$ to induce protein expression. Three days after $CuSO_4$ induction the cells were harvested and lysed. Expression of fusion proteins was determined by Western blot analysis of cell lysates using anti-NTAP (Rockland Immunochemicals) and anti-Myc (Santa Cruz Biotechnology) antibodies for proteins expressed from pHZ13 and pHZ12, respectively. For lysates with detectable expression of both the Myc-tagged and NTAP-tagged proteins, IgG beads were used to purify the NTAP-tagged protein complexes, the complexes were run on SDS-PAGE gels, and the gels were immunoblotted with anti-Myc to probe whether the Myc-tagged protein was co-purified. For interactions involving proteins with undetectable expression, the co-AP assay was attempted by swapping the inserts between pHZ12 and

pHZ13. Interactions were only considered tested if both tagged proteins could be detected in cell lysates.

Computational analyses

For enrichment analysis of *Aedes aegypti* mosquito dengue interactors, a gene ontology annotation (GOA) file was downloaded from UniProt-GOA (ftp.ebi.ac.uk/pub/databases/GO/goa/proteomes/31436.A_aegypti.goa) [158], and an OBO file version 1.2 was downloaded from The Gene Ontology project (www.geneontology.org/ontology/obo_format_1_2/gene_ontology_ext.obo) [159]. A tree for InterPro domains was downloaded from EMBL-EBI (ftp.ebi.ac.uk/pub/databases/interpro/interpro.xml.gz) [160]. Cytoscape [161] with the BINGO plug-in [162] was used to analyze GO annotation and InterPro domain enrichments of dengue interactors. For analysis of the degree of dengue-interacting host proteins we assemble a human PPI interactome by combining the unique PPI collected from IntAct [163], MINT [164], BioGRID [165], Reactome [166] and HPRD [167] on 11 November 2010. The identifiers of the interacting proteins from these databases were all converted to Ensembl gene identifiers and a human PPI network having 134,050 unique interactions among proteins from 11,982 genes. Three gene sets were considered: human interactors identified in our screen, human interactors identified in other screens, and human orthologs of *Aedes aegypti* interactors identified in our screen. For each gene set the degrees of all the genes were calculated and averaged. Next, random sets of genes of equal size to each of the three gene sets were picked and the average degrees for each random set were calculated. This was repeated for one million random sets. The distribution of these averages is plotted in Figure S2. The *p*-value significance of the average degree for each gene set was calculated from the respective distribution using a two-tailed test.

Supporting Information

Table S1 Dengue-mosquito interactions found in this study.
(XLSX)

Table S2 Mosquito proteins with human orthologs that interact with proteins from other viruses.
(XLSX)

Table S3 NS3 domain analysis.
(XLSX)

Table S4 Gene Ontology (GO) and domain enrichment for mosquito proteins.
(XLSX)

Table S5 Capsid domain analysis.
(XLSX)

Table S6 Dengue-human interactions found in this study.
(XLSX)

Table S7 Human proteins that interact with proteins from other viruses.
(XLSX)

Table S8 Gene Ontology (GO) and domain enrichment for human proteins.
(XLSX)

Table S9 Oligonucleotide primers used in this study.
(XLSX)

Table S10 Dengue-host protein-protein interactions supported by multiple forms of evidence.
(XLSX)

Table S11 Yeast two-hybrid screen details.
(XLSX)

Figure S1 Intraviral protein-protein interactions. Interactions were identified by the galactose-dependent growth of diploid yeast expressing two dengue proteins. Each panel is a group of four indicator plates: Glucose complete minimal (CM) lacking leucine (–leucine) (top-left), Galactose CM –leucine (top-right), Glucose CM +X-gal (bottom-left) and Galactose CM +X-gal (bottom-left). An interaction is indicated by galactose-dependent growth on the plates lacking leucine (top two plates in each panel) or galactose-dependent blue colony color on the X-Gal plates (bottom two plates in each panel). *Drosophila melanogaster* Cyclin Y and Eip63E were used as a positive interaction control while *D. melanogaster* Cyclin Y and Cyclin J were used as a negative control. All media lack uracil, histidine, and tryptophan to select the two-hybrid plasmids.
(PDF)

Figure S2 Dengue-interacting host proteins are enriched for hubs. Enrichment analyses for proteins with many interacting partners in three dengue-host interactomes: human interactors of dengue baits identified in this screen (A), human interactors of dengue baits identified in other screens (B), and *Aedes aegypti* interactors of dengue baits in this screen (C).
(PDF)

Figure S3 Co-AP assays for dengue-host protein interactions. Additional co-AP results that were not shown in Figure 3. Details are as described in Figure 3.
(PDF)

Data S1 Cytoscape files containing all of the interaction data. Four Cytoscape files combined into a single compressed file include all of the two-hybrid interactions detected between dengue and either human or mosquito proteins, as well as the interactions supported by multiple forms of evidence. The interactions are annotated with information about how they were detected, whether they were conserved among the four serotypes, and whether they were confirmed by co-affinity purification assays.
(ZIP)

Acknowledgments

We thank Dr. Mark Brown (University of Georgia, Athens, Georgia) for providing *Aedes aegypti* eggs, Dr. Eduardo Palomino (Wayne State University) for mouse blood, and Dr. Nopporn Sittisombut (Chiang Mai University, Chiang Mai, Thailand) for dengue cDNA. We also thank Drs. Dongmei Liu and Steve Guest for reagents and helpful discussions.

Author Contributions

Conceived and designed the experiments: DM RLF. Performed the experiments: DM HZ. Analyzed the data: DM HZ TM RLF. Contributed reagents/materials/analysis tools: AS PS PM TL. Wrote the paper: DM RLF.

References

1. Gubler DJ (1998) Dengue and dengue hemorrhagic fever. Clin Microbiol Rev 11: 480–496.
2. Guzman MG, Halstead SB, Artsob H, Buchy P, Farrar J, et al. (2010) Dengue: a continuing global threat. Nat Rev Microbiol 8: S7–16.

3. Murphy BR, Whitehead SS (2011) Immune response to dengue virus and prospects for a vaccine. Annu Rev Immunol 29: 587–619.
4. Schmitz J, Roehrig J, Barrett A, Hombach J (2011) Next generation dengue vaccines: a review of candidates in preclinical development. Vaccine 29: 7276–7284.
5. CDC (2010) Locally acquired Dengue–Key West, Florida, 2009–2010. MMWR 59: 577–581.
6. Guzman A, Isturiz RE (2010) Update on the global spread of dengue. Int J Antimicrob Agents 36 Suppl 1: S40–42.
7. Black WCt, Bennett KE, Gorrochotegui-Escalante N, Barillas-Mury CV, Fernandez-Salas I, et al. (2002) Flavivirus susceptibility in Aedes aegypti. Arch Med Res 33: 379–388.
8. Watts DM, Burke DS, Harrison BA, Whitmire RE, Nisalak A (1987) Effect of temperature on the vector efficiency of Aedes aegypti for dengue 2 virus. Am J Trop Med Hyg 36: 143–152.
9. Najera JA, Gonzalez-Silva M, Alonso PL (2011) Some lessons for the future from the Global Malaria Eradication Programme (1955–1969). PLoS Med 8: e1000412.
10. Dame DA, Curtis CF, Benedict MQ, Robinson AS, Knols BG (2009) Historical applications of induced sterilisation in field populations of mosquitoes. Malar J 8 Suppl 2: S2.
11. Hoffmann AA, Montgomery BL, Popovici J, Iturbe-Ormaetxe I, Johnson PH, et al. (2011) Successful establishment of Wolbachia in Aedes populations to suppress dengue transmission. Nature 476: 454–457.
12. Phuc HK, Andreasen MH, Burton RS, Vass C, Epton MJ, et al. (2007) Late-acting dominant lethal genetic systems and mosquito control. BMC Biol 5: 11.
13. Wise de Valdez MR, Nimmo D, Betz J, Gong HF, James AA, et al. (2011) Genetic elimination of dengue vector mosquitoes. Proc Natl Acad Sci U S A 108: 4772–4775.
14. Westaway EG, and J. Blok (1997) Taxonomy and evolutionary relationships of flaviviruses. In: Gubler DJ, editor. Dengue and dengue hemorrhagic fever. London: CAB International. pp. 147–173.
15. Holmes EC, Twiddy SS (2003) The origin, emergence and evolutionary genetics of dengue virus. Infect Genet Evol 3: 19–28.
16. Kuno G, Chang GJ, Tsuchiya KR, Karabatsos N, Cropp CB (1998) Phylogeny of the genus Flavivirus. J Virol 72: 73–83.
17. Lindenbach BD, Thiel HJ, Rice CM (2006) Flaviviridae: The Viruses and Their Replication. In: Knipe DMH, Peter M., editor. Fields Virology. 5 ed. Philadelphia, USA.: Lippincott Williams & Wilkins. pp. 1153–1252.
18. Sampath A, Padmanabhan R (2009) Molecular targets for flavivirus drug discovery. Antiviral Res 81: 6–15.
19. Ackermann M, Padmanabhan R (2001) De novo synthesis of RNA by the dengue virus RNA-dependent RNA polymerase exhibits temperature dependence at the initiation but not elongation phase. J Biol Chem 276: 39926–39937.
20. Egloff MP, Benarroch D, Selisko B, Romette JL, Canard B (2002) An RNA cap (nucleoside-2′-O-)-methyltransferase in the flavivirus RNA polymerase NS5: crystal structure and functional characterization. EMBO J 21: 2757–2768.
21. Munoz-Jordan JL, Sanchez-Burgos GG, Laurent-Rolle M, Garcia-Sastre A (2003) Inhibition of interferon signaling by dengue virus. Proc Natl Acad Sci U S A 100: 14333–14338.
22. Mazzon M, Jones M, Davidson A, Chain B, Jacobs M (2009) Dengue virus NS5 inhibits interferon-alpha signaling by blocking signal transducer and activator of transcription 2 phosphorylation. J Infect Dis 200: 1261–1270.
23. Avirutnan P, Fuchs A, Hauhart RE, Somnuke P, Youn S, et al. (2010) Antagonism of the complement component C4 by flavivirus nonstructural protein NS1. J Exp Med 207: 793–806.
24. Avirutnan P, Hauhart RE, Somnuke P, Blom AM, Diamond MS, et al. (2011) Binding of flavivirus nonstructural protein NS1 to C4b binding protein modulates complement activation. J Immunol 187: 424–433.
25. Netsawang J, Noisakran S, Puttikhunt C, Kasinrerk W, Wongwiwat W, et al. (2010) Nuclear localization of dengue virus capsid protein is required for DAXX interaction and apoptosis. Virus Res 147: 275–283.
26. Bhuvanakantham R, Chong MK, Ng ML (2009) Specific interaction of capsid protein and importin-alpha/beta influences West Nile virus production. Biochem Biophys Res Commun 389: 63–69.
27. Johansson M, Brooks AJ, Jans DA, Vasudevan SG (2001) A small region of the dengue virus-encoded RNA-dependent RNA polymerase, NS5, confers interaction with both the nuclear transport receptor importin-beta and the viral helicase, NS3. J Gen Virol 82: 735–745.
28. Rawlinson SM, Pryor MJ, Wright PJ, Jans DA (2009) CRM1-mediated nuclear export of dengue virus RNA polymerase NS5 modulates interleukin-8 induction and virus production. J Biol Chem 284: 15589–15597.
29. Colpitts TM, Barthel S, Wang P, Fikrig E (2011) Dengue virus capsid protein binds core histones and inhibits nucleosome formation in human liver cells. PLoS One 6: e24365.
30. Limjindaporn T, Netsawang J, Noisakran S, Thiemmeca S, Wongwiwat W, et al. (2007) Sensitization to Fas-mediated apoptosis by dengue virus capsid protein. Biochem Biophys Res Commun 362: 334–339.
31. Nagila A, Netsawang J, Srisawat C, Noisakran S, Morchang A, et al. (2011) Role of CD137 signaling in dengue virus-mediated apoptosis. Biochem Biophys Res Commun 410: 428–433.
32. Ashour J, Laurent-Rolle M, Shi PY, Garcia-Sastre A (2009) NS5 of dengue virus mediates STAT2 binding and degradation. J Virol 83: 5408–5418.
33. Khadka S, Vangeloff AD, Zhang C, Siddavatam P, Heaton NS, et al. (2011) A physical interaction network of dengue virus and human proteins. Mol Cell Proteomics 10: M111 012187.
34. Le Breton M, Meyniel-Schicklin L, Deloire A, Coutard B, Canard B, et al. (2011) Flavivirus NS3 and NS5 proteins interaction network: a high-throughput yeast two-hybrid screen. BMC Microbiol 11: 234.
35. Folly BB, Weffort-Santos AM, Fathman CG, Soares LR (2011) Dengue-2 structural proteins associate with human proteins to produce a coagulation and innate immune response biased interactome. BMC Infect Dis 11: 34.
36. Colpitts TM, Cox J, Nguyen A, Feitosa F, Krishnan MN, et al. (2011) Use of a tandem affinity purification assay to detect interactions between West Nile and dengue viral proteins and proteins of the mosquito vector. Virology 417: 179–187.
37. Doolittle JM, Gomez SM (2011) Mapping protein interactions between Dengue virus and its human and insect hosts. PLoS 5: e954.
38. Guo X, Xu Y, Bian G, Pike AD, Xie Y, et al. (2010) Response of the mosquito protein interaction network to dengue infection. BMC Genomics 11: 380.
39. Cusick ME, Yu H, Smolyar A, Venkatesan K, Carvunis AR, et al. (2009) Literature-curated protein interaction datasets. Nat Methods 6: 39–46.
40. Huang H, Jedynak BM, Bader JS (2007) Where have all the interactions gone? Estimating the coverage of two-hybrid protein interaction maps. PLoS Comput Biol 3: e214.
41. Hart GT, Ramani AK, Marcotte EM (2006) How complete are current yeast and human protein-interaction networks? Genome Biol 7: 120.
42. Giot L, Bader JS, Brouwer C, Chaudhuri A, Kuang B, et al. (2003) A protein interaction map of Drosophila melanogaster. Science 302: 1727–1736.
43. von Mering C, Krause R, Snel B, Cornell M, Oliver SG, et al. (2002) Comparative assessment of large-scale data sets of protein-protein interactions. Nature 417: 399–403.
44. Deane CM, Salwinski L, Xenarios I, Eisenberg D (2002) Protein interactions: two methods for assessment of the reliability of high throughput observations. Mol Cell Proteomics 1: 349–356.
45. Ito T, Chiba T, Ozawa R, Yoshida M, Hattori M, et al. (2001) A comprehensive two-hybrid analysis to explore the yeast protein interactome. Proc Natl Acad Sci U S A 98: 4569–4574.
46. Uetz P, Giot L, Cagney G, Mansfield TA, Judson RS, et al. (2000) A comprehensive analysis of protein-protein interactions in Saccharomyces cerevisiae. Nature 403: 623–627.
47. Stanyon CA, Liu G, Mangiola BA, Patel N, Giot L, et al. (2004) A Drosophila protein-interaction map centered on cell-cycle regulators. Genome Biol 5: R96.
48. Braun P, Tasan M, Dreze M, Barrios-Rodiles M, Lemmens I, et al. (2009) An experimentally derived confidence score for binary protein-protein interactions. Nat Methods 6: 91–97.
49. Chen YC, Rajagopala SV, Stellberger T, Uetz P (2010) Exhaustive benchmarking of the yeast two-hybrid system. Nat Methods 7: 667–668; author reply 668.
50. Yu J, Finley RL Jr (2009) Combining multiple positive training sets to generate confidence scores for protein-protein interactions. Bioinformatics 25: 105–111.
51. Rice CM, Lenches EM, Eddy SR, Shin SJ, Sheets RL, et al. (1985) Nucleotide sequence of yellow fever virus: implications for flavivirus gene expression and evolution. Science 229: 726–733.
52. Smith GW, Wright PJ (1985) Synthesis of proteins and glycoproteins in dengue type 2 virus-infected vero and Aedes albopictus cells. J Gen Virol 66 (Pt 3): 559–571.
53. Lobigs M (1993) Flavivirus premembrane protein cleavage and spike heterodimer secretion require the function of the viral proteinase NS3. Proc Natl Acad Sci U S A 90: 6218–6222.
54. Dejnirattisai W, Jumnainsong A, Onsirisakul N, Fitton P, Vasanawathana S, et al. (2010) Cross-reacting antibodies enhance dengue virus infection in humans. Science 328: 745–748.
55. Brooks AJ, Johansson M, John AV, Xu Y, Jans DA, et al. (2002) The interdomain region of dengue NS5 protein that binds to the viral helicase NS3 contains independently functional importin beta 1 and importin alpha/beta-recognized nuclear localization signals. J Biol Chem 277: 36399–36407.
56. Kapoor M, Zhang L, Ramachandra M, Kusukawa J, Ebner KE, et al. (1995) Association between NS3 and NS5 proteins of dengue virus type 2 in the putative RNA replicase is linked to differential phosphorylation of NS5. J Biol Chem 270: 19100–19106.
57. Vasudevan SG, Johansson M, Brooks AJ, Llewellyn LE, Jans DA (2001) Characterisation of inter- and intra-molecular interactions of the dengue virus RNA dependent RNA polymerase as potential drug targets. Farmaco 56: 33–36.
58. Arias CF, Preugschat F, Strauss JH (1993) Dengue 2 virus NS2B and NS3 form a stable complex that can cleave NS3 within the helicase domain. Virology 193: 888–899.
59. Wang S, He R, Anderson R (1999) PrM- and cell-binding domains of the dengue virus E protein. J Virol 73: 2547–2551.
60. Clum S, Ebner KE, Padmanabhan R (1997) Cotranslational membrane insertion of the serine proteinase precursor NS2B-NS3(Pro) of dengue virus type 2 is required for efficient in vitro processing and is mediated through the hydrophobic regions of NS2B. J Biol Chem 272: 30715–30723.
61. Rual JF, Venkatesan K, Hao T, Hirozane-Kishikawa T, Dricot A, et al. (2005) Towards a proteome-scale map of the human protein-protein interaction network. Nature 437: 1173–1178.

62. Jensen LJ, Bork P (2008) Biochemistry. Not comparable, but complementary. Science 322: 56–57.
63. Dyer MD, Murali TM, Sobral BW (2008) The landscape of human proteins interacting with viruses and other pathogens. PLoS Pathog 4: e32.
64. de Chassey B, Navratil V, Tafforeau L, Hiet MS, Aublin-Gex A, et al. (2008) Hepatitis C virus infection protein network. Mol Syst Biol 4: 230.
65. Sim S, Dimopoulos G (2010) Dengue virus inhibits immune responses in Aedes aegypti cells. PLoS One 5: e10678.
66. Xi Z, Ramirez JL, Dimopoulos G (2008) The Aedes aegypti toll pathway controls dengue virus infection. PLoS Pathog 4: e1000098.
67. Sim S, Ramirez JL, Dimopoulos G (2012) Dengue virus infection of the Aedes aegypti salivary gland and chemosensory apparatus induces genes that modulate infection and blood-feeding behavior. PLoS Pathog 8: e1002631.
68. Ye Y, Meyer HH, Rapoport TA (2001) The AAA ATPase Cdc48/p97 and its partners transport proteins from the ER into the cytosol. Nature 414: 652–656.
69. Meyer HH, Shorter JG, Seemann J, Pappin D, Warren G (2000) A complex of mammalian ufd1 and npl4 links the AAA-ATPase, p97, to ubiquitin and nuclear transport pathways. EMBO J 19: 2181–2192.
70. Arita M, Wakita T, Shimizu H (2012) Valosin-containing protein (VCP/p97) is required for poliovirus replication and is involved in cellular protein secretion pathway in poliovirus infection. J Virol 86: 5541–5553.
71. Jiao BY, Lin WS, She FF, Chen WN, Lin X (2011) Hepatitis B virus X protein enhances activation of nuclear factor kappaB through interaction with valosin-containing protein. Arch Virol 156: 2015–2021.
72. Randow F, Seed B (2001) Endoplasmic reticulum chaperone gp96 is required for innate immunity but not cell viability. Nat Cell Biol 3: 891–896.
73. Yang Y, Liu B, Dai J, Srivastava PK, Zammit DJ, et al. (2007) Heat shock protein gp96 is a master chaperone for toll-like receptors and is important in the innate function of macrophages. Immunity 26: 215–226.
74. Morales C, Wu S, Yang Y, Hao B, Li Z (2009) Drosophila glycoprotein 93 Is an ortholog of mammalian heat shock protein gp96 (grp94, HSP90b1, HSPC4) and retains disulfide bond-independent chaperone function for TLRs and integrins. J Immunol 183: 5121–5128.
75. Reyes-Del Valle J, Chavez-Salinas S, Medina F, Del Angel RM (2005) Heat shock protein 90 and heat shock protein 70 are components of dengue virus receptor complex in human cells. J Virol 79: 4557–4567.
76. Xu XF, Chen ZT, Gao N, Zhang JL, An J (2009) Myosin Vc, a member of the actin motor family associated with Rab8, is involved in the release of DV2 from HepG2 cells. Intervirology 52: 258–265.
77. Aravind L, Koonin EV (1999) G-patch: a new conserved domain in eukaryotic RNA-processing proteins and type D retroviral polyproteins. Trends Biochem Sci 24: 342–344.
78. Gifford R, Kabat P, Martin J, Lynch C, Tristem M (2005) Evolution and distribution of class II-related endogenous retroviruses. J Virol 79: 6478–6486.
79. Ma L, Jones CT, Groesch TD, Kuhn RJ, Post CB (2004) Solution structure of dengue virus capsid protein reveals another fold. Proc Natl Acad Sci U S A 101: 3414–3419.
80. Kou Z, Quinn M, Chen H, Rodrigo WW, Rose RC, et al. (2008) Monocytes, but not T or B cells, are the principal target cells for dengue virus (DV) infection among human peripheral blood mononuclear cells. J Med Virol 80: 134–146.
81. Pham AM, Langlois RA, TenOever BR (2012) Replication in cells of hematopoietic origin is necessary for Dengue virus dissemination. PLoS Pathog 8: e1002465.
82. Balsitis SJ, Coloma J, Castro G, Alava A, Flores D, et al. (2009) Tropism of dengue virus in mice and humans defined by viral nonstructural protein 3-specific immunostaining. Am J Trop Med Hyg 80: 416–424.
83. Chua JJ, Ng MM, Chow VT (2004) The non-structural 3 (NS3) protein of dengue virus type 2 interacts with human nuclear receptor binding protein and is associated with alterations in membrane structure. Virus Res 102: 151–163.
84. Lamesch P, Li N, Milstein S, Fan C, Hao T, et al. (2007) hORFeome v3.1: a resource of human open reading frames representing over 10,000 human genes. Genomics 89: 307–315.
85. Fu W, Sanders-Beer BE, Katz KS, Maglott DR, Pruitt KD, et al. (2009) Human immunodeficiency virus type 1, human protein interaction database at NCBI. Nucleic Acids Res 37: D417–422.
86. Pinney JW, Dickerson JE, Fu W, Sanders-Beer BE, Ptak RG, et al. (2009) HIV-host interactions: a map of viral perturbation of the host system. Aids 23: 549–554.
87. Ptak RG, Fu W, Sanders-Beer BE, Dickerson JE, Pinney JW, et al. (2008) Cataloguing the HIV type 1 human protein interaction network. AIDS Res Hum Retroviruses 24: 1497–1502.
88. Shapira SD, Gat-Viks I, Shum BO, Dricot A, de Grace MM, et al. (2009) A physical and regulatory map of host-influenza interactions reveals pathways in H1N1 infection. Cell 139: 1255–1267.
89. Kwofie SK, Schaefer U, Sundararajan VS, Bajic VB, Christoffels A (2011) HCVpro: hepatitis C virus protein interaction database. Infect Genet Evol 11: 1971–1977.
90. Calderwood MA, Venkatesan K, Xing L, Chase MR, Vazquez A, et al. (2007) Epstein-Barr virus and virus human protein interaction maps. Proc Natl Acad Sci U S A 104: 7606–7611.
91. Chatr-aryamontri A, Ceol A, Peluso D, Nardozza A, Panni S, et al. (2009) VirusMINT: a viral protein interaction database. Nucleic Acids Res 37: D669–673.
92. Ellencrona K, Syed A, Johansson M (2009) Flavivirus NS5 associates with host-cell proteins zonula occludens-1 (ZO-1) and regulating synaptic membrane exocytosis-2 (RIMS2) via an internal PDZ binding mechanism. Biol Chem 390: 319–323.
93. Limjindaporn T, Wongwiwat W, Noisakran S, Srisawat C, Netsawang J, et al. (2009) Interaction of dengue virus envelope protein with endoplasmic reticulum-resident chaperones facilitates dengue virus production. Biochem Biophys Res Commun 379: 196–200.
94. Bhattacharya D, Mayuri, Best SM, Perera R, Kuhn RJ, et al. (2009) Protein kinase G phosphorylates mosquito-borne flavivirus NS5. J Virol 83: 9195–9205.
95. Garcia-Montalvo BM, Medina F, del Angel RM (2004) La protein binds to NS5 and NS3 and to the 5′ and 3′ ends of Dengue 4 virus RNA. Virus Res 102: 141–150.
96. Noisakran S, Sengsai S, Thongboonkerd V, Kanlaya R, Sinchaikul S, et al. (2008) Identification of human hnRNP C1/C2 as a dengue virus NS1-interacting protein. Biochem Biophys Res Commun 372: 67–72.
97. Chang CJ, Luh HW, Wang SH, Lin HJ, Lee SC, et al. (2001) The heterogeneous nuclear ribonucleoprotein K (hnRNP K) interacts with dengue virus core protein. DNA Cell Biol 20: 569–577.
98. Jiang L, Yao H, Duan X, Lu X, Liu Y (2009) Polypyrimidine tract-binding protein influences negative strand RNA synthesis of dengue virus. Biochem Biophys Res Commun 385: 187–192.
99. Hershkovitz O, Rosental B, Rosenberg LA, Navarro-Sanchez ME, Jivov S, et al. (2009) NKp44 receptor mediates interaction of the envelope glycoproteins from the West Nile and dengue viruses with NK cells. J Immunol 183: 2610–2621.
100. Brault JB, Kudelko M, Vidalain PO, Tangy F, Despres P, et al. (2011) The interaction of flavivirus M protein with light chain Tctex-1 of human dynein plays a role in late stages of virus replication. Virology 417: 369–378.
101. Kurosu T, Chaichana P, Yamate M, Anantapreecha S, Ikuta K (2007) Secreted complement regulatory protein clusterin interacts with dengue virus nonstructural protein 1. Biochem Biophys Res Commun 362: 1051–1056.
102. Chua JJ, Bhuvanakantham R, Chow VT, Ng ML (2005) Recombinant non-structural 1 (NS1) protein of dengue-2 virus interacts with human STAT3beta protein. Virus Res 112: 85–94.
103. Chiu MW, Shih HM, Yang TH, Yang YL (2007) The type 2 dengue virus envelope protein interacts with small ubiquitin-like modifier-1 (SUMO-1) conjugating enzyme 9 (Ubc9). J Biomed Sci 14: 429–444.
104. Lozach PY, Burleigh L, Staropoli I, Navarro-Sanchez E, Harriague J, et al. (2005) Dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN)-mediated enhancement of dengue virus infection is independent of DC-SIGN internalization signals. J Biol Chem 280: 23698–23708.
105. Heaton NS, Perera R, Berger KL, Khadka S, Lacount DJ, et al. (2010) Dengue virus nonstructural protein 3 redistributes fatty acid synthase to sites of viral replication and increases cellular fatty acid synthesis. Proc Natl Acad Sci U S A 107: 17345–17350.
106. Yook SH, Oltvai ZN, Barabasi AL (2004) Functional and topological characterization of protein interaction networks. Proteomics 4: 928–942.
107. Everett RD, Chelbi-Alix MK (2007) PML and PML nuclear bodies: implications in antiviral defence. Biochimie 89: 819–830.
108. Alcock F, Swanton E (2009) Mammalian OS-9 is upregulated in response to endoplasmic reticulum stress and facilitates ubiquitination of misfolded glycoproteins. J Mol Biol 385: 1032–1042.
109. Umareddy I, Pluquet O, Wang QY, Vasudevan SG, Chevet E, et al. (2007) Dengue virus serotype infection specifies the activation of the unfolded protein response. Virol J 4: 91.
110. Thompson MR, Kaminski JJ, Kurt-Jones EA, Fitzgerald KA (2011) Pattern recognition receptors and the innate immune response to viral infection. Viruses 3: 920–940.
111. Le Negrate G (2012) Viral interference with innate immunity by preventing NF-kappaB activity. Cell Microbiol 14: 168–181.
112. Gilmore TD, Wolenski FS (2012) NF-kappaB: where did it come from and why? Immunol Rev 246: 14–35.
113. Valanne S, Wang JH, Ramet M (2011) The Drosophila Toll signaling pathway. J Immunol 186: 649–656.
114. Tanigawa K, Suzuki K, Kimura H, Takeshita F, Wu H, et al. (2009) Tryptophan aspartate-containing coat protein (CORO1A) suppresses Toll-like receptor signalling in Mycobacterium leprae infection. Clin Exp Immunol 156: 495–501.
115. Arakawa R, Bagashev A, Song L, Maurer K, Sullivan KE (2010) Characterization of LRRFIP1. Biochem Cell Biol 88: 899–906.
116. Johnsen IB, Nguyen TT, Ringdal M, Tryggestad AM, Bakke O, et al. (2006) Toll-like receptor 3 associates with c-Src tyrosine kinase on endosomes to initiate antiviral signaling. EMBO J 25: 3335–3346.
117. Tadano M, Makino Y, Fukunaga T, Okuno Y, Fukai K (1989) Detection of dengue 4 virus core protein in the nucleus. I. A monoclonal antibody to dengue 4 virus reacts with the antigen in the nucleus and cytoplasm. J Gen Virol 70 (Pt 6): 1409–1415.
118. Wang SH, Syu WJ, Huang KJ, Lei HY, Yao CW, et al. (2002) Intracellular localization and determination of a nuclear localization signal of the core protein of dengue virus. J Gen Virol 83: 3093–3102.

119. Sangiambut S, Keelapang P, Aaskov J, Puttikhunt C, Kasinrerk W, et al. (2008) Multiple regions in dengue virus capsid protein contribute to nuclear localization during virus infection. J Gen Virol 89: 1254–1264.
120. Hiscox JA, Whitehouse A, Matthews DA (2010) Nucleolar proteomics and viral infection. Proteomics 10: 4077–4086.
121. Hiscox JA (2007) RNA viruses: hijacking the dynamic nucleolus. Nat Rev Microbiol 5: 119–127.
122. Yang MR, Lee SR, Oh W, Lee EW, Yeh JY, et al. (2008) West Nile virus capsid protein induces p53-mediated apoptosis via the sequestration of HDM2 to the nucleolus. Cell Microbiol 10: 165–176.
123. Xu Z, Anderson R, Hobman TC (2011) The capsid-binding nucleolar helicase DDX56 is important for infectivity of West Nile virus. J Virol 85: 5571–5580.
124. Tsuda Y, Mori Y, Abe T, Yamashita T, Okamoto T, et al. (2006) Nucleolar protein B23 interacts with Japanese encephalitis virus core protein and participates in viral replication. Microbiol Immunol 50: 225–234.
125. Lilley BN, Gilbert JM, Ploegh HL, Benjamin TL (2006) Murine polyomavirus requires the endoplasmic reticulum protein Derlin-2 to initiate infection. J Virol 80: 8739–8744.
126. Oda Y, Okada T, Yoshida H, Kaufman RJ, Nagata K, et al. (2006) Derlin-2 and Derlin-3 are regulated by the mammalian unfolded protein response and are required for ER-associated degradation. J Cell Biol 172: 383–393.
127. Wang M, Ye R, Barron E, Baumeister P, Mao C, et al. (2010) Essential role of the unfolded protein response regulator GRP78/BiP in protection from neuronal apoptosis. Cell Death Differ 17: 488–498.
128. Malhotra JD, Kaufman RJ (2007) The endoplasmic reticulum and the unfolded protein response. Semin Cell Dev Biol 18: 716–731.
129. Pena J, Harris E (2011) Dengue virus modulates the unfolded protein response in a time-dependent manner. J Biol Chem 286: 14226–14236.
130. Yu CY, Hsu YW, Liao CL, Lin YL (2006) Flavivirus infection activates the XBP1 pathway of the unfolded protein response to cope with endoplasmic reticulum stress. J Virol 80: 11868–11880.
131. Fischl W, Bartenschlager R (2011) Exploitation of cellular pathways by Dengue virus. Curr Opin Microbiol 14: 470–475.
132. Balmaseda A, Hammond SN, Perez L, Tellez Y, Saborio SI, et al. (2006) Serotype-specific differences in clinical manifestations of dengue. Am J Trop Med Hyg 74: 449–456.
133. Yu H, Braun P, Yildirim MA, Lemmens I, Venkatesan K, et al. (2008) High-quality binary protein interaction map of the yeast interactome network. Science 322: 104–110.
134. Yu H, Tardivo L, Tam S, Weiner E, Gebreab F, et al. (2011) Next-generation sequencing to generate interactome datasets. Nat Methods 8: 478–480.
135. Lim J, Hao T, Shaw C, Patel AJ, Szabo G, et al. (2006) A protein-protein interaction network for human inherited ataxias and disorders of Purkinje cell degeneration. Cell 125: 801–814.
136. Yu J, Murali T, Finley RL Jr (2012) Assigning confidence scores to protein-protein interactions. Methods Mol Biol 812: 161–174.
137. Schwartz AS, Yu J, Gardenour KR, Finley RL Jr, Ideker T (2009) Cost-effective strategies for completing the interactome. Nat Methods 6: 55–61.
138. Yu H, Luscombe NM, Lu HX, Zhu X, Xia Y, et al. (2004) Annotation transfer between genomes: protein-protein interologs and protein-DNA regulogs. Genome Res 14: 1107–1118.
139. Tarassov K, Messier V, Landry CR, Radinovic S, Serna Molina MM, et al. (2008) An in vivo map of the yeast protein interactome. Science 320: 1465–1470.
140. Babu M, Vlasblom J, Pu S, Guo X, Graham C, et al. (2012) Interaction landscape of membrane-protein complexes in Saccharomyces cerevisiae. Nature 489: 585–589.
141. Krishnan MN, Ng A, Sukumaran B, Gilfoy FD, Uchil PD, et al. (2008) RNA interference screen for human genes associated with West Nile virus infection. Nature 455: 242–245.
142. Sessions OM, Barrows NJ, Souza-Neto JA, Robinson TJ, Hershey CL, et al. (2009) Discovery of insect and human dengue virus host factors. Nature 458: 1047–1050.
143. Bischof J, Maeda RK, Hediger M, Karch F, Basler K (2007) An optimized transgenesis system for Drosophila using germ-line-specific phiC31 integrases. Proc Natl Acad Sci U S A 104: 3312–3317.
144. Liu D, Finley RL, Jr. (2010) Cyclin Y is a novel conserved cyclin essential for development in Drosophila. Genetics 184: 1025–1035.
145. Parrish JR, Limjindaporn T, Hines JA, Liu J, Liu G, et al. (2004) High-throughput cloning of Campylobacter jejuni ORFs by in vivo recombination in Escherichia coli. J Proteome Res 3: 582–586.
146. Veraksa A, Bauer A, Artavanis-Tsakonas S (2005) Analyzing protein complexes in Drosophila with tandem affinity purification-mass spectrometry. Dev Dyn 232: 827–834.
147. Stanyon CA, Limjindaporn T, Finley RL, Jr. (2003) Simultaneous cloning of open reading frames into several different expression vectors. Biotechniques 35: 520–522, 524–526.
148. Munstermann LE (1997) Care and maintenance of Aedes mosquito colonies. In: Crampton JMB, C. B.;Louis, C., editor. The molecular biology of insect disease vectors: a methods manual Springer. pp. 13–20
149. Sodja A, Fujioka H, Lemos FJ, Donnelly-Doman M, Jacobs-Lorena M (2007) Induction of actin gene expression in the mosquito midgut by blood ingestion correlates with striking changes of cell shape. J Insect Physiol 53: 833–839.
150. Finley RL, Jr., Brent R (1994) Interaction mating reveals binary and ternary connections between Drosophila cell cycle regulators. Proc Natl Acad Sci U S A 91: 12980–12984.
151. Golemis EA, Serebriiskii I, Finley RL Jr, Kolonin MG, Gyuris J, et al. (2001) Interaction trap/two-hybrid system to identify interacting proteins. Curr Protoc Mol Biol Chapter: Unit 20.21.
152. Kolonin MG, Zhong J, Finley RL (2000) Interaction mating methods in two-hybrid systems. Methods Enzymol 328: 26–46.
153. Gietz D, St Jean A, Woods RA, Schiestl RH (1992) Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res 20: 1425.
154. Gyuris J, Golemis E, Chertkov H, Brent R (1993) Cdi1, a human G1 and S phase protein phosphatase that associates with Cdk2. Cell 75: 791–803.
155. Orr-Weaver TL, Szostak JW (1983) Yeast recombination: the association between double-strand gap repair and crossing-over. Proc Natl Acad Sci U S A 80: 4417–4421.
156. Zhong J, Zhang H, Stanyon CA, Tromp G, Finley RL Jr (2003) A strategy for constructing large protein interaction maps using the yeast two-hybrid system: regulated expression arrays and two-phase mating. Genome Res 13: 2691–2699.
157. Klueg KM, Alvarado D, Muskavitch MA, Duffy JB (2002) Creation of a GAL4/UAS-coupled inducible gene expression system for use in Drosophila cultured cell lines. Genesis 34: 119–122.
158. Barrell D, Dimmer E, Huntley RP, Binns D, O'Donovan C, et al. (2009) The GOA database in 2009–an integrated Gene Ontology Annotation resource. Nucleic Acids Res 37: D396–403.
159. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, et al. (2000) Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet 25: 25–29.
160. Hunter S, Jones P, Mitchell A, Apweiler R, Attwood TK, et al. (2012) InterPro in 2011: new developments in the family and domain prediction database. Nucleic Acids Res 40: D306–312.
161. Smoot ME, Ono K, Ruscheinski J, Wang PL, Ideker T (2011) Cytoscape 2.8: new features for data integration and network visualization. Bioinformatics 27: 431–432.
162. Maere S, Heymans K, Kuiper M (2005) BiNGO: a Cytoscape plugin to assess overrepresentation of gene ontology categories in biological networks. Bioinformatics 21: 3448–3449.
163. Aranda B, Achuthan P, Alam-Faruque Y, Armean I, Bridge A, et al. (2009) The IntAct molecular interaction database in 2010. Nucleic Acids Res 38: 22.
164. Ceol A, Chatr Aryamontri A, Licata L, Peluso D, Briganti L, et al. (2009) MINT, the molecular interaction database: 2009 update. Nucleic Acids Res 38: 6.
165. Stark C, Breitkreutz BJ, Chatr-Aryamontri A, Boucher L, Oughtred R, et al. (2011) The BioGRID Interaction Database: 2011 update. Nucleic Acids Res 39: D698–704.
166. Haw R, Hermjakob H, D'Eustachio P, Stein L (2011) Reactome Pathway Analysis to Enrich Biological Discovery in Proteomics Datasets. Proteomics 1002: 201100066.
167. Goel R, Muthusamy B, Pandey A, Prasad TS (2011) Human protein reference database and human proteinpedia as discovery resources for molecular biotechnology. Mol Biotechnol 48: 87–95.

Biochemical and Biophysical Research Communications 410 (2011) 428–433

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Role of CD137 signaling in dengue virus-mediated apoptosis

Amar Nagila [a,b], Janjuree Netsawang [c], Chatchawan Srisawat [b], Sansanee Noisakran [i,d], Atthapan Morchang [a,e], Umpa Yasamut [a,e], Chunya Puttikhunt [i,d], Watchara Kasinrerk [f,g], Prida Malasit [i,d], Pa-thai Yenchitsomanus [a,d], Thawornchai Limjindaporn [h,*]

[a] Medical Molecular Biology Unit, Office for Research and Development, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand
[b] Department of Biochemistry, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand
[c] Faculty of Medical Technology, Rangsit University, Bangkok, Thailand
[d] Medical Biotechnology Unit, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Bangkok, Thailand
[e] Department of Immunology, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand
[f] Division of Clinical Immunology, Department of Medical Technology, Faculty of Associated Medical Sciences, Chiang Mai University, Chiang Mai, Thailand
[g] Biomedical Technology Research Center, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency at Chiang Mai University, Chiang Mai, Thailand
[h] Department of Anatomy, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand
[i] Dengue Hemorrhagic Fever Research Unit, Office for Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

ARTICLE INFO

Article history:
Received 24 May 2011
Available online 6 June 2011

Keywords:
Dengue virus
Dengue virus capsid protein
Apoptosis
CD137 signaling

ABSTRACT

Hepatic dysfunction is a well recognized feature of dengue virus (DENV) infection. However, molecular mechanisms of hepatic injury are still poorly understood. A complex interaction between DENV and the host immune response contributes to DENV-mediated tissue injury. DENV capsid protein (DENV C) physically interacts with the human death domain-associated protein Daxx. A double substitution mutation in DENV C (R85A/K86A) abrogates Daxx interaction, nuclear localization and apoptosis. Therefore we compared the expression of cell death genes between HepG2 cells expressing DENV C and DENV C (R85A/K86A) using a real-time PCR array. Expression of CD137, which is a member of the tumor necrosis factor receptor family, increased significantly in HepG2 cells expressing DENV C compared to HepG2 cells expressing DENV C (R85A/K86A). In addition, CD137-mediated apoptotic activity in HepG2 cells expressing DENV C was significantly increased by anti-CD137 antibody compared to that of HepG2 cells expressing DENV C (R85A/K86A). In DENV-infected HepG2 cells, CD137 mRNA and CD137 positive cells significantly increased and CD137-mediated apoptotic activity was increased by anti-CD137 antibody. This work is the first to demonstrate the contribution of CD137 signaling to DENV-mediated apoptosis.

1. Introduction

DENV infection is an important mosquito-borne viral disease and its clinical symptoms range from a predominantly febrile disease, dengue fever (DF), to dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), which typically occurs in cases of subsequent infection with a different serotype of DENV. Patients with DHF present with hemorrhagic tendencies, plasma leakage, thrombocytopenia, and hemoconcentration [1]. Hepatic dysfunction in DENV infection is demonstrated by hepatomegaly and increases in transaminase levels [2,3]. Clinically severe liver involvement results in severe bleeding [4]. Hepatic biopsy specimens obtained from fatal cases of DSS show cellular apoptosis, which may relate to the pathogenesis of DSS [5–7].

A complex interaction between DENV and the host immune response contributes to DENV-mediated apoptosis [8–10]. Both the cell death receptor and the mitochondrial apoptotic pathways are affected [11–17]. Using a yeast two-hybrid screen, we found that DENV C physically interacts with the human death domain-associated protein Daxx. The interaction between DENV C and Daxx in DENV-infected liver cells was demonstrated by co-immunoprecipitation and double immunofluorescence staining. The two proteins were predominantly co-localized in cellular nuclei [12]. DENV C contains three nuclear localization signals (NLS), ^{6}KKAR^{9}, ^{73}KKSK^{76}, and the bipartite signal ^{85}RKeigrmlnilnRRRR^{100}. Substitution mutations in DENV C (R85A/K86A) result in loss of nuclear localization, Daxx interaction and apoptosis [13].

In this study, we compared the expression of cell death genes between HepG2 cells expressing DENV C and DENV C (R85A/K86A) using a real-time PCR array. Expression of CD137 increased significantly in HepG2 cells expressing DENV C compared to HepG2 cells expressing DENV C (R85A/K86A). CD137-mediated apoptotic activity in HepG2 cells expressing DENV C was significantly increased by anti-CD137 antibody compared to HepG2 cells expressing DENV C (R85A/K86A). CD137-mediated apoptotic

* Corresponding author. Fax: +66 2 418 4793.
E-mail address: limjindaporn@yahoo.com (T. Limjindaporn).

0006-291X/$ - see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2011.05.151

Table 1
Genes exhibiting a change in expression between HepG2 cells expressing DENVC and HepG2 cells expressing DENVC (R85A/K86A).

Gene no.	Fold change	Gene name	Gene description
1	3.12	TNF-α	Tumor necrosis factor-α (TNF superfamily, member 2)
2	3.12	TP73	Tumor protein p73
3	2.46	CD38	CD38 molecule
4	2.22	PLG	Plasminogen
5	1.99	PRKCE	Protein kinase C, epsilon
6	1.85	NLRP2	NLR family, pyrin domain containing 2
7	1.79	LCK	Lymphocyte-specific protein tyrosine kinase
8	1.71	HRK	Harakiri, BCL2 interacting protein
9	1.64	PDCD5	Programmed cell death 5
10	1.62	IFI16	Interferon, gamma-inducible protein 16
11	1.61	SIPA1	Signal-induced proliferation-associated 1
12	1.56	CUL3	Cullin 3
13	1.56	PMAIP1	Phorbol-12-myristate-13-acetate-induced protein 1
14	1.45	TRAIL	Tumor necrosis factor ligand superfamily, member 10
15	1.41	CD137	Tumor necrosis factor receptor superfamily, member 9

activity in DENV-infected HepG2 cells was also increased by anti-CD137 antibody indicating for the first time the apoptotic role of CD137 signaling in DENV infection.

2. Materials and methods

2.1. Apoptotic gene expression profiling using a real-time PCR array

The transient transfection of plasmid pcDNA3.1/His C-DENV C or pcDNA3.1/His C-DENV C (R85A/K86A) or pcDNA3.1/His C in HepG2 cells was performed using FuGene HD (Roche-applied science, Germany). Up to 1×10^6 HepG2 cells were seeded into a 6-well plate for 24 h prior to transfection. Four micrograms of plasmid DNA were pre-incubated with 12 μl of FuGene HD reagent at a ratio 1:3 to generate a complex in serum-free media, Optimem (Gibco-BRL, USA). This complex was then added to the HepG2 cells in 3% FBS culture medium. Twenty-four hours post-transfection, the old medium was removed and transfected cells were triggered with 0.5 μg/ml anti-Fas mAb (Sigma) and 1 μg/ml cycloheximide (CHX) containing 2% FBS culture medium for 24 h. Subsequently, both detached and adherent cells were harvested and stained for cell surface phosphatidyl serine (Annexin V assay) to assess apoptosis. Total RNA was isolated from transfected cells using the High Pure RNA isolation kit (Roche). RNAs with an OD_{260nm}/OD_{280nm} absorbance ratio of at least 2.0 were used. Total RNA was reverse-transcribed into cDNA using the RT^2 First strand Kit (Qiagen), mixed with RT^2 qPCR mastermix containing SYBR Green (Qiagen), and aliquoted in equal volumes to each well of the real-time PCR arrays. The Human Apoptosis RT^2 Profiler™ PCR Array (Qiagen) interrogates 384 genes related to the apoptotic pathway. The real-time PCR cycling program was run on a Roche Light Cycler 480. The threshold cycle (Ct) of each gene was determined and subsequently analyzed by RT^2 Profiler PCR Array data Analysis software; http://pcrdataanalysis.sabiosciences.com/pcr/arrayanalysis.php.

2.2. Verification of apoptotic gene expression profiling using quantitative real-time PCR and flow cytometry analysis

The mRNA expression changes of TNF-α, TRAIL and CD137 in HepG2 cells expressing DENV C and HepG2 cells expressing DENV C (R85A/K86A) were confirmed by quantitative real-time PCR using a different set of gene-specific primers. The primers used were as follows: for TNF-α: 5′-TGCTTGTTCCTCAGCCTCTT-3′ (forward) and 5′-ATGGGCTACA GGCTTGTCACT-3′ (reverse), for TRAIL: 5′-TGGGACAGACCTGC GTGCTGAT-3′ (forward) and 5′-ACGAGCTGAC GGAGTTGCCACT-3′ (reverse), for CD137: 5′-TCACTGCCTGGGG GCAGGAT-3′ (forward) and 5′-GGCGGGGTCACAGAGGATGC-3′ (reverse), and for ACTB; 5′-ACAGAGCCTCGCCTTTGCCGATC-3′ (forward) and 5′-CCCCGTCACCG GAGTCCATCA-3′ (reverse), respectively. Amplification was monitored using SYBR Green I reaction mix (Roche) and Roche Light Cycler 480. The Ct of each mRNA and ACTB control was measured and the difference between their ΔCt was calculated. The relative expression values ($2^{-\Delta\Delta Ct}$) was then determined. Results were obtained from three independent experiments. Unpaired *t*-test was used for statistical analysis by StatView program and *p* value less than 0.05 was considered significant.

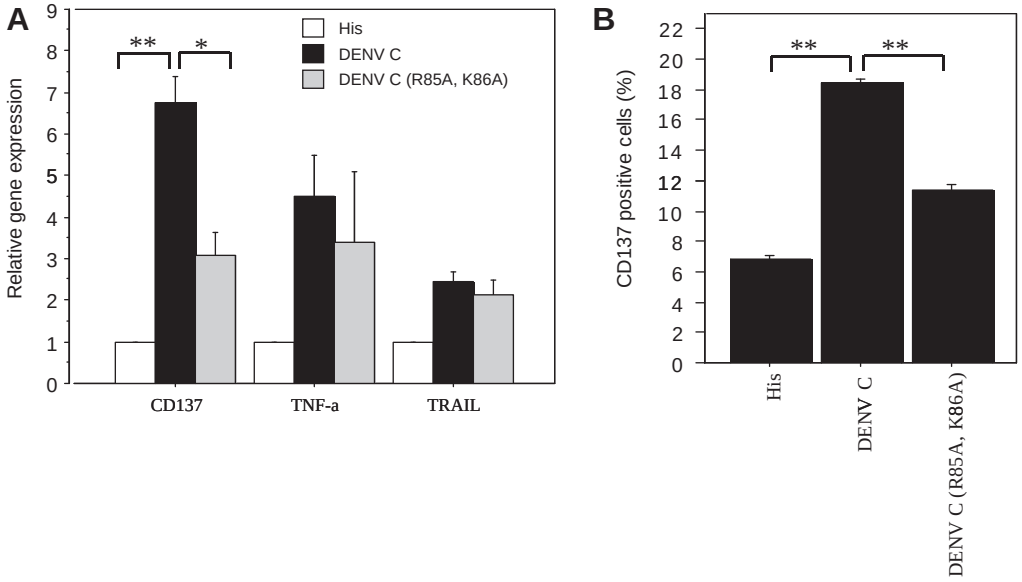

Fig. 1. CD137 mRNA and CD137 positive cells (%) in HepG2 cells expressing DENV C or DENV C (R85A/K86A). (A) Relative gene expression (fold change) was determined by real-time PCR using specific primers to CD137, TNF-α, TRAIL, and ACTB. (B) CD137 positive cells (%) were subsequently measured by flow cytometric analysis using antibody to CD137. The bar graph is the average of three independent experiments. Asterisks indicate a significant difference among two groups ($^*p < 0.01$, $^{**}p < 0.001$).

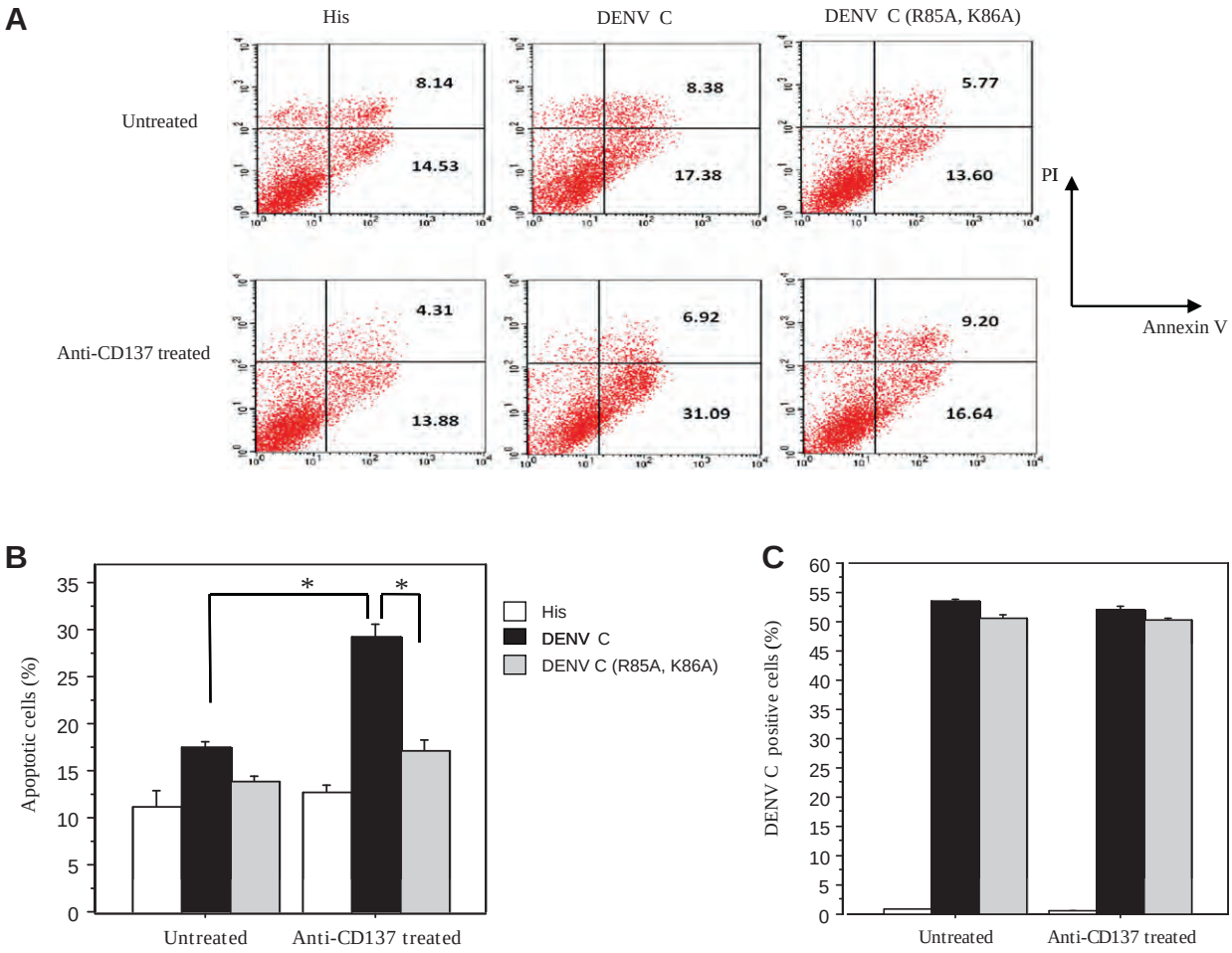

Fig. 2. Induction of DENV C-mediated apoptosis by CD137 signaling. (A) Forty-eight hours post transfection, HepG2 cells expressing DENV C, or DENV C (R85A/K86A) or pcDNA3.1/His C were treated either with 2 μg/ml antibody to CD137 or an isotype control antibody for 6 h and examined for the presence of apoptotic cells by annexin V/FITC and PI double staining and quantitation by flow cytometry. (B) Plotted is the average from three independent experiments in A. (C) The expression of DENV C was determined by DENV C staining with mAb to DENV C [18] and quantitation by flow cytometry. The bar graph is the average of three independent experiments. Asterisks indicate a significant difference among two groups ($^*p < 0.01$).

CD137 positive cells (%) were subsequently measured in both HepG2 cells expressing DENV C and HepG2 cells expressing DENV C (R85A/K86A) by flow cytometric analysis using antibody to CD137 (Santa Cruz Biotechnology). Unpaired *t*-test was used for statistical analysis by StatView program and *p* value less than 0.05 was considered significant.

2.3. Induction of apoptosis in HepG2 cells expressing DENV C by antibody to CD137

The transient transfection of plasmid pcDNA3.1/His C-DENV C or pcDNA3.1/His C-DENV C (R85A/K86A) or pcDNA3.1/His C in HepG2 cells was performed using FuGene HD. Up to 1×10^6 HepG2 cells were seeded into a 6-well plate for 24 h prior to transfection. After 48 h post transfection, up to 1×10^6 HepG2 cells expressing DENV C, or DENV C (R85A/K86A) or pcDNA3.1/His C were treated either with 2 μg/ml anti-CD137 antibody or an isotype control antibody for 6 h in culture medium containing 2% FBS and examined for the presence of apoptotic cells using annexinV/PI staining (BD Biosciences). Briefly, detached and adherent cells were harvested, suspended in annexin V buffer and incubated on ice with FITC-conjugated annexin V for 15 min. Immediately prior to analysis, propidium iodide was added and samples were analyzed by flow cytometry. In addition, the expression of DENV C was determined by DENV C staining with mAb to DENV C [18] and quantitation by flow cytometry. Results were obtained from three independent experiments. Unpaired *t*-test was used for statistical analysis by StatView program and *p* value less than 0.05 was considered significant.

2.4. Expression of CD137 in DENV-infected HepG2 cells

Up to 1×10^6 HepG2 cells were seeded in a 60 mm dish and cultured for 24 h before infection. HepG2 cells were grown in DMEM medium (Gibco-BRL) supplemented with 10% FBS, 2 mM L-glutamine, 1% non-essential amino acids, 1 mM sodium pyruvate, 100U/ml of penicillin and streptomycin at 37 °C in a humidified atmosphere containing 5% CO_2. Cells were infected with DENV serotype 2 strain 16681 at a multiplicity of infection (MOI) of 5. Mock and DENV-infected HepG2 cells were harvested at 36, 48, and 60 h post infection and analyzed for CD137 mRNA expression by quantitative real-time PCR, for CD137 positive cells (%) by flow cytometric analysis using antibody to CD137 and for apoptosis using annexinV/PI staining. DENV E (3H5) staining was used to demonstrate DENV infection [19,20]. Results were obtained from three independent experiments. Unpaired *t*-test was used for statistical analysis by StatView program and *p* value less than 0.05 was considered significant.

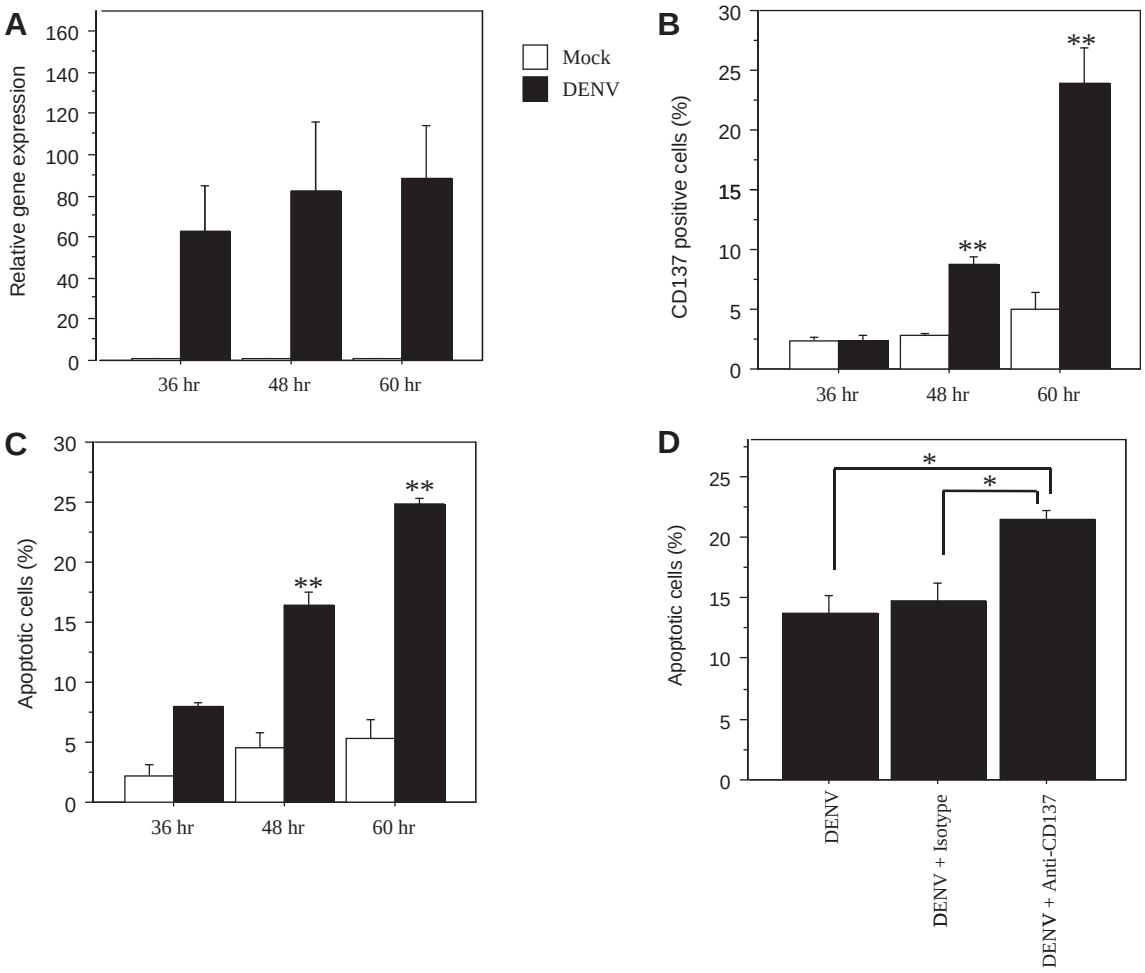

Fig. 3. Induction of DENV-mediated apoptosis by CD137 signaling. HepG2 Cells were infected with DENV at a MOI of 5 and incubated for 36, 48 and 60 h post infection. (A) Relative gene expression (fold change) was determined by real-time PCR using specific primers to CD137 and ACTB. (B) CD137 positive cells (%) were subsequently measured by flow cytometric analysis using antibody to CD137. (C) Apoptotic cells (%) were examined by annexin V/FITC and PI double staining and quantitation by flow cytometry. (D) HepG2 cells infected with DENV at a MOI of 5 and incubated for 48 h were treated either with 2 μg/ml antibody to CD137 or isotype control antibody for 6 h. The bar graph is the average of three independent experiments. Asterisks indicate a significant difference among two groups ($^{*}p < 0.01$, $^{**}p < 0.001$).

2.5. Induction of apoptosis in DENV-infected HepG2 cells by antibody to CD137

Up to 2×10^5 HepG2 cells infected with DENV serotype 2 strain 16681 at MOI of 5 for 48 h were treated either with 2 μg/ml antibody to CD137 or isotype control antibody for 6 h. Both detached and adherent cells were collected to examine the effect of CD137 antibody on apoptosis of mock and DENV-infected HepG2 cells. Apoptosis (%) was examined by flow cytometry after staining with annexinV/PI. Results were obtained from three independent experiments. Unpaired *t*-test was used for statistical analysis by StatView program and *p* value less than 0.05 was considered significant.

2.6. Results and discussion

Our previous work showed that DENV C interacts with at least one death pathway associated protein, Daxx (13), and raised the possibility that it may have other death pathway interactions. To explore this possibility total RNA was prepared from HepG2 cells expressing either DENV C or mutated DENV C (R85A/K86A) and used to probe real-time PCR arrays containing death pathway targets. Table 1 lists gene exhibiting a change in expression between HepG2 cells expressing DENV C and HepG2 cells expressing DENV C (R85A/K86A). Death ligands and receptors including TNF-α, TRAIL, and CD137 were up-regulated in HepG2 cells expressing DENV C compared to cells expressing the mutated DENV C. However, the role of CD137(4-1BB), which is a member of the tumor necrosis factor receptor family [21,22], in DENV infection has not been previously investigated. Therefore, mRNA expression of CD137 was confirmed using quantitative real-time PCR. CD137 mRNA levels were increased 6.5-fold in DENV C expressing cells relative to control cells and over 2-fold compared to cells transfected with mutant DENV C (Fig. 1A). Similarly, CD137 positive cells (%) increased 2-fold in HepG2 cells expressing DENV C compared to cells expressing mutated DENV C (Fig. 1B). These results demonstrate that DENV C affects CD137 transcription and the failure of the R85A/K86A mutant to elicit a similar enhancement suggests that increased expression of CD137 requires nuclear localization of DENV C. In contrast, expression of TNF-α and TRAIL were also increase several folds in DENV C expressing cells, but the mutation of the nuclear localization signal had no effect on this induction (Fig. 1A). The differences in CD137 expression were not due to differences in transfection efficiency or expression of the DENV C proteins, as demonstrated in the following experiment.

The potential effects of enhanced CD137 expression in DENV C-expressing cells were assessed by examining the ability of anti-CD137 mAb treatment to induce apoptosis in these cells.

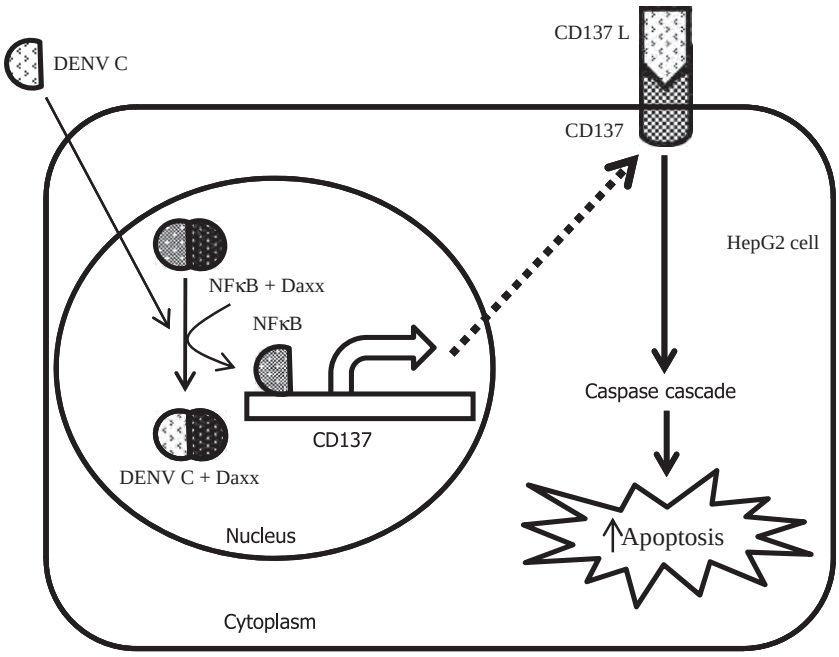

Fig. 4. Proposed model for sensitization to CD137-mediated apoptosis by DENV C. The competitive binding between DENV C and NF-κβ with Daxx limits the interaction between Daxx and NF-κβ thereby releasing NF-κβ to activate CD137 promoter, which eventually activate caspase cascades and apoptosis.

Anti-CD137 antibody stimulated apoptosis nearly 2-fold in cells expressing DENV C (Fig. 2A and B). In contrast, anti-CD137 mAb had little or no effect on DENV C (R85A/K86A) transfected cells or HepG2 His control cells (Fig. 2A and B). This difference was not due to differences in transfection efficiency or expression since DENV C and DENV C (R85A/K86A) were expressed at similar levels (Fig. 2C). These data indicate that CD137 may play a signaling role in DENV C-mediated apoptosis.

To determine if similar effects occur during DENV infection, CD137 expression was examined in DENV-infected HepG2 cells by quantitative real-time PCR and flow cytometric analysis. CD137 mRNA expression increased 60-fold by 36 h post DENV infection and 80-fold by 48 h post DENV infection (Fig. 3A). CD137 positive cells also increased, but less dramatically, 3-fold by 48 h post infection and 5-fold by 60 h (Fig. 3B and Fig. S1). This correlates with the level of apoptosis induced by DENV infection, which increased 4-fold by 48 h post DENV infection and 5-fold by 60 h post DENV infection (Fig. 3C and Fig. S2). As CD137 signaling induces cell death by apoptosis [23], we asked whether CD137 signaling contributed to DENV-induced apoptosis by examining the effect of anti-CD137 mAb on apoptosis of DENV-infected HepG2 cells. Anti-CD137 mAb increased apoptosis by about 50% (Fig. 3D) indicating a role of CD137 signaling in DENV-mediated apoptosis.

We propose that CD137 mediates apoptosis in DENV-infected liver cells, which may partly explain the mechanism of hepatocyte injury during DENV infection (Fig. 4). In human hepatoma cells, DENV infection induces cell death typical of apoptosis late in the virus cycle. The transcription factor NF-κβ is activated before apoptosis [24] and NF-κβ is known to activate CD137 promoter. DENV C may facilitate this by binding Daxx, [12,13], which normally interacts with NF-κβ [25], thereby releasing NF-κβ to activate CD137 promoter [26]. As CD137 ligand was also expressed in HepG2 cells [27] and CD137 ligand mRNA expression increased 3-fold by 60 h post DENV infection (data not shown), CD137 ligand and CD137 interaction may eventually activate caspase cascades and apoptosis in DENV-infected liver cells. How DENV C affects NF-κβ, and Daxx requires further investigation. However, this work is the first to demonstrate that CD137 signaling contributes to DENV-mediated apoptosis.

Acknowledgments

This work was financially supported by Siriraj Research Development Fund to T.L. We appreciate Dr. William A Fonzi, Georgetown University, USA, for critical reading and editing this manuscript. A.N. was supported by Siriraj Graduate Thesis Scholarship. A.M. and U.Y. are Ph.D. students supported by the Royal Golden Jubilee-Ph.D. program. T.L. is a Thailand Research Fund (TRF) Research Scholar and P.Y. is a TRF-Senior Research Scholar.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.05.151.

References

[1] S.B. Halstead, Pathogenesis of dengue: challenges to molecular biology, Science 239 (1988) 476–481.
[2] C.H. Kuo, D.I. Tai, C.S. Chang-Chien, C.K. Lan, S.S. Chiou, Y.F. Liaw, Liver biochemical tests and dengue fever, Am. J. Trop. Med. Hyg. 47 (1992) 265–270.
[3] L.J. Souza, J.G. Alves, R.M. Nogueira, C. Gicovate Neto, D.A. Bastos, E.W. Siqueira, J.T. Souto Filho, A. Cezario Tde, C.E. Soares, C. Carneiro Rda, Aminotransferase changes and acute hepatitis in patients with dengue fever: analysis of 1,585 cases, Braz. J. Infect. Dis. 8 (2004) 156–163.
[4] D.T. Trung, T.T. Thao le, T.T. Hien, N.T. Hung, N.N. Vinh, P.T. Hien, et al., Liver involvement associated with dengue infection in adults in Vietnam, Am. J. Trop. Med. Hyg. 83 (2010) 774–780.
[5] N. Bhamarapravati, Hemostatic defects in dengue hemorrhagic fever, Rev. Infect. Dis. 11 (Suppl. 4) (1989) S826–S829.
[6] M.R. Huerre, N.T. Lan, P. Marianneau, N.B. Hue, H. Khun, N.T. Hung, N.T. Khen, M.T. Drouet, V.T. Huong, D.Q. Ha, Y. Buisson, V. Deubel, Liver histopathology and biological correlates in five cases of fatal dengue fever in Vietnamese children, Virchows Arch. 438 (2001) 107–115.
[7] D. Limonta, V. Capo, G. Torres, A.B. Perez, M.G. Guzman, Apoptosis in tissues from fatal dengue shock syndrome, J. Clin. Virol. 40 (2007) 50–54.
[8] S.J. Gagnon, F.A. Ennis, A.L. Rothman, Bystander target cell lysis and cytokine production by dengue virus-specific human CD4(+) cytotoxic T-lymphocyte clones, J. Virol. 73 (1999) 3623–3629.
[9] G.F. Silveira, F. Meyer, A. Delfraro, A.L. Mosimann, N. Coluchi, C. Vasquez, C.M. Probst, A. Bafica, J. Bordignon, C.N. Dos Santos, Dengue virus type 3 isolated from a fatal case with visceral complications induces enhanced proinflammatory responses and apoptosis of human dendritic cells, J. Virol. 85 (2011) 5374–5383.

[10] A. Morchang, U. Yasamut, J. Netsawang, S. Noisakran, W. Wongwiwat, P. Songprakhon, C. Srisawat, C. Puttikhunt, W. Kasinrerk, P. Malasit, P.T. Yenchitsomanus, T. Limjindaporn, Cell death gene expression profile: role of RIPK2 in dengue virus-mediated apoptosis, Virus Res. 156 (2011) 25–34.
[11] H. Liao, J. Xu, J. Huang, FasL/Fas pathway is involved in dengue virus induced apoptosis of the vascular endothelial cells, J. Med. Virol. 82 (2010) 1392–1399.
[12] T. Limjindaporn, J. Netsawang, S. Noisakran, S. Thiemmeca, W. Wongwiwat, S. Sudsaward, P. Avirutnan, C. Puttikhunt, W. Kasinrerk, R. Sriburi, N. Sittisombut, P.T. Yenchitsomanus, P. Malasit, Sensitization to Fas-mediated apoptosis by dengue virus capsid protein, Biochem. Biophys. Res. Commun. 362 (2007) 334–339.
[13] J. Netsawang, S. Noisakran, C. Puttikhunt, W. Kasinrerk, W. Wongwiwat, P. Malasit, P.T. Yenchitsomanus, T. Limjindaporn, Nuclear localization of dengue virus capsid protein is required for DAXX interaction and apoptosis, Virus Res. 147 (2010) 275–283.
[14] S. Wati, S.M. Rawlinson, R.A. Ivanov, L. Dorstyn, M.R. Beard, D.A. Jans, S.M. Pitson, C.J. Burrell, P. Li, J.M. Carr, Tumour necrosis factor alpha (TNF-{alpha}) stimulation of cells with established dengue virus type 2 infection induces cell death that is accompanied by a reduced ability of TNF-{alpha} to activate nuclear factor {kappa}B and reduced sphingosine kinase-1 activity, J. Gen. Virol. 92 (2011) 807–818.
[15] A.M. Nasirudeen, D.X. Liu, Gene expression profiling by microarray analysis reveals an important role for caspase-1 in dengue virus-induced p53-mediated apoptosis, J. Med. Virol. 81 (2009) 1069–1081.
[16] T. El-Bacha, V. Midlej, A.P. Pereira, L. da Silva, M. Silva da Costa, A. Benchimol, A.T. Galina, Da. Poian, Mitochondrial and bioenergetic dysfunction in human hepatic cells infected with dengue 2 virus, Biochim. Biophys. Acta 1772 (2007) 1158–1166.
[17] P. Klomporn, M. Panyasrivanit, N. Wikan, D.R. Smith, Dengue infection of monocytic cells activates ER stress pathways, but apoptosis is induced through both extrinsic and intrinsic pathways, Virology 409 (2010) 189–197.
[18] C. Puttikhunt, P. Ong-Ajchaowlerd, T. Prommool, S. Sangiambut, J. Netsawang, T. Limjindaporn, P. Malasit, W. Kasinrerk, Production and characterization of anti-dengue capsid antibodies suggesting the N terminus region covering the first 20 amino acids of dengue virus capsid protein is predominantly immunogenic in mice, Arch. Virol. 154 (2009) 1211–1221.
[19] M.K. Gentry, E.A. Henchal, J.M. McCown, W.E. Brandt, J.M. Dalrymple, Identification of distinct antigenic determinants on dengue-2 virus using monoclonal antibodies, Am. J. Trop. Med. Hyg. 31 (1982) 548–555.
[20] T. Limjindaporn, W. Wongwiwat, S. Noisakran, C. Srisawat, J. Netsawang, C. Puttikhunt, W. Kasinrerk, P. Avirutnan, S. Thiemmeca, R. Sriburi, N. Sittisombut, P. Malasit, P.T. Yenchitsomanus, Interaction of dengue virus envelope protein with endoplasmic reticulum-resident chaperones facilitates dengue virus production, Biochem. Biophys. Res. Commun. 379 (2009) 196–200.
[21] I.K. Jang, Z.H. Lee, Y.J. Kim, S.H. Kim, B.S. Kwon, Human 4-1BB (CD137) signals are mediated by TRAF2 and activate nuclear factor-kappa B, Biochem. Biophys. Res. Commun. 242 (1998) 613–620.
[22] D.S. Vinay, B.S. Kwon, Role of 4-1BB in immune responses, Semin. Immunol. 10 (1998) 481–489.
[23] C. Gullo, L.K. Koh, W.L. Pang, K.T. Ho, S.H. Tan, H. Schwarz, Inhibition of proliferation and induction of apoptosis in multiple myeloma cell lines by CD137 ligand signaling, PLoS One 5 (2010) e10845.
[24] P. Marianneau, A. Cardona, L. Edelman, V. Deubel, P. Despres, Dengue virus replication in human hepatoma cells activates NF-kappaB which in turn induces apoptotic cell death, J. Virol. 71 (1997) 3244–3249.
[25] J. Park, J.H. Lee, M. La, M.J. Jang, G.W. Chae, S.B. Kim, H. Tak, Y. Jung, B. Byun, J.K. Ahn, C.O. Joe, Inhibition of NF-kappaB acetylation and its transcriptional activity by Daxx, J. Mol. Biol. 368 (2007) 388–397.
[26] J.O. Kim, H.W. Kim, K.M. Baek, C.Y. Kang, NF-kappaB and AP-1 regulate activation-dependent CD137 (4-1BB) expression in T cells, FEBS Lett. 541 (2003) 163–170.
[27] Q. Wang, P. Zhang, Q. Zhang, X. Wang, J. Li, C. Ma, W. Sun, L. Zhang, Analysis of CD137 and CD137L expression in human primary tumor tissues, Croat. Med. J. 49 (2008) 192–200.

VIROLOGY JOURNAL

SHORT REPORT Open Access

Inhibition of p38MAPK and CD137 signaling reduce dengue virus-induced TNF-α secretion and apoptosis

Amar Nagila[1,2†], Janjuree Netsawang[5†], Aroonroong Suttitheptumrong[1,4], Atthapan Morchang[1,4], Sasiprapa Khunchai[1,4], Chatchawan Srisawat, Chunya Puttikhunt[6], Sansanee Noisakran[6], Pa-thai Yenchitsomanus[1] and Thawornchai Limjindaporn[1,3*]

Abstract

Background: Hepatic injury in dengue virus (DENV) infection is authenticated by hepatomegaly and an upsurge in transaminase levels. DENV replicates in hepatocytes and causes hepatocyte apoptosis both *in vitro* and *in vivo*. Understanding the molecular mechanisms of DENV-induced hepatic injury could facilitate the development of alternate chemotherapeutic agents and improved therapies.

Findings: The p38 mitogen-activated protein kinase (MAPK) participates in both apoptosis-related signaling and pro- inflammatory cytokine production. The role of p38 MAPK in DENV-infected HepG2 cells was examined using RNA interference. The results showed that DENV infection activated p38 MAPK and induced apoptosis. The p38 MAPK activation and TNF-α production were controlled by p38 MAPK and CD137 signaling in DENV-infected HepG2 cells as activated p38 MAPK, TNF-α and apoptosis were significantly decreased in p38 MAPK and CD137 depleted DENV-infected HepG2 cells. Addition of exogenous TNF-α to p38 MAPK depleted DENV-infected HepG2 cells restored DENV-induced apoptosis in HepG2 cells.

Conclusion: DENV induces CD137 signaling to enhance apoptosis by increasing TNF-α production via activation of p38 MAPK.

Keywords: Dengue virus, Apoptosis, TNF-α, p38MAPK, CD137

Findings

Hepatic dysfunction is a crucial feature of DENV infection. Hepatic biopsy specimens obtained from fatal cases of dengue shock syndrome (DSS) show cellular apoptosis, which may relate to the pathogenesis of DSS [1,2]. DENV replicates in hepatocytes and apoptosis of DENV-infected hepatic cells has been observed both *in vitro* and *in vivo*. Both the cell death receptor and the mitochondrial apoptotic pathways are affected [3-9].

DENV capsid protein (DENV C) activates both extrinsic and intrinsic apoptotic pathways in hepatic cell lines [10-12]. DENV C localizes to both the cytoplasm and nucleus of DENV-infected HepG2 cells. DENV C contains three nuclear localization signals (NLS), ^{6}KKAR^{9}, ^{73}KKSK^{76} and the bipartite signal ^{85}RKeigrmlnilnRRRR^{100}. Substitution mutations in DENV C (R85A/K86A) result in loss of nuclear localization, Daxx interaction, and apoptosis [11]. Comparison of the apoptotic gene expression profile of DENV C and DENV C (R85A/K86A) transfected HepG2 cells showed a significant increase in expression of CD137, a member of TNF receptor family, in HepG2 cells expressing DENV C. In DENV-infected HepG2 cells, CD137 and CD137 ligand mRNA expression increased 60-fold and 3-fold post DENV infection, respectively. CD137 positive cells increased, but less dramatically, about 3-fold post infection and correlates with the level of apoptosis induced by DENV infection of HepG2 cells, which increased 4-fold post DENV infection [13].

* Correspondence: thawornchai.lim@mahidol.ac.th
†Equal contributors
[1]Division of Molecular Medicine, Department of Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand
[3]Department of Anatomy, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand
Full list of author information is available at the end of the article

CD137 recruits TNF receptor associated factor 2 (TRAF2) and activates apoptosis signal regulating kinase 1 (ASK1), resulting in activation of cJun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) [14]. p38 MAPK is primarily implicated in apoptosis-related signaling [15]. However, activation of p38 MAPK increases inflammatory cytokine production [16]. Activation of p38 MAPK may contribute to the pathogenesis of DENV-induced apoptosis via both mechanisms.

Inhibition of DENV-induced apoptosis in HepG2 cells by siRNA against p38 MAPK

We first asked whether RNAi knockdown of p38α MAPK, which is a crucial mediator of proinflammatory cytokine production, would inhibit DENV-induced apoptosis in HepG2 cells, an approach that might alleviate DENV-mediated hepatic injury. At 24 h after seeding, HepG2 cells were transfected with 200 pmol of siRNA directed against p38α MAPK, 5′CAGACCATATTAACCAGCTTC AGCA3′ or with siControl, 5′ CACGCCTCTTTGTCTT GTTTCGAAA 3′ (Invitrogen), using lipofectamine 2000 (Invitrogen). Twenty-four hours post transfection, the cells were infected with DENV serotype 2 strain 16681 at an MOI of 5 using the methods described previously [13]. The percentage of apoptotic cells was determined by annexin V/FITC and PI double staining (BD Biosciences) and quantitation by flow cytometry. The efficiency of p38α MAPK knockdown by siRNA was examined by real-time RT-PCR using p38α MAPK-specific primers (p38α MAPK-F, 5′CGACTTGCTGGAGAAGATGC3′, and p38α MAPK-R, 5′TCCATCTCTTCTTGGTCAAGG3′) and by Western blot analysis using primary antibodies against p38 MAPK and β-actin (Santa Cruz Biotechnology). About 30% decreases in both mRNA and protein expression levels of p38 MAPK were observed (Figure 1A, 1B). In addition, treatment with siRNA against p38 MAPK

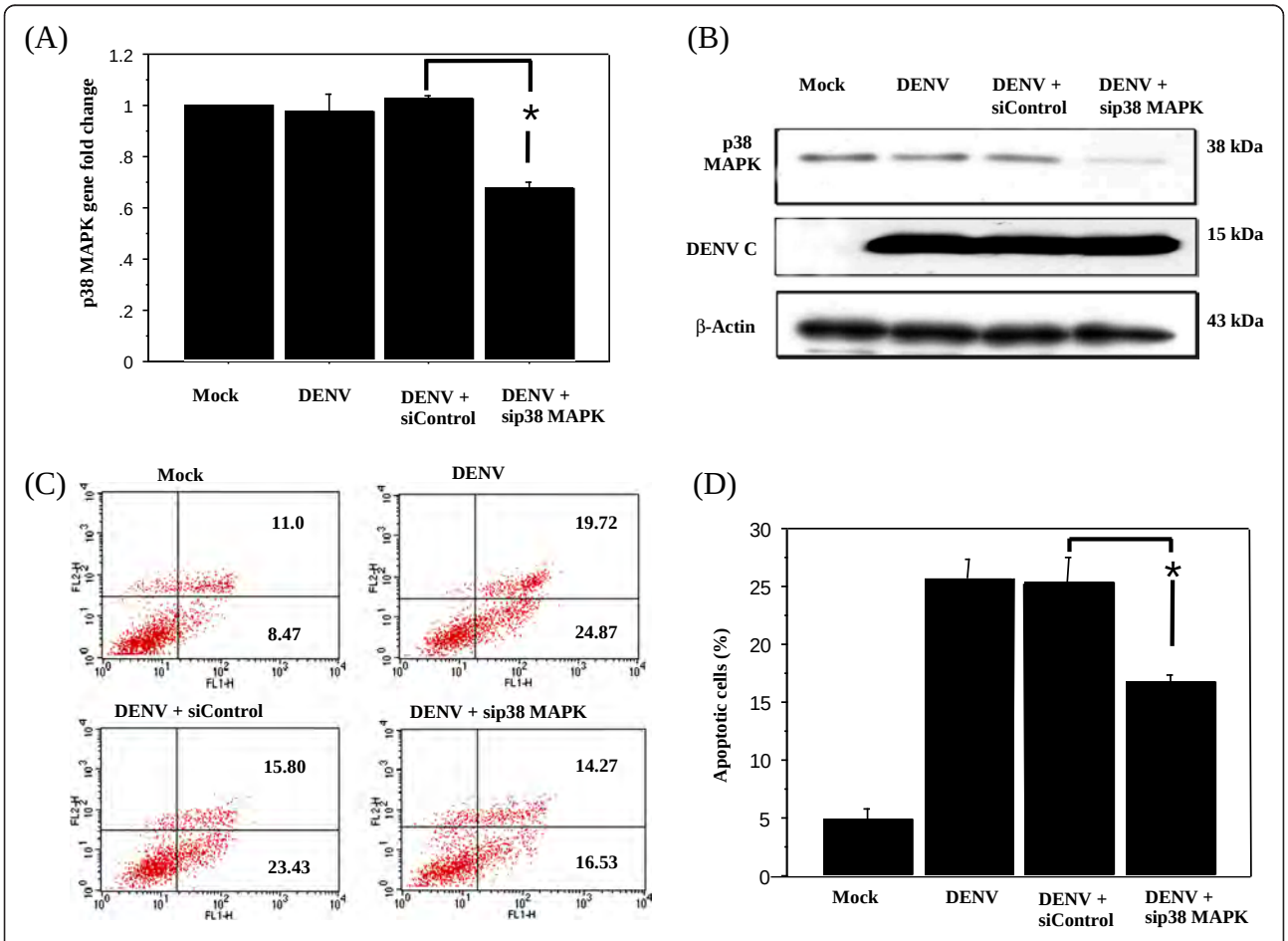

Figure 1 Decreased apoptosis in p38 MAPK knockdown DENV-infected HepG2 cells. HepG2 cells were transfected either with siRNA directed against p38 MAPK or with control siRNA. Twenty-four hours post transfection, cells were infected with DENV at MOI 5 for 60 h. Cells were harvested and analyzed for (**A**) mRNA level of p38 MAPK (**B**) protein level of p38 MAPK. (**C**) Apoptosis by flow cytometry. Apoptosis cells have annexin V+/PI- staining. (**D**) Bar graph represents apoptosis experiments. All data were obtained from three independent experiments and reported as the mean ± SEM. Statistical differences between the groups were tested with an unpaired *t*-test using StatView version 5.0 and *P* value less than 0.05 was considered significant.

reduced apoptosis from 23.43% to 16.53% in DENV-infected HepG2 cells (Figure 1C, D) suggesting a role for p38 MAPK in DENV-mediated apoptosis. The result of siRNA directed against p38 MAPK in DENV-infected HepG2 cells is agreeable to our previous study of pharmacological inhibition of p38 MAPK by SB203580 in DENV-infected HepG2 cells [17]. Therefore, genetic inhibition of p38 activity reproduces pharmacological inhibition of the enzyme and confirms the contribution of p38 MAPK to apoptotic events induced by DENV [17]. DENV infected HepG2 cells equally in the presence or absence of SB203580 [17] but DENV production in BHK-21 cells was decreased in the presence of SB203580 [18].

Inhibition of DENV C-induced apoptosis in stable HepG2 cell expressing DENV C by siRNA against p38 MAPK

We next asked whether DENV C activates p38 MAPK-induced apoptosis by examining the effect of siRNA against p38α MAPK on apoptosis of HepG2 cells stably expressing DENV C. HepG2 cells stably expressing DENV C were constructed in this study using retroviral system (Stratagene). Briefly, 70% confluent of HEK 293T cells was transfected with p-VPack-GP, p-VPack-VSV-G and pFB-Neo-DENV C using lipofectamine 2000 (Invitrogen). The culture supernatant containing infectious viral particles was collected after 24 h post transfection and added to HepG2 cells, which were pre-incubated with 8 μg / ml of polybrene. Twenty-four h after incubation, stable HepG2 cells expressing DENV C were selected with media containing 0.5 mg/ml G418 (Calbiochem). The G418-resistant cells were grown and maintained in DMEM medium containing 0.5 mg/ml G418, and the expression of DENV C was examined by flow cytometry and Western blot analysis using antibody to DENV C [19]. Up to 5×10^5 the stably expressing cells were plated for 24 h prior to transfection. The cells were then transfected with siRNAs as described in the preceding experiment. Knock-down efficiency was assessed by Western blot analysis. The 15 kDa capsid protein was expressed in stable HepG2 cells expressing DENV but not in HepG2 cells expressing control plasmid (Figure 2A). To activate the extrinsic apoptotic

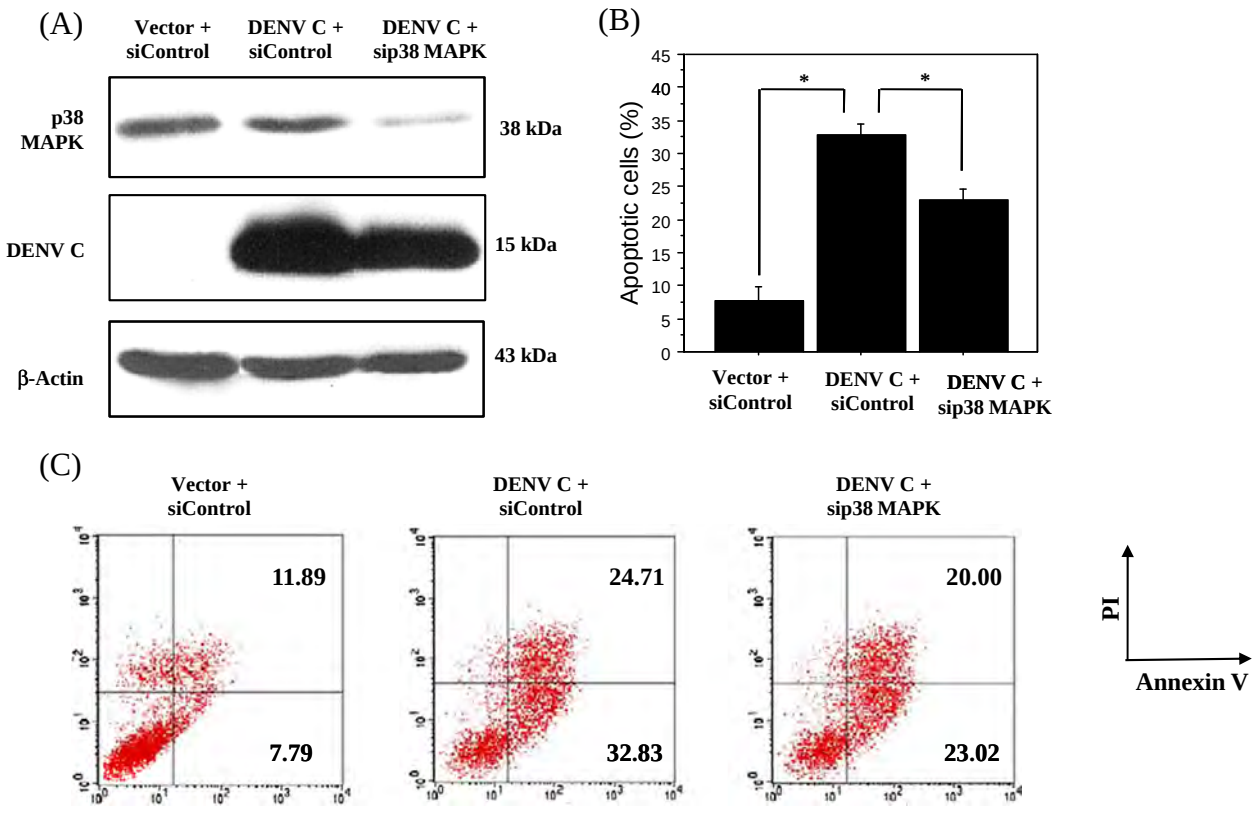

Figure 2 Decreased apoptosis in p38 MAPK knockdown HepG2 cells expressing DENV C. HepG2 cells expressing DENV C or control plasmid were transfected either with siRNA directed against p38 MAPK or with control siRNA and then treated with 0.5 μg/ml anti-Fas mAb and 1 ug/ml cycloheximide for 24 h. Cells were collected and analyzed for (**A**) p38 MAPK, DENV C and actin (**B**) Bar graph represents apoptosis experiments. All data were obtained from three independent experiments and reported as the mean ± SEM. Statistical differences between the groups were tested with an unpaired *t*-test using StatView version 5.0 and *P* value less than 0.05 was considered significant. (**C**) Apoptosis by flow cytometry.

pathway, cells were treated with 0.5 μg/ml anti-Fas mAb (Sigma) and 1 ug/ml cycloheximide (Sigma) for 24 h in culture medium. Both adherent and floating cells, as well as culture supernatants, were collected and assessed for apoptosis by annexinV/PI staining (BD Biosciences). As with DENV-infected cells, siRNA p38α MAPK resulted in a substantial reduction in p38 MAPK protein, but had no effect on DENV C expression (Figure 2A). In the presence of anti-Fas and cycloheximide, apoptosis of HepG2 cells expressing DENV C increased from 7.79% to 32.83% compared to that of HepG2 cells expressing control plasmid. HepG2 cells expressing DENV C transfected with p38 MAPK siRNA reduced apoptosis from 32.83% to 23% (Figure 2B, 2C). In our previous study without anti-Fas mAb and cycloheximide treatment, HepG2 cells were transiently transfected with a DENV C or control plasmid and incubated in the presence of DMSO or 10 μM of SB203580 for 24 h. The percentage of apoptotic cells was then determined by annexin V/FITC and PI double staining and quantitation by flow cytometry. Comparable to apoptosis of HepG2 cells expressing control plasmid, apoptosis of HepG2 cells expressing DENV C increased from 10.11% to 24.40%. Apoptosis of HepG2 cells expressing DENV C decreased from 24.40% to 5.69% in medium with SB203580 [17]. Therefore, genetic inhibition of p38 activity reproduces pharmacological inhibition of the enzyme and verifies the contribution of p38 MAPK to apoptotic events induced specifically by DENV C. However, not all DENV-induced cell death is caused by DENV C, other DENV proteins including M, NS3 protease and NS2B-NS3 precursor also induces apoptosis [20,21].

Inhibition of DENV-induced phosphorylated p38 MAPK, TNF-α production and apoptosis in HepG2 cells by siRNA against CD137

To further define the molecular mechanisms involved, we asked whether CD137 signaling regulates p38 MAPK activation and apoptosis in DENV-infected HepG2 cells. siRNA knockdown of CD137 was performed as described for p38 MAPK using the CD137-specific oligo 5′CACGCTCCGTTTCTCTGTTGTTAAA 3′ (Invitrogen). The efficiency of knockdown was examined by real-time RT-PCR using CD137-specific primers CD137-F, 5′CCA AAA TGT TCT GCT GAT CG3′ and CD137-R, 5′ AAG ACT GTG GCG CCC TG3′. The number of CD 137 positive cells was measured by flow cytometry using a primary antibody against CD137 (Santa Cruz Biotechnology). Transfection of HepG2 cells with siRNA against CD137 resulted in a nearly 2-fold reduction in CD137 mRNA and CD137-positive cells (Figure 3A, 3B). The effect of CD137 depletion on p38 MAPK activation during DENV infection and apoptosis were measured by Western blot analysis using primary antibody against phosphorylated p38 MAPK (Santa Cruz Biotechnology), and by annexinV/PI staining (BD Biosciences), respectively. Knockdown of CD137 expression reduced the amount of phosphorylated p38 MAPK (Figure 3C) and apoptosis (Figure 3D). These results indicate a role of CD137 signaling in regulation of p38 MAPK activation and apoptosis in DENV-infected HepG2 cells. As DENV induced CD137 expression only 30% of the infected cells (Figure 3B) and expressed at the late stage of infection [13], CD137 and p38 MAPK signaling may support other apoptotic signaling pathways [3-9] to aggravate apoptosis at the late stage of DENV infection. Multiple studies *in vitro* and *in vivo* models implicate TNF-α in DENV-induced tissue damage [8,22]. In addition, TNF-α induced apoptosis via p38 MAPK activation was shown in pseudorabies virus infection [23].

We next asked whether TNF-α mediated DENV-induced apoptosis via CD137 signaling. TNF-α expression in CD137 depleted DENV-infected HepG2 cells was examined by real-time RT-PCR using TNF-α-specific primers TNF-α-F, 5′TGCTTGTTCCTCAGCCTCTT3′,and TNF-α-R, 5′ ATGGGCTACAGGCTTGTCACT3′ and by ELISA (R&D Systems). DENV infection of HepG2 cells resulted in a dramatic increase in TNF-α mRNA and protein (Figure 3E, 3F). However, CD137 siRNA treatment reduced of TNF-α production about 30% (Figure 3E, 3F), which correlates well with the comparable reduction in apoptosis of CD137 knockdown cells (Figure 3D).

Inhibition of p38 MAPK activity by SB203580 decreased TNF-α production in DENV-infected HepG2 cells

As p38 MAPK is one of the downstream targets of CD137 signaling, it is important to assess whether inhibition of p38 MAPK activity in DENV-infected HepG2 cells would reduce TNF-α expression. HepG2 cells were infected with DENV serotype 2 at a MOI 5 and incubated 48 h in the presence of either DMSO or 10 μM SB203580 (Santa Cruz Biotechnology). TNF-α mRNA expression of DENV-infected HepG2 cells and TNF-α protein expression in supernatant of DENV-infected HepG2 cells were subsequently examined by real-time RT-PCR and by ELISA, respectively. As expected, treatment with 10 μM SB203580 significantly reduced TNF-α expression in DENV-infected HepG2 cells (Figure 4A, 4B) indicating that pharmacological inhibition of p38 MAPK reduced TNF-α secretion in DENV-infected HepG2 cells. Whether inhibition of both p38 and CD137 signalings has the additive effect on TNF-α production merits further investigation.

The role of TNF-α expression in apoptosis of DENV-infected HepG2 cells

It would be important to evaluate the effect of TNF-α expression in apoptosis of DENV-infected HepG2 cells. Supernatants containing DENV from mock-infected or DENV-infected HepG2 cells in the presence of either

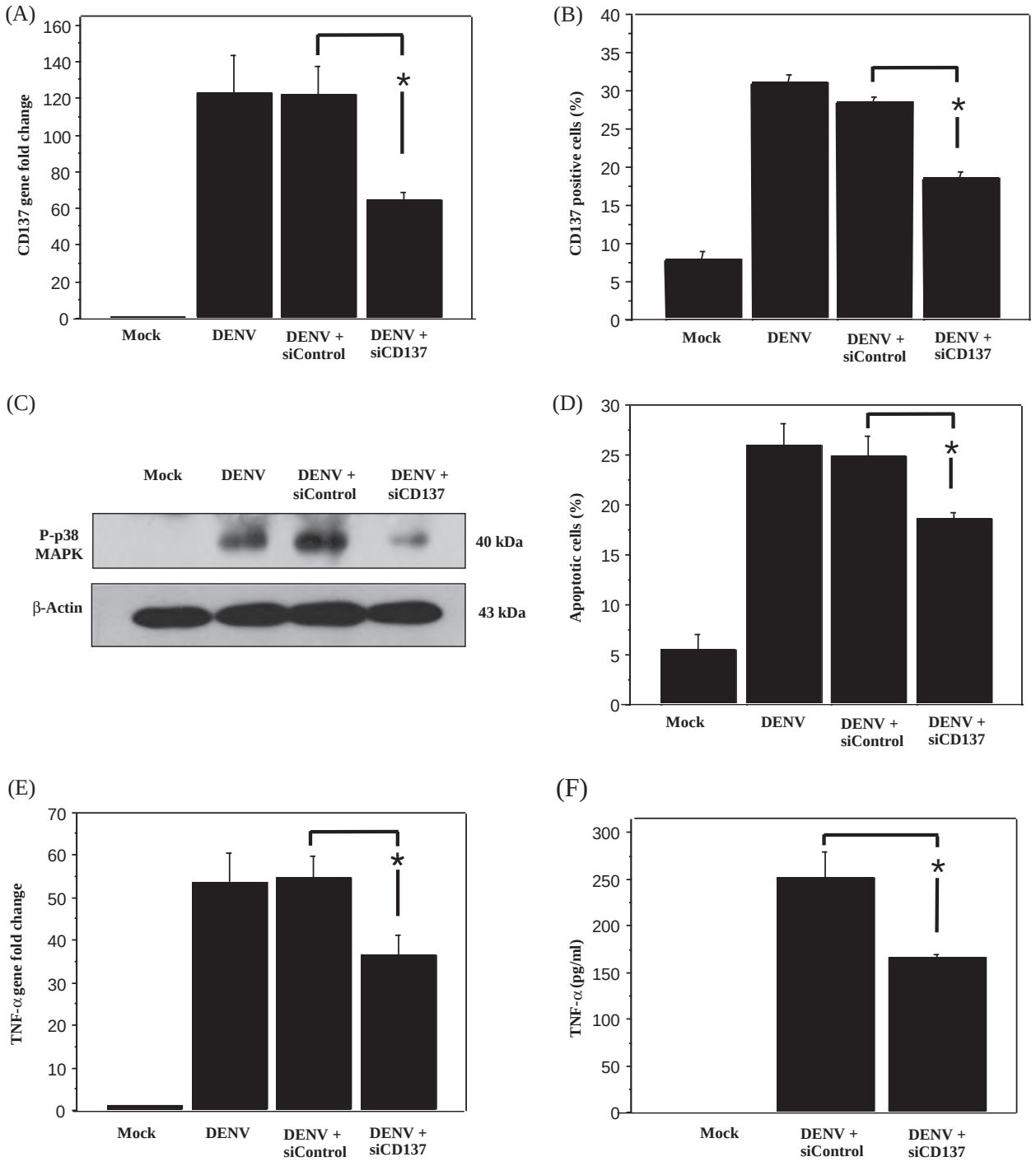

Figure 3 Reduced DENV-induced phosphorylated p38 MAPK, TNF-α and apoptosis in HepG2 cells by siRNA against CD137. HepG2 cells were transfected with either CD137 siRNA or control siRNA. Twenty-four hours post transfection, cells were infected with DENV at MOI 5 for 60 h. Cells were collected and analyzed for (**A**) CD137 mRNA (**B**) CD137 positive cells (**C**) phosphorylation of p38 and (**D**) Apoptosis by flow cytometry. The cells were collected and analyzed for TNF-α mRNA (**E**) and the culture supernatant was analyzed for TNF-α protein (**F**). All data were obtained from three independent experiments and reported as the mean ± SEM. Statistical differences between the groups were tested with an unpaired *t*-test using StatView version 5.0 and *P* value less than 0.05 was considered significant.

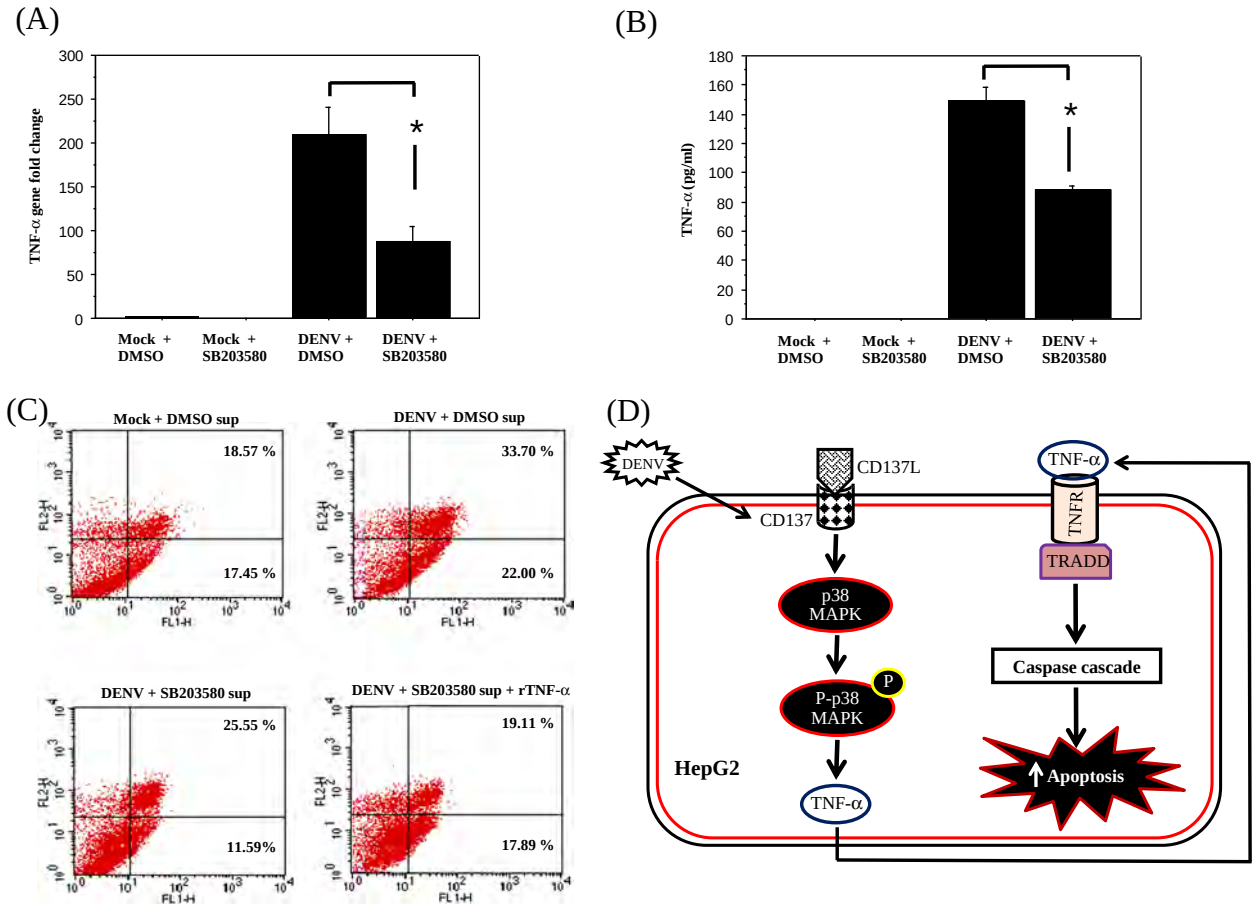

Figure 4 Reduced DENV-induced TNF-α production and apoptosis in SB203580 treated DENV-infected HepG2 cells and exogenous TNF-α restored the apoptosis. (**A**) HepG2 cells were infected with DENV serotype 2 at a MOI 5 and incubated 48 h in the presence of either DMSO or 10 μM SB203580. TNF-α mRNA (A) and TNF-α protein (**B**) expression of DENV-infected HepG2 cells was subsequently examined by real-time RT-PCR and by ELISA, respectively. All data were obtained from three independent experiments and reported as the mean ± SEM. Statistical differences between the groups were tested with an unpaired *t*-test using StatView version 5.0 and *P* value less than 0.05 was considered significant. (**C**) Supernatants containing DENV from mock-infected or DENV-infected HepG2 cells in the presence of either DMSO or 10 μM SB203580 was isolated and incubated with HepG2 cells in the presence or absence of recombinant TNF-α. The percentage of apoptotic cells was then determined at 72 h by annexin V/FITC and PI double staining and quantitation by flow cytometry. Apoptosis cells have annexin V+/PI- staining. (**D**) Model representing the augmentation of DENV-induced apoptosis mediated by TNF-α via the activation of p38 MAPK and CD137 signaling.

DMSO or 10 μM SB203580 was isolated and further incubated with HepG2 cells in the presence or absence of recombinant TNF-α (250 ng/ml)(Sigma). The percentage of apoptotic cells was then determined at 72 h by annexin V/FITC and PI double staining and quantitation by flow cytometry. The result in Figure 4C showed that apoptosis of DENV-infected HepG2 cells decreased from 22% to 11.59% when HepG2 cells were incubated with supernatant containing virus from SB203580 treated DENV-infected HepG2 cells. Addition of recombinant TNF-α to supernatant from SB203580 treated DENV-infected HepG2 cells increased apoptosis of DENV-infected HepG2 cells from 11.59% to 17.89% (Figure 4C) suggesting that TNF-α production affects DENV-induced apoptosis of HepG2 cells. Our result supports the previous studies for the role of TNF-α expression in DENV-induced apoptosis in other cell lines. TNF-α produced from macrophage was shown to enhance DENV-induced endothelial cell death [24] and inhibition of peripheral blood mononuclear cell (PBMC) apoptosis by etanercept, which is the antibody to TNF-α, was also reported [25]. In summary, we propose in Figure 4D that DENV induces phosphorylated p38 MAPK to stimulate apoptosis and inhibition of p38 MAPK and CD137 pathway reduce DENV-induced TNF-α secretion and apoptosis of HepG2 cells.

Competing interests

The authors declare that they have no competing interest.

Authors' contributions

TL, PY and SN conceived of the study. AN, JN, AM and CS carried out experiments in RNA interference and apoptosis. AS and SK carried out cytokine assay. CP participated in antibody production. All authors have read and approved the final version of the manuscript.

Acknowledgements

We appreciate Dr. William A Fonzi, Georgetown University, USA, for the critical reading and editing of this manuscript. This work was financially supported by Mahidol University Grant and Siriraj Research Developmental Fund to T.L., who is a Mid-Career Thailand Research Fund–Commission of Higher Education (TRF-CHE) Research Scholar. PY is a TRF-Senior Research Scholar. JN is a TRF-CHE Young Research Scholar. AM was supported by TRF-Royal Golden Jubilee (TRF-RGJ) Ph.D. Program. SK was supported by CHE under the program strategic scholarship for frontier research network for the join Ph.D. program. AS was supported by Siriraj Developmental Scholarship and AN was supported by Siriraj Graduate Thesis Scholarship. All authors obtained permission to acknowledge the source of funding.

Author details

[1]Division of Molecular Medicine, Department of Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. [2]Department of Biochemistry, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. [3]Department of Anatomy, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. [4]Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. [5]Faculty of Medical Technology, Rangsit University, Bangkok, Thailand. [6]Medical Biotechnology Research Unit, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Bangkok, Thailand.

Received: 14 December 2012 Accepted: 2 April 2013
Published: 4 April 2013

References

1. Bhamarapravati N: **Hemostatic defects in dengue hemorrhagic fever.** *Rev Infect Dis* 1989, **11**(Suppl 4):S826–S829.
2. Limonta D, Capo V, Torres G, Perez AB, Guzman MG: **Apoptosis in tissues from fatal dengue shock syndrome.** *J Clin Virol* 2007, **40:**50–54.
3. Marianneau P, Cardona A, Edelman L, Deubel V, Despres P: **Dengue virus replication in human hepatoma cells activates NF-kappaB which in turn induces apoptotic cell death.** *J Virol* 1997, **71:**3244–3249.
4. Thongtan T, Panyim S, Smith DR: **Apoptosis in dengue virus infected liver cell lines HepG2 and Hep3B.** *J Med Virol* 2004, **72:**436–444.
5. Nasirudeen AM, Liu DX: **Gene expression profiling by microarray analysis reveals an important role for caspase-1 in dengue virus-induced p53-mediated apoptosis.** *J Med Virol* 2009, **81:**1069–1081.
6. El-Bacha T, Midlej V, da Silva Pereira AP, da Costa Silva L, Benchimol M, Galina A, Da Poian AT: **Mitochondrial and bioenergetic dysfunction in human hepatic cells infected with dengue 2 virus.** *Biochim Biophys Acta* 2007, **1772:**1158–1166.
7. Matsuda T, Almasan A, Tomita M, Tamaki K, Saito M, Tadano M, Yagita H, Ohta T, Mori N: **Dengue virus-induced apoptosis in hepatic cells is partly mediated by Apo2 ligand/tumour necrosis factor-related apoptosis-inducing ligand.** *J Gen Virol* 2005, **86:**1055–1065.
8. Wati S, Rawlinson SM, Ivanov RA, Dorstyn L, Beard MR, Jans DA, Pitson SM, Burrell CJ, Li P, Carr JM: **Tumour necrosis factor alpha (TNF-{alpha}) stimulation of cells with established dengue virus type 2 infection induces cell death that is accompanied by a reduced ability of TNF-{alpha} to activate nuclear factor {kappa}B and reduced sphingosine kinase-1 activity.** *J Gen Virol* 2011, **92:**807–818.
9. Liao H, Xu J, Huang J: **FasL/Fas pathway is involved in dengue virus induced apoptosis of the vascular endothelial cells.** *J Med Virol* 2010, **82:**1392–1399.
10. Limjindaporn T, Netsawang J, Noisakran S, Thiemmeca S, Wongwiwat W, Sudsaward S, Avirutnan P, Puttikhunt C, Kasinrerk W, Sriburi R, *et al*: **Sensitization to Fas-mediated apoptosis by dengue virus capsid protein.** *Biochem Biophys Res Commun* 2007, **362:**334–339.
11. Netsawang J, Noisakran S, Puttikhunt C, Kasinrerk W, Wongwiwat W, Malasit P, Yenchitsomanus PT, Limjindaporn T: **Nuclear localization of dengue virus capsid protein is required for DAXX interaction and apoptosis.** *Virus Res* 2010, **147:**275–283.
12. Nasirudeen AM, Wang L, Liu DX: **Induction of p53-dependent and mitochondria-mediated cell death pathway by dengue virus infection of human and animal cells.** *Microbes Infect* 2008, **10:**1124–1132.
13. Nagila A, Netsawang J, Srisawat C, Noisakran S, Morchang A, Yasamut U, Puttikhunt C, Kasinrerk W, Malasit P, Yenchitsomanus PT, Limjindaporn T: **Role of CD137 signaling in dengue virus-mediated apoptosis.** *Biochem Biophys Res Commun* 2011, **410:**428–433.
14. Sollner L, Shaqireen DOKMM, Wu JT, Schwarz H: **Signal transduction mechanisms of CD137 ligand in human monocytes.** *Cell Signal* 2007, **19:**1899–1908.
15. Xia Z, Dickens M, Raingeaud J, Davis RJ, Greenberg ME: **Opposing effects of ERK and JNK-p38 MAP kinases on apoptosis.** *Science* 1995, **270:**1326–1331.
16. Ono K, Han J: **The p38 signal transduction pathway: activation and function.** *Cell Signal* 2000, **12:**1–13.
17. Morchang A, Yasamut U, Netsawang J, Noisakran S, Wongwiwat W, Songprakhon P, Srisawat C, Puttikhunt C, Kasinrerk W, Malasit P, *et al*: **Cell death gene expression profile: role of RIPK2 in dengue virus-mediated apoptosis.** *Virus Res* 2011, **156:**25–34.
18. Ceballos-Olvera I, Chavez-Salinas S, Medina F, Ludert JE, del Angel RM: **JNK phosphorylation, induced during dengue virus infection, is important for viral infection and requires the presence of cholesterol.** *Virology* 2009, **396:**30–36.
19. Puttikhunt C, Ong-Ajchaowlerd P, Prommool T, Sangiambut S, Netsawang J, Limjindaporn T, Malasit P, Kasinrerk W: **Production and characterization of anti-dengue capsid antibodies suggesting the N terminus region covering the first 20 amino acids of dengue virus capsid protein is predominantly immunogenic in mice.** *Arch Virol* 2009, **154:**1211–1221.
20. Catteau A, Kalinina O, Wagner MC, Deubel V, Courageot MP, Despres P: **Dengue virus M protein contains a proapoptotic sequence referred to as ApoptoM.** *J Gen Virol* 2003, **84:**2781–2793.
21. Shafee N, AbuBakar S: **Dengue virus type 2 NS3 protease and NS2B-NS3 protease precursor induce apoptosis.** *J Gen Virol* 2003, **84:**2191–2195.
22. Yen YT, Chen HC, Lin YD, Shieh CC, Wu-Hsieh BA: **Enhancement by tumor necrosis factor alpha of dengue virus-induced endothelial cell production of reactive nitrogen and oxygen species is key to hemorrhage development.** *J Virol* 2008, **82:**12312–12324.
23. Yeh CJ, Lin PY, Liao MH, Liu HJ, Lee JW, Chiu SJ, Hsu HY, Shih WL: **TNF-alpha mediates pseudorabies virus-induced apoptosis via the activation of p38 MAPK and JNK/SAPK signaling.** *Virology* 2008, **381:**55–66.
24. Chen HC, Hofman FM, Kung JT, Lin YD, Wu-Hsieh BA: **Both virus and tumor necrosis factor alpha are critical for endothelium damage in a mouse model of dengue virus-induced hemorrhage.** *J Virol* 2007, **81:**5518–5526.
25. Jaiyen Y, Masrinoul P, Kalayanarooj S, Pulmanausahakul R, Ubol S: **Characteristics of dengue virus-infected peripheral blood mononuclear cell death that correlates with the severity of illness.** *Microbiol Immunol* 2009, **53:**442–450.

doi:10.1186/1743-422X-10-105
Cite this article as: Nagila *et al.*: Inhibition of p38MAPK and CD137 signaling reduce dengue virus-induced TNF-α secretion and apoptosis. *Virology Journal* 2013 **10**:105.

Biochemical and Biophysical Research Communications 438 (2013) 20–25

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Role of cathepsin B in dengue virus-mediated apoptosis

Atthapan Morchang [a,b], Jutatip Panaampon [a], Aroonroong Suttitheptumrong [a,b], Umpa Yasamut [a,b], Sansanee Noisakran [c], Pa-thai Yenchitsomanus [a], Thawornchai Limjindaporn [a,d,*]

[a] Division of Molecular Medicine, Department of Research and Development, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand
[b] Graduate Program in Immunology, Department of Immunology, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand
[c] Medical Biotechnology Unit, National Center for Genetic Engineering and Biotechnology, National Science and Technology Development Agency, Thailand
[d] Department of Anatomy, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand

ARTICLE INFO

Article history:
Received 18 June 2013
Available online 15 July 2013

Keywords:
Apoptosis
Dengue virus
Cathepsins
Lysosome

ABSTRACT

Dengue virus (DENV) infection is one of the most important mosquito-borne viral diseases, which is endemic in the tropical and sub-tropical regions. Patients with dengue hemorrhagic fever (DHF) generally present hemorrhagic tendencies, plasma leakage, thrombocytopenia, and hemoconcentration. Hepatic dysfunction is also a crucial feature of DENV infection. Hepatic biopsy specimens obtained from fatal cases of DENV infection show cellular apoptosis, which apparently relate to the pathogenesis. Cathepsins, which are cysteine proteases inside the lysosome, were previously reported to be up-regulated in patients with DHF. However, their functions during DENV infection have not been thoroughly investigated. We show for the first time that DENV induces lysosomal membrane permeabilization. The resulting cytosolic cathepsin B and S contributed to apoptosis via caspase activation. The activity of caspase 3 was significantly reduced in DENV-infected HepG2 cells treatedwith cathepsin B or S inhibitors. Treatment with cathepsin B inhibitor also reduced the activity of caspase 9, suggesting that cathepsin B activates both caspase-9 and caspase-3. Reduced cathepsin B expression, effected by RNA interference, mimicked pharmacological inhibition of the enzyme and confirmed the contribution of cathepsin B to apoptotic events induced by DENV in HepG2 cells.

1. Introduction

Dengue virus (DENV) infection is one of the most important mosquito-borne viral diseases and is endemic in several countries. DENV has four serotypes (DENV-1, -2, -3 and -4). Clinical manifestations of infection include febrile disease, dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS). The last occurs in cases of subsequent infection with a different serotype of DENV [1]. Patients with DHF present with hemorrhagic tendencies, plasma leakage, thrombocytopenia, and hemoconcentration. Evidence of hepatic injury is also demonstrated by hepatomegaly and increases in transaminase levels [2–4]. Hepatic biopsy specimens obtained from fatal cases of DSS show cellular apoptosis, which may relate to the pathogenesis of DSS [5–7].

Following DENV infection, apoptosis of hepatic cells was observed both *in vitro* and *in vivo* [6–17]. DENV infection promotes apoptosis in the hepatoma cell line, HepG2, partly through the induction of TRAIL, a member of the death receptor pathway [18]. TNFR and Fas signaling also contribute to DENV mediated apoptosis [11,15]. Changes in mitochondria typical of the apoptotic process have also been demonstrated in DENV-infected HepG2 cells, indicating a role of the mitochondrial pathway [16]. In Huh-7 cells, another hepatoma cell line, DENV infection also increases mitochondrial membrane potential and p53 expression [19,20]. In a mouse model of DENV infection, intrahepatic infiltrating $CD8^+$ T cells cause liver cell death [21]. A contribution of apoptosis to the pathogenesis of fatal DHF/DSS in humans is suggested by immunolocalization studies [7]. Apoptotic cells were present in the livers offive of six patients from a Cuban dengue epidemic [7].

Lysosomes are membrane-bound organelles filled with hydrolytic enzymes including cysteine proteases and cathepsins [22]. Whereas intra-lysosomal cathepsins can degrade proteins and participate in several cellular processes, such as autophagy, antigen presentation and cytokine maturation, cytosolic cathepsins mediate apoptosis. [23,26–28]. In hepatocytes, cathepsins process Bid or caspase 2, degrade Bcl-2, and trigger the intrinsic apoptosis pathway. Although cathepsin genes have been shown to be up-regulated in DENV-infected HepG2 cells [13] and in patients with DHF [29], the role of cathepsins in DENV-mediatedapoptosis has not been investigated.

This study aims to characterize the role of cathepsins in DENV-mediated apoptosis. We show here that DENV induces lysosomal

* Corresponding author at: Department of Anatomy, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand.
E-mail address: thawornchai.lim@mahidol.ac.th (T. Limjindaporn).

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.07.009

membrane permeabilization thereby releasing cathepsins to induce apoptosis and that cathepsin B contributes to DENV-mediated apoptosis in HepG2 cells.

2. Materials and methods

2.1. Culture and infection of HepG2 cells

HepG2 cells were cultured for 24 h before infection in DMEM medium (Gibco-BRL), supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 1% non-essential amino acids, 1 mM sodium pyruvate, 100 U/ml of penicillin and streptomycin at37 °C in a humidified atmosphere containing 5% CO_2. Cells were infected with DENV serotype 2 (DENV-2) strain 16681 at a multiplicity of infection (MOI) of 5. The fraction of cells infected was measured by flow cytometry following immunofluorescent staining of DENV envelope protein (DENV E) [30,31].

2.2. Apoptosis assay

About 1 to 2 × 10^5 HepG2 cells were seeded in a 12-well culture plate, infected with DENV-2 for 2 h, washed with PBS, and incubated for 24 h, 36 h and 48 h, respectively. Following incubation, cells were stained with annexin V/FITC and propidium iodide (PI) (BD Biosciences). Apoptotic cells, which were annexin V+/PI−, werequantitated by flow cytometry.

To assess the caspase activity, up to 2 × 10^4 HepG2 cells were seeded in a 96-well plate and pre-treated with 50 μM cathepsin B inhibitor (CA-074 Me, Calbiochem), 10 μM cathepsin L inhibitor (Z-FY (t-Bu)-DMK, Calbiochem) or 5 μM cathepsin S inhibitor (Z-FL-COCHO, Calbiochem) for 3 h before infection with DENV-2. All concentrations tested in this study were proven to be non-toxic to the HepG2 cells (data not shown). An equal volume of DMSO (Sigma) was used as a vehicle control. The cells were infected with DENV-2 for 48 h. The activity of caspases 3 and 7 was measured using the fluorescence-based assay, Apo-One® Homogeneous Caspase-3/7 assay (Promega). In addition, the activity of caspase 8 and caspase 9 was separately measured using luminescence-based assays, Caspase-Glo™ 8 Assay andCaspase-Glo™ 9 Assay (Promega), respectively.

2.3. Lysosome permeability assay

About 1 to 2 × 10^5 HepG2 cells were seeded in a 12-well culture plate, infected with DENV-2 for 2 h, washed with PBS, and incubated for 24 h, 36 h and 48 h, respectively. At the indicated time points, lysosomes were labeled by incubation of cells with 0.5 μg/ml of acridine orange (Molecular Probes) for 15 min at 37 °C using the protocol described previously [32,33]. Acridine orange accumulates in intact lysosomes and fluoresces red. Fluorescence was analyzed with flow cytometry. The reduced red fluorescence of DENV-infected cells relative tomock-infected cells was taken as a measure of lysosome permeabilization. The role of reactive oxygen species (ROS) in lysosomal membrane permeabilization was assessed. HepG2 cells were seeded in a 12-well cul-

Table 1
Primers used for quantitative real time PCR.

Gene	Primer sequence (5′–3′)		Product size (bp)
	Forward	Reverse	
CTSB	TGTGTATTCGGACTTCCTGCT	GTGTGCCATTCTCCACTCC	113
CTSS	GGGTACCTCATGTGACAAG	TCACTTCTTCACTGGTCATG	400
CTSL	CAGTGTGGGAGAAGAACATG	TGATTCTTCACAGGAGTCAC	247
CTSD	GTTTCGTGCATTGGAAGACC	GGTTTTGTCCCCTCTCACTC	302
ACTB	AGAAAATCTGGCACCACACC	CTCCTTAATGTCACGCACGA	395

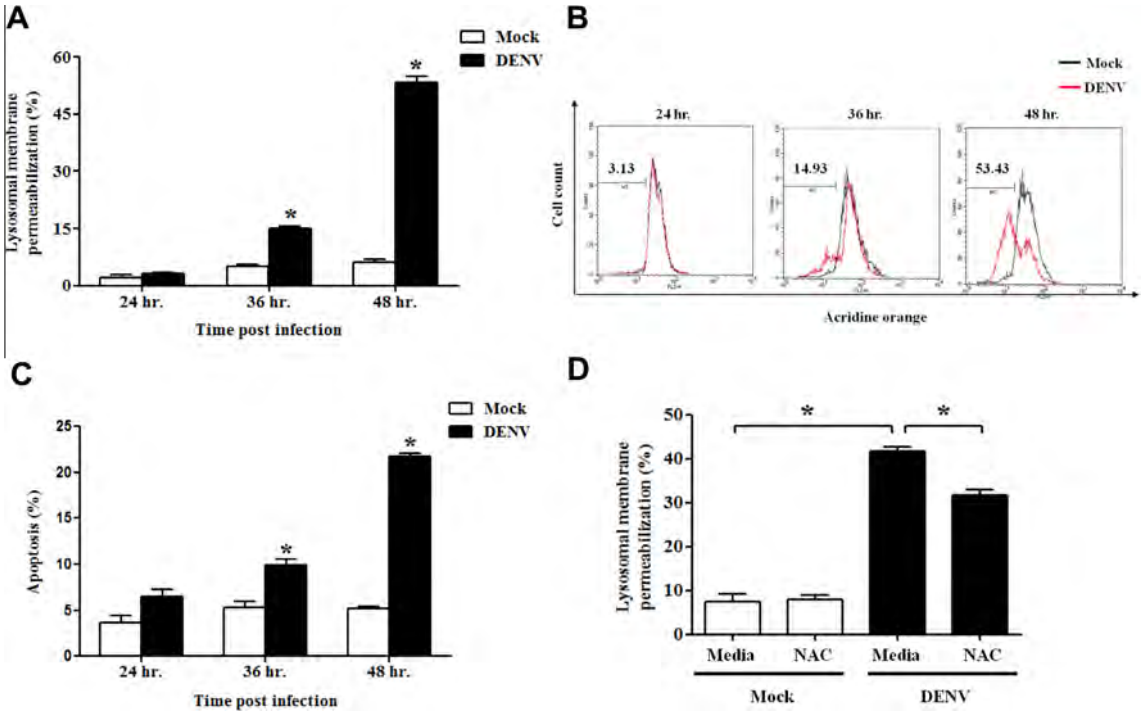

Fig. 1. DENV induced lysosomal membrane permeabilization, partly mediated by ROS in HepG2 cells. HepG2 cells were infected with DENV-2 at a MOI of 5 for 24, 36 and 48 h. Apoptosis was determined by annexin V/PI staining (C). Lysosomal membrane permeabilization was determined by acridine orange staining (A and B). To determine the role of ROS in mediating lysosomal membrane permeabilization, HepG2 cells were infected with DENV-2 at a MOI of 5 and cultured in the presence or the absence of 5 mM of NAC for 48 h (D). The results were obtained from flow cytometry analysis and expressed as the average of three independent experiments ± SEM. The asterisks indicate statistically significant differences between groups ($p < 0.05$).

ture plate, infected with DENV-2 for 2 h, washed with PBS, and incubated in the presence or the absence of 5 mM N-acetyl cysteine (NAC) (Calbiochem) for 24 h, 36 h and 48 h, respectively. At the indicated time points, lysosomes were labeled by incubation of cells with 0.5 μg/ml of acridine orange (Molecular Probes) for 15 min at 37 °C using the protocol described previously [32,33].

2.4. Real-time PCR

Up to 2×10^6 HepG2 cells were seeded in a 60 mm dish and were infected with DENV-2 for 24 h and 48 h, respectively. Total RNA from mock-infected or DENV-infected cells was isolated and reverses transcribed using the High Pure RNA isolation kit (Roche) and SuperScript® III First-Strand Synthesis System (Invitrogen), respectively. *CTSB*, *CTSD*, *CTSL*, *CTSS* or *ACTB* mRNA were PCR amplified using gene-specific primers (Table 1). Amplification was monitored using SYBR Green I Reaction Mix (Roche) in a Roche Light Cycler 480. The *Ct* of each mRNA and ACTB control was measured and the difference between their ΔCt was calculated. The relative expression values ($2^{-\Delta\Delta Ct}$) between mock-infected and DENV-infected HepG2 cells was then determined.

2.5. RNA interference

Up to 1.5×10^5 HepG2 cells were seeded in a 12-well culture plate and transfected either with 200 nM of small interfering RNA (siRNA) directed against *CTSB* (5′ UCU CUU UGA UGG UGG GAC ACU GUG G 3′)(Invitrogen) or with siControl (stealth RNAi negative control Hi GC) (Invitrogen) using lipofectamine 2000 (Invitrogen). At 24 h post transfection, cells were infected with DENV-2 and harvested 48 h later. The cells were lysed in RIPA buffer and subjected to Western blot analysis [34] using primary antibodies against CTSB(Cell Signaling), cleaved caspase 3 (Cell signaling), DENV E [30, 31] or β-actin (Santa Cruz Biotechnology), respectively.

2.6. Statistical analysis

All data were obtained from three independent experiments and reported as the mean ± SEM. Statistical differences between the groups were tested with an unpaired *t*-test using StatView version 5.0 and *P* values less than 0.05 were considered statistically significant.

3. Results and discussion

3.1. DENV induced lysosomal membrane permeabilization in HepG2cells

Following DENV infection, apoptosis of hepatic cells was observed both *in vitro* and *in vivo* [6–17] However, the role of cathepsins in DENV-mediated apoptosis has not been investigated. Different stimuli have been shown to cause lysosomal membrane

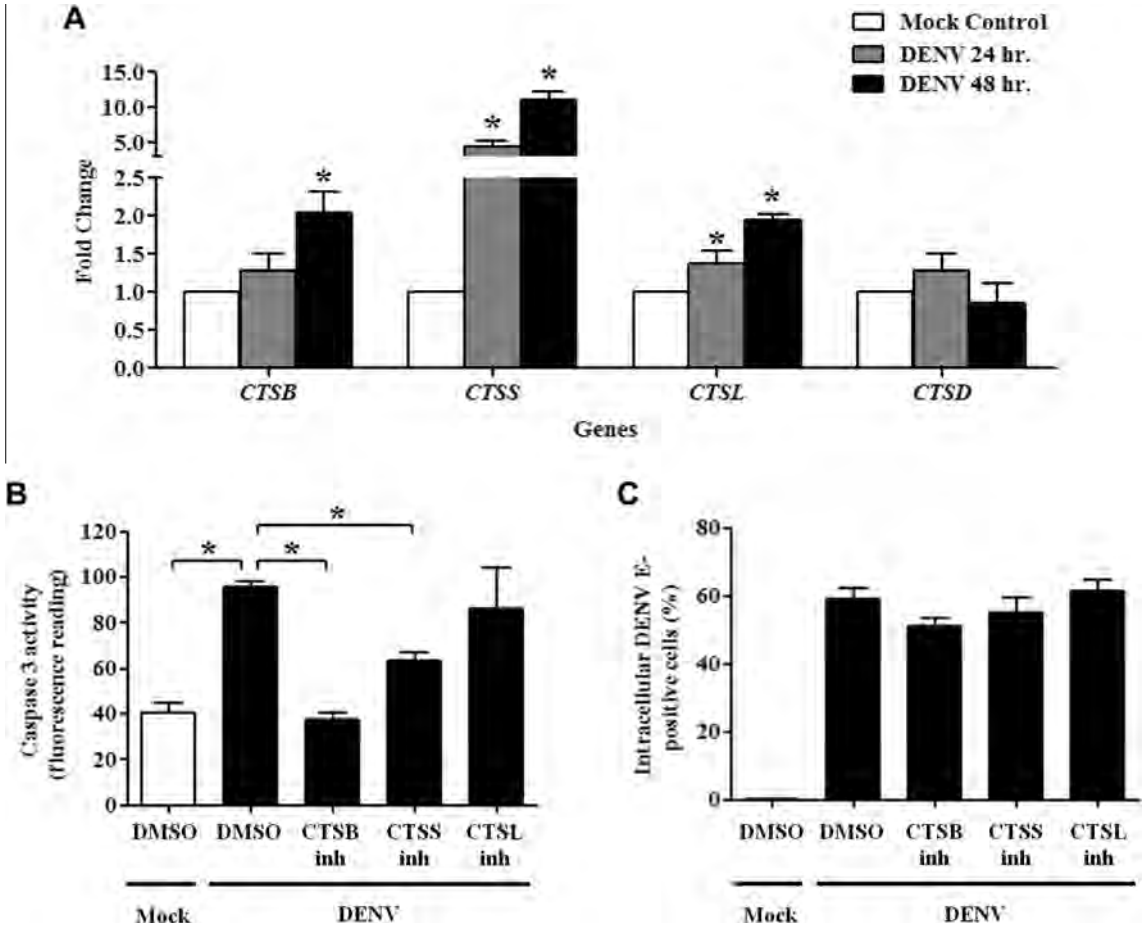

Fig. 2. DENV mediated up-regulation of cathepsin genes, which involved in apoptosis in HepG2 cells. HepG2 cells were infected with DENV-2 at a MOI of 5 for 24 and 48 h. *CTSB*, *CTSS*, *CTSL* and *CTSD* gene expression was determined by real time PCR using specific primers (A). To determine the role of cathepsins in mediating apoptosis, HepG2 cells were pre-treated with the specific inhibitors of CTSB (50 μM), CTSS (5 μM) and CTSL (10 μM) or an equal volume of DMSO vehicle control (0.2%) for 3 h before infection with DENV-2 at a MOI of 5. The cells were cultured in the presence of the inhibitors or DMSO for 48 h post infection before measurement of caspase 3 activity (B) and the percentage of infection (C). The results are expressed as the average of three independentexperiments ± SEM. The asterisks indicate statistically significant differences between groups ($p < 0.05$).

permeabilization and the released cytosolic cathepsin can mediate apoptosis [24–28]. We first asked whether DENV infection induced lysosomal membrane permeabilization. At 24 h post infection, 6% of the cell population exhibited an apoptotic phenotype and this increased to 10% at 36 h and 21% at 48 h (Fig. 1C). Theincrease in apoptotic cells was paralleled by an increase in the number of cells with reduced acridine orange red fluorescence. As show in Fig. 1A and B, reduced lysosomal fluorescence was evident in 3% of cells at 24 h, increasing to 14% and 53% at 36 and 48 h, respectively. The data suggested that DENV-induced apoptosis was accompanied by lysosomal membrane permeabilization.

DENV infection is known to induce ROS accumulation [36] and increased ROS has been shown to mediate lysosome destabilization [35]. Consequently, we next asked whether ROS contributed to lysosomal destabilization during DENV infection. HepG2 cells were infected with DENV in the presence or the absence of 5 mM N-acetyl cysteine (NAC), which confers antioxidant effect and isable to reduce free radicals. As expected, NAC partially reversed the effects of DENV-induced lysosomal destabilization (Fig. 1D) implying that DENV-induced ROS contributes to lysosomal membrane permeabilization. However, NAC reduced permeabilization by only 25%, implying that factors other than ROS make a significant contribution to this effect [37]. In other stimuli, death receptor engagements by their ligands, including TNF-α or TRAIL, result in activation of caspase 8 and lysosomal destabilization in mouse hepatocytes [27,38].

3.2. DENV-induced apoptosis in HepG2 cells is mediated by cathepsin B

Previous gene expression studies from our laboratory and others identified cathepsin B and Sas differentially expressed during DENV infection [13,29,39]. However, a role of cathepsins in DENV-mediated apoptosis has not been investigated. Cathepsin B, D, L and S were selected for further study as they were shown to contribute to apoptosis of hepatocytes [27, 40–42]. HepG2 cells were infected with DENV-2 for 24 h and 48 h and the change in cathepsin mRNAs were evaluated. The average percent of HepG2 cells infected with DENV-2 were 80 and 90, respectively (data not shown). Our results (Fig. 2A) showed that *CTSB*, *CTSL*, *CTSS* but not *CTSD* mRNA expression increased in DENV-infected cells. *CTSS* expressionincreased most dramatically, 5-fold by 24 h post infection and 10-fold by 48 h. *CTSB* and *CTSL* mRNA expression each increased 2-fold by 48 h post infection while *CTSD* was not increased at both time points. We then tested whether cathepsin B, L, and S were involved in DENV-mediated apoptosis. HepG2 cells were pre-treated with cathepsin B, L, and S inhibitors before infection with DENV-2 and incubating for 48 h. The results showed that caspase 3 activity was significantly decreased in DENV-infected cells treated with cathepsin B and S inhibitors but not with cathepsin L inhibitor (Fig. 2B). The decrease was not due to a reduction in DENV infection since similar levels of infection were seen between vehicle- and inhibitor-treated cells (Fig 2C).This data suggest that DENV-induced apoptosis was mediated by cathepsin B and S in HepG2 cells.

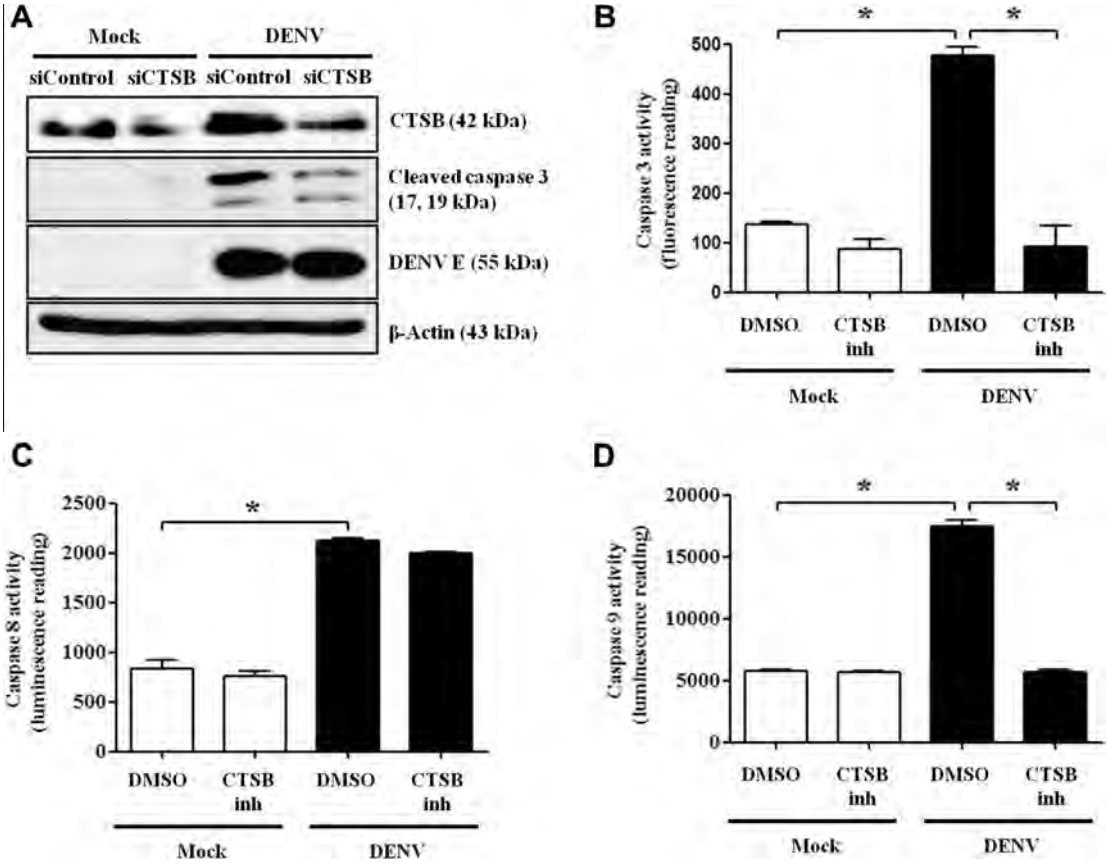

Fig. 3. Cathepsin B mediated caspase 9 and caspase 3 activation in DENV-infected HepG2 cells. HepG2 cells were transfected either with 200 nM of siRNA directed against *CTSB* or siControl Hi GC for 24 h before infection with DENV-2 at a MOI of 5 for 48 h. Cell lysates were subjected to Western blot analysis to determine the knock down efficiency and the effect to apoptosis using CTSB and cleaved caspase 3 antibodies, respectively (A). To determine the apoptosis pathway influenced by cathepsin B, HepG2 cells were pre-treated with the specific inhibitors of CTSB (50 μM) or equal volume of DMSO vehicle control (0.2%) for 3 h before infection with DENV-2 at a MOI of 5. The cells were cultured in the presence of the inhibitors or DMSO for 48 h post infection before measurement of caspase 3 activity (B), caspase 8 activity (C) and caspase 9activity (D). The results are expressed as the average of three independent experiments ± SEM. The asterisks indicate statistically significant differences between groups ($p < 0.05$).

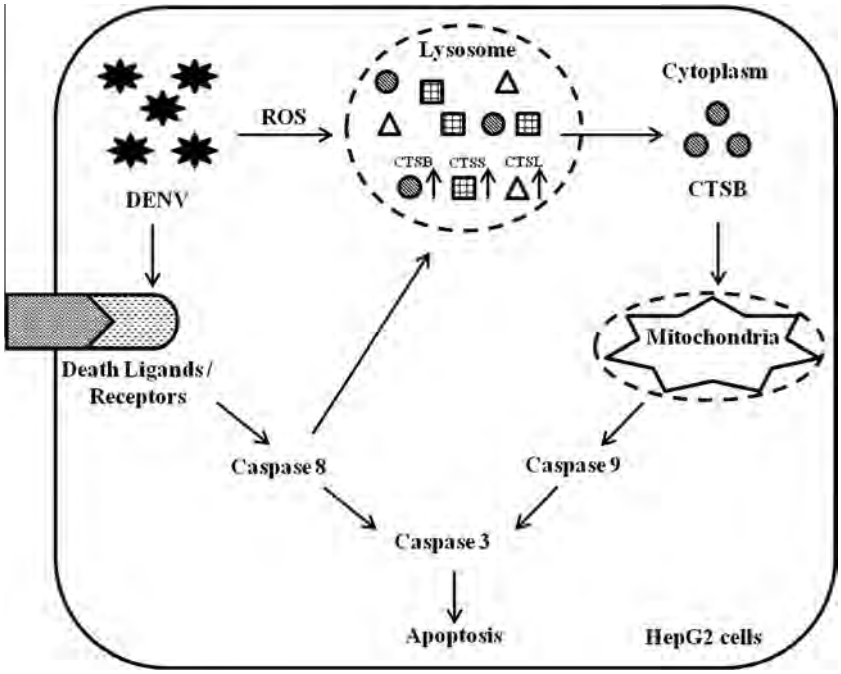

Fig. 4. The proposed model of the lysosomal pathway of apoptosis after DENV infection in HepG2 cells. DENV infection of HepG2 cells induces lysosomal membrane permeabilization, and up-regulation of cathepsin B, which turns on the intrinsic pathway of apoptosis by activation of caspase 9 and caspase 3. Lysosomal membrane permeabilization is partly mediated by ROS and possibly by caspase 8 through ligands/receptors signaling.

Since treatment with cathepsin B inhibitor strongly inhibited activation of caspase 3 activity, we next tested the specificity of this effect by RNAi knockdown of cathepsin B. HepG2 cells were transfected with siRNA directed against *CTSB* before infection with DENV. The efficiency of cathepsin B knockdown by siRNA and the effect on apoptosis were examined by Western blot analysis using primary antibodies against CTSB and cleaved caspase 3, respectively. CTSB protein expression was up-regulated in DENV-infected HepG2 cells (Fig. 3A, siControl, Mock vs. DENV) and siRNA against CTSB prevented this increase (Fig. 3A, DENV, siControl vs. siCTSB). In addition, treatment with siRNA directed against*CTSB* reduced the amount of cleaved caspase 3 in DENV-infected cells (Fig. 3A, DENV, siControl vs. siCTSB) suggesting a role for CTSB in DENV-mediated apoptosis. The result of siRNA directed against *CTSB* in DENV-infected HepG2 cells is in agreement with the result of pharmacological inhibition of CTSB (Fig. 3B). Therefore, genetic inhibition of CTSB expression reproduces pharmacological inhibition of the enzyme and confirms the contribution of CTSB to apoptotic event induced by DENV. Cathepsin B was previously shown to involve in cell death in herpes simplex virus-infected monocytic cells[33]. In addition, cathepsin B secreted from HIV-infected macrophages contributes to neuronal apoptosis [43]. Interestingly, inhibition ofcathepsin B was shown to protect glioma cells from pavovirus-induced cytolysis [44].

3.3. Cathepsin B mediates caspase 9 activation in DENV-infected HepG2 cells

The initiator caspase 8 of the death receptor pathway and the initiator caspase 9 of the mitochondrial pathway activate the effector caspase 3. Activated caspase 3 then cleaves cellular target proteins leading to cell death [45]. We next asked whether caspase 8 or caspase 9 pathways were activated by cathepsin B. HepG2 cells pre-treated with cathepsin B inhibitor were infected with DENV-2 and the activity of caspase 8 and 9 were measured using a luminescence-based assay. In the absence of cathepsin B inhibitor, the activities of both caspase 8 and caspase 9 were drastically increased in DENV-infected cells suggesting the involvement of both extrinsic and intrinsic pathways (Fig. 3C and D). However, treatment with cathepsin B inhibitor significantly prevented the increase of caspase 9, but not caspase 8 (Fig. 3C and D). These results suggested that caspase 8 is not the downstream of cathepsin B and that cathepsin B activation of caspase 3 occurs via caspase 9. The data are in agreement with *in vivo* studies demonstrating that cathepsin B inhibition suppresses LPS/D-GalN-induced caspase-9 and caspase-3 activation and prevents hepatic failure in mice [46]. Furthermore, caspase 8 was previously shown to be the upstream of cathepsin B, which subsequently induced downstream caspase 9 and caspase 3 in TNF-alpha-mediated hepatocyte apoptosis [27].

We propose a lysosomal pathway of apoptosis after DENV infection in HepG2 cells (Fig. 4). DENV induces lysosomal membrane permeabilization partly via ROS thereby releasing cathepsins to induce apoptosis and cathepsin B contributes to DENV-mediated apoptosis in HepG2 cells via caspase-9 and caspase-3 activation. The molecular mechanisms by which cathepsin B mediates apoptosis merit further investigation.

Acknowledgments

This work was supported by Mahidol University Grant to TL. We would like to thank Thailand Research Fund (TRF)-Royal Golden Jubilee Ph.D. program forsupporting to AM and UY. Currently, TL and SN are TRF Research Scholars and PY is a TRF Senior Research Scholar. We appreciate the kind assistance from Professor William A. Fonzi, Georgetown University, USA, for the critical reading and editing of this manuscript.

References

[1] S.B. Halstead, Dengue, Lancet 370 (2007) 1644–1652.
[2] C.H. Kuo, D.I. Tai, C.S. Chang-Chien, C.K. Lan, S.S. Chiou, Y.F. Liaw, Liver biochemical tests and dengue fever, Am. J. Trop. Med.Hyg. 47 (1992) 265–270.

[3] L.J. Souza, J.G. Alves, R.M. Nogueira, C. Gicovate Neto, D.A. Bastos, E.W. Siqueira, J.T. Souto Filho, A. Cezario Tde, C.E. Soares, C. CarneiroRda, Aminotransferase changes and acute hepatitis in patients with dengue fever: analysis of 1585 cases, Braz. J. Infect. Dis. 8 (2004) 156–163.
[4] S.B. Halstead, Pathogenesis of dengue: challenges to molecular biology, Science 239 (1988) 476–481.
[5] N. Bhamarapravati, Hemostatic defects in dengue hemorrhagic fever, Rev. Infect. Dis. 11 (Suppl. 4) (1989) S826–829.
[6] M.R. Huerre, N.T. Lan, P. Marianneau, N.B. Hue, H. Khun, N.T. Hung, N.T. Khen, M.T. Drouet, V.T. Huong, D.Q. Ha, Y. Buisson, V. Deubel, Liverhistopathology and biological correlates in five cases of fatal dengue fever in Vietnamese children, Virchows Arch. 438 (2001) 107–115.
[7] D. Limonta, V. Capo, G. Torres, A.B. Perez, M.G. Guzman, Apoptosis in tissues from fatal dengue shock syndrome, J. Clin. Virol. 40 (2007) 50–54.
[8] P. Marianneau, A.M. Steffan, C. Royer, M.T. Drouet, A. Kirn, V. Deubel, Differing infection patterns of dengue and yellow fever viruses in a human hepatoma cell line, J. Infect.Dis. 178 (1998) 1270–1278.
[9] A. Couvelard, P. Marianneau, C. Bedel, M.T. Drouet, F. Vachon, D. Henin, V. Deubel, Report of a fatal case of dengue infection with hepatitis: demonstration of dengue antigens in hepatocytes and liver apoptosis, Hum.Pathol. 30 (1999) 1106–1110.
[10] T. Thongtan, S. Panyim, D.R. Smith, Apoptosis in dengue virus infected liver cell lines HepG2 and Hep3B, J. Med. Virol. 72 (2004) 436–444.
[11] T. Limjindaporn, J. Netsawang, S. Noisakran, S. Thiemmeca, W. Wongwiwat, S. Sudsaward, P. Avirutnan, C. Puttikhunt, W. Kasinrerk, R. Sriburi, N. Sittisombut, P.T. Yenchitsomanus, P. Malasit, Sensitizationto Fas-mediated apoptosis by dengue virus capsid protein, Biochem. Biophys. Res. Commun. 362 (2007) 334–339.
[12] J. Netsawang, S. Noisakran, C. Puttikhunt, W. Kasinrerk, W. Wongwiwat, P. Malasit, P.T. Yenchitsomanus, T. Limjindaporn, Nuclear localization of dengue virus capsid protein is required for DAXX interaction andapoptosis, Virus Res. 147 (2010) 275–283.
[13] A. Morchang, U. Yasamut, J. Netsawang, S. Noisakran, W. Wongwiwat, P. Songprakhon, C. Srisawat, C. Puttikhunt, W. Kasinrerk, P. Malasit, P.T. Yenchitsomanus, T. Limjindaporn, Celldeath gene expression profile: role of RIPK2 in dengue virus-mediated apoptosis, Virus Res. 156 (2011) 25–34.
[14] A. Nagila, J. Netsawang, C. Srisawat, S. Noisakran, A. Morchang, U. Yasamut, C. Puttikhunt, W. Kasinrerk, P. Malasit, P.T. Yenchitsomanus, T. Limjindaporn, Roleof CD137 signaling in dengue virus-mediated apoptosis, Biochem. Biophys. Res. Commun. 410 (2011) 428–433.
[15] A. Nagila, J. Netsawang, A. Suttitheptumrong, A. Morchang, S. Khunchai, C. Srisawat, C. Puttikhunt, S. Noisakran, P.T. Yenchitsomanus, T. Limjindaporn, Inhibition of p38MAPKand CD137 signaling reduce dengue virus-induced TNF-alpha secretion and apoptosis, Virol. J. 10 (2013) 105.
[16] T. El-Bacha, V. Midlej, A.P. Pereira da Silva, L. Silva da Costa, M. Benchimol, A. Galina, A.T. Da Poian, Mitochondrial and bioenergetic dysfunction in human hepatic cells infected with dengue 2 virus, Biochim. Biophys.Acta 1772 (2007) 1158–1166.
[17] C.F. Lin, S.W. Wan, M.C. Chen, S.C. Lin, C.C. Cheng, S.C. Chiu, Y.L. Hsiao, H.Y. Lei, H.S. Liu, T.M. Yeh, Y.S. Lin, Liverinjury caused by antibodies against dengue virus nonstructural protein 1 in a murine model, Lab. Invest. 88 (2008) 1079–1089.
[18] T. Matsuda, A. Almasan, M. Tomita, K. Tamaki, M. Saito, M. Tadano, H. Yagita, T. Ohta, N. Mori, Dengue virus-induced apoptosis in hepatic cells is partly mediated by Apo2ligand/tumour necrosis factor-related apoptosis-inducing ligand, J. Gen. Virol. 86 (2005) 1055–1065.
[19] A.M. Nasirudeen, D.X. Liu, Gene expression profiling by microarray analysis reveals an important role for caspase-1 in dengue virus-induced p53-mediated apoptosis, J. Med. Virol. 81 (2009) 1069–1081.
[20] A.M. Nasirudeen, L. Wang, D.X. Liu, Induction of p53-dependent and mitochondria-mediated cell death pathway by dengue virus infection of human and animal cells, Microbes Infect. 10 (2008) 1124–1132.
[21] J.M. Sung, C.K. Lee, B.A. Wu-Hsieh, Intrahepatic infiltrating NK and CD8 T cells cause liver cell death in different phases of dengue virus infection, PLoS ONE 7 (2012) e46292.
[22] C. de Duve, Lysosomes revisited, Eur. J. Biochem. 137 (1983) 391–397.
[23] U. Repnik, V. Stoka, V. Turk, B. Turk, Lysosomes and lysosomal cathepsins in cell death, Biochim. Biophys. Acta 2012 (1824) 22–33.
[24] R. Goldman, A. Kaplan, Rupture of rat liver lysosomes mediated by L-amino acid esters, Biochim. Biophys. Acta 318 (1973) 205–216.
[25] D.L. Thiele, P.E. Lipsky, Regulation of cellular function by products of lysosomal enzyme activity: elimination of human natural killer cells by a dipeptide methyl ester generated from L-leucine methyl ester by monocytes or polymorphonuclear leukocytes, Proc. Natl. Acad. Sci. USA 82 (1985) 2468–2472.
[26] L.P. Deiss, H. Galinka, H. Berissi, O. Cohen, A. Kimchi, Cathepsin D protease mediates programmed cell death induced by interferon-gamma, Fas/APO-1 and TNF-alpha, EMBOJ. 15 (1996) 3861–3870.
[27] M.E. Guicciardi, J. Deussing, H. Miyoshi, S.F. Bronk, P.A. Svingen, C. Peters, S.H. Kaufmann, G.J. Gores, Cathepsin B contributes to TNF-alpha-mediated hepatocyte apoptosis by promoting mitochondrial release of cytochromec, J. Clin. Invest. 106 (2000) 1127–1137.
[28] V. Stoka, B. Turk, S.L. Schendel, T.H. Kim, T. Cirman, S.J. Snipas, L.M. Ellerby, D. Bredesen, H. Freeze, M. Abrahamson, D. Bromme, S. Krajewski, J.C. Reed, X.M. Yin, V. Turk, G.S. Salvesen, Lysosomalprotease pathways to apoptosis, J. Biol. Chem. 276 (2001) 3149–3157.
[29] S. Ubol, P. Masrinoul, J. Chaijaruwanich, S. Kalayanarooj, T. Charoensirisuthikul, J. Kasisith, Differences in global gene expression in peripheral blood mononuclear cells indicate a significant role of the innate responses in progression of dengue fever but not dengue hemorrhagic fever, J. Infect.Dis. 197 (2008) 1459–1467.
[30] M.K. Gentry, E.A. Henchal, J.M. McCown, W.E. Brandt, J.M. Dalrymple, Identification of distinct antigenic determinants on dengue-2 virus using monoclonal antibodies, Am. J. Trop. Med.Hyg. 31 (1982) 548–555.
[31] E.A. Henchal, J.M. McCown, D.S. Burke, M.C. Seguin, W.E. Brandt, Epitopic analysis of antigenic determinants on the surface of dengue-2 virions using monoclonal antibodies, Am. J. Trop. Med.Hyg. 34 (1985) 162–169.
[32] J. Nylandsted, M. Gyrd-Hansen, A. Danielewicz, N. Fehrenbacher, U. Lademann, M. Hoyer-Hansen, E. Weber, G. Multhoff, M. Rohde, M. Jaattela, Heat shock protein 70 promotescell survival by inhibiting lysosomal membrane permeabilization, J. Exp. Med. 200 (2004) 425–435.
[33] P. Peri, K. Nuutila, T. Vuorinen, P. Saukko, V. Hukkanen, Cathepsins are involved in virus-induced cell death in ICP4 and Us3 deletion mutant herpes simplex virus type 1-infected monocytic cells, J. Gen.Virol. 92 (2011) 173–180.
[34] H. Towbin, T. Staehelin, J. Gordon, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications, Proc. Natl. Acad. Sci. USA 76 (1979) 4350–4354.
[35] A. Terman, T. Kurz, B. Gustafsson, U.T. Brunk, Lysosomal labilization, IUBMB Life 58 (2006) 531–539.
[36] Y.T. Yen, H.C. Chen, Y.D. Lin, C.C. Shieh, B.A. Wu-Hsieh, Enhancement by tumor necrosis factor alpha of dengue virus-induced endothelial cell production of reactive nitrogen and oxygen species is key to hemorrhage development, J.Virol. 82 (2008) 12312–12324.
[37] P. Boya, G. Kroemer, Lysosomal membrane permeabilization in cell death, Oncogene 27 (2008) 6434–6451.
[38] N.W. Werneburg, M.E. Guicciardi, S.F. Bronk, S.H. Kaufmann, G.J. Gores, Tumor necrosis factor-related apoptosis-inducing ligand activates a lysosomal pathway of apoptosis that is regulated by Bcl-2 proteins, J. Biol.Chem. 282 (2007) 28960–28970.
[39] S. Sim, J.L. Ramirez, G. Dimopoulos, Dengue virus infection of the *Aedes aegypti* salivary gland and chemosensory apparatus induces genes that modulate infection and blood-feeding behavior, PLoS Pathog. 8 (2012) e1002631.
[40] G.S. Wu, P. Saftig, C. Peters, W.S. El-Deiry, Potential role for cathepsin D in p53-dependent tumor suppression and chemosensitivity, Oncogene 16 (1998) 2177–2183.
[41] R. Ishisaka, T. Utsumi, T. Kanno, K. Arita, N. Katunuma, J. Akiyama, K. Utsumi, Participation of a cathepsin L-type protease in the activation of caspase-3, Cell Struct.Funct. 24 (1999) 465–470.
[42] T. Zheng, M.J. Kang, K. Crothers, Z. Zhu, W. Liu, C.G. Lee, L.A. Rabach, H.A. Chapman, R.J. Homer, D. Aldous, G.T. DeSanctis, S. Underwood, M. Graupe, R.A. Flavell, J.A. Schmidt, J.A. Elias, Role of cathepsin S-dependent epithelial cell apoptosis in IFN-gamma-induced alveolar remodeling and pulmonary emphysema, J. Immunol. 174 (2005) 8106–8115.
[43] E.J. Rodriguez-Franco, Y.M. Cantres-Rosario, M. Plaud-Valentin, R. Romeu, Y. Rodriguez, R. Skolasky, V. Melendez, C.L. Cadilla, L.M. Melendez, Dysregulation ofmacrophage-secreted cathepsin B contributes to HIV-1-linked neuronal apoptosis, PLoS ONE 7 (2012) e36571.
[44] M. Di Piazza, C. Mader, K. Geletneky, Y.C.M. Herrero, E. Weber, J. Schlehofer, L. Deleu, J. Rommelaere, Cytosolic activation of cathepsins mediates parvovirus H-1-induced killing of cisplatin and TRAIL-resistant gliomacells, J. Virol. 81 (2007) 4186–4198.
[45] M.O. Hengartner, The biochemistry of apoptosis, Nature 407 (2000) 770–776.
[46] B.Z. Yan, L.Y. Chen, L. Kang, X.R. Wang, M.R. Bi, W. Wang, B.S. Yang, Hepatoprotective effects of cathepsin B inhibitor on acute hepatic failure induced by lipopolysaccharide/D-galactosamine inmice, Hepatobiliary Pancreat. Dis. Int. 12 (2013) 80–86.