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Anti–Dengue Virus Nonstructural Protein 1 Antibodies Cause NO-Mediated Endothelial Cell Apoptosis via Ceramide-Regulated Glycogen Synthase Kinase-3β and NF-κB Activation

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Immunopathogenetic mechanisms of dengue virus (DENV) infection are involved in hemorrhagic syndrome resulting from thrombocytopenia, coagulopathy, and vasculopathy. We have proposed a mechanism of molecular mimicry in which Abs against DENV nonstructural protein 1 (NS1) cross-react with human endothelial cells and cause NF-κB-regulated immune activation and NO-mediated apoptosis. However, the signaling pathway leading to NF-κB activation after the binding of anti-DENV NS1 Abs to endothelial cells is unresolved. In this study, we found that anti-DENV NS1 Abs caused the formation of lipid rafts, and that disrupting lipid raft formation by methyl-β-cyclodextrin decreased NO production and apoptosis. Treatment with anti-DENV NS1 Abs elevated ceramide generation in lipid rafts. Pharmacological inhibition of acid sphingomyelinase (aSMase) decreased anti-DENV NS1 Ab-mediated ceramide and NO production, as well as apoptosis. Exogenous ceramide treatment induced biogenesis of inducible NO synthase (iNOS)/NO and apoptosis through an NF-κB–regulated manner. Furthermore, activation of glycogen synthase kinase-3β (GSK-3β) was required for ceramide-induced NF-κB activation and iNOS expression. Notably, anti-DENV NS1 Abs caused GSK-3β–mediated NF-κB activation and iNOS expression, which were regulated by aSMapse. Moreover, pharmacological inhibition of GSK-3β reduced hepatic endothelial cell apoptosis in mice passively administered anti-DENV NS1 Abs. These results suggest that anti-DENV NS1 Abs bind to the endothelial cell membrane and cause NO production and apoptosis via a mechanism involving the aSMapse/ceramide/GSK-3β/NF-κB/iNOS/NO signaling pathway.

fever patients (20, 22, 23). For AECAs in dengue autoimmunity, DENV nonstructural protein 1 (NS1) Abs in patient sera account, at least in part, for the endothelial cell cross-reactivity and apoptosis induction (22). In vitro studies demonstrated that anti-DENV NS1 Abs induce endothelial cells to undergo apoptosis (26) and also regulate inflammatory activation (27). Moreover, in vivo studies revealed pathogenic effects of anti-DENV NS1 Abs on hepatitis through endothelial cell injury (28). These findings suggest that anti-DENV NS1 Abs may act as AECAs, which promote DENV-induced autoimmune responses (4). In conclusion, molecular mimicry-based autoimmune mechanisms initiated by the immunogenicity of DENV NS1 protein are likely involved in dengue disease pathogenesis (29, 30).

Anti-DENV NS1 Abs cause inducible NO synthase (iNOS) expression and NO production in endothelial cells, which lead to NO-mediated cell apoptosis (4, 26). Anti-DENV NS1 Abs also induce NF-κB-regulated inflammatory activation, including cytokine production and adhesion molecule expression (27). However, the upstream signaling leading to these effects after anti-DENV NS1 binding to the cell surface is not well characterized. In this study, we aimed to elucidate the relevant signaling pathway(s) in particular, the role of lipid rafts and ceramide. The formation of lipid rafts, the membrane microdomains that are enriched in cholesterol and glycosphingolipids, play an important role in cellular signaling in response to various stimuli (31–35). Ceramide, which is enriched in lipid rafts, acts as a second messenger of multiple extracellular stimuli in cell survival, inflammation, and apoptotic cell death (36–41). We investigated the formation of raft structure and the generation of cellular ceramide after binding of anti-DENV NS1 Abs to endothelial cells. We determined critical roles for lipid raft formation and ceramide generation in anti-DENV NS1 Ab-induced glycosylin synthase kinase-3β (GSK-3β)–regulated NF-κB activation followed by NF-κB–regulated iNOS/NO biosynthesis in endothelial cells.

Materials and Methods
Cell cultures and reagents

The human microvascular endothelial cell line 1 (HMEC-1) was obtained from the Centers for Disease Control and Prevention (42) and passaged in culture plates containing endothelial cell growth medium M200 (Cascade Biologies, Portland, OR) supplemented with 2% FBS, 1 μg/ml hydrocortisone, 10 ng/ml epidermal growth factor, 3 ng/ml basic fibroblast growth factor, 10 μg/ml heparin, and antibiotics. C2-ceramide (BioMol, Plymouth Meeting, PA), pyrroloidine dithiocarbamate (PDTC), BAY 11-7085 (Sigma-Aldrich, St. Louis, MO), and SB415286 (TOCRIS, Ellisville, MO) were dissolved in DMSO. Lithium chloride (LiCl), methyl-β-cyclodextrin (MβCD), and chlorpromazine hydrochloride (CHL) (Sigma-Aldrich) were dissolved in serum-free medium.

Mice

C3H/HeN breeder mice were obtained from Charles River Breeding Laboratories (Kanagawa, Japan) and maintained on standard laboratory food and water in the Laboratory Animal Center of National Cheng Kung University Medical College. Their male 8-wk-old progeny were used for the experiments. The animal use protocol was reviewed and approved by the Institutional Animal Care and Use Committee. An in vivo experiment for examining the pathogenic effects was performed according to previous studies (28). Mice were injected i.v. with control IgG (500 μg), anti-DENV NS1 (500 μg), control IgG plus LiCl (20 mg/kg), or anti-DENV NS1 plus LiCl for 48 h.

Preparation of recombinant DENV NS1 and anti-DENV NS1 Abs

The recombinant protein expression and purification followed the procedures described previously for DENV2 NS1 preparation (26). For Ab generation, BALB/c mice were i.p. immunized with PBS or 25 μg purified DENV NS1 Ab–induced NO production and apoptosis. (A) Confocal microscopic detection of FITC-labeled anti-DENV NS1 Abs (green) on HMEC-1 cells at both 4˚C and 37˚C, and clustering with Texas Red–labeled cavinolin-1 (red) in raflike structure (yellow, see Merge) at 37˚C, but not 4˚C. Scale bar is shown. (B) Fluorescence tracings from (A) were analyzed for the colocalization coefficients of anti-DENV NS1 and caveolin-1 by green and red fluorescence intensity using Image-Pro Plus version 6.0 software. (C) In the presence or absence of 2 mM lipid raft inhibitor MβCD, HMEC-1 cells were treated with 10 μg control IgG, anti-DENV NS1, or anti-pan cadherin at 37˚C for 1 h. The formation of lipid raft structure in cell membranes was determined by affinity dye staining using FITC-conjugated cholera toxin B, and the cross-binding of control IgG, anti-DENV NS1, and anti-pan cadherin was stained using Texas Red–conjugated anti-mouse and anti-rabbit Abs, followed by confocal microscopic analysis. Scale bar is shown. (D) The fluorescence tracing from (C) was analyzed for the colocalization coefficients of anti-DENV NS1 Abs and cholera toxin B by red and green fluorescence intensity using Image-Pro Plus version 6.0 software. (E) In the presence or absence of 2 mM MβCD, the generation of NO by 10 μg anti-DENV NS1 stimulation in HMEC-1 cells was determined by flow cytometry. The time-kinetic responses of NO production by anti-DENV NS1 are shown as the means ± SD of triplicate cultures. (F) TUNEL assay was performed to detect anti-DENV NS1 Ab–induced cell apoptosis followed by cytometric analysis. The percentages of apoptotic cells are shown as the means ± SD of triplicate cultures. *p < 0.05 as compared with the groups without MβCD treatment.
recombinant DENV NS1 protein emulsified in CFA (Sigma-Aldrich) followed by four times in IFA. The IgG fractions from both PBS-immunized control and NS1-immunized mouse sera were purified using a protein G-Sepharose affinity chromatography column (Amersham Pharmacia Biotech, Piscataway, NJ). All procedures for control IgG and anti-DENV NS1 purification were the same and performed simultaneously. The preparations were subjected to testing for endotoxin contamination using a Limulus amebocyte lysate assay (Pyrotell, Associates of Cape Cod, Falmouth, MA); the endotoxin concentrations of anti-DENV NS1 and control IgG were both <0.03 endotoxin unit/ml.

**Immunostaining**

Mouse anti-DENV NS1 or control IgG were incubated with cells at 4°C or 37°C for 1 h. Cells were washed briefly in PBS, fixed with 1% formaldehyde in PBS at room temperature for 10 min, and washed again with PBS followed by FITC-conjugated or Alexa 594–conjugated goat anti-mouse IgG (Invitrogen, Camarillo, CA) and Alexa 594–conjugated cholera toxin subunit B (Invitrogen) was used for confocal microscopy analysis (Leica TCS SPII, Nussloch, Germany; Olympus FV-1000, Tokyo, Japan). For the detection of acid sphingomyelinase (aSMase) and ceramide generation, fixed or nonfixed HMEC-1 cells were incubated respectively with specific Abs against aSMase (Santa Cruz Biotechnology, Santa Cruz, CA), FITC-conjugated cholera toxin subunit B, and Alexa 594–conjugated cholera toxin subunit B (Invitrogen) were used followed by confocal microscopy analysis (Leica TCS SPII, Nussloch, Germany; Olympus FV-1000, Tokyo, Japan). For the detection of acid sphingomyelinase (aSMase) and ceramide generation, fixed or nonfixed HMEC-1 cells were incubated respectively with specific Abs against aSMase (Santa Cruz Biotechnology) and ceramide (GlycoTech Productions and Handels, Kükels, Germany) at 4°C or 37°C for 1 h followed by FITC-conjugated goat anti-rabbit IgG or anti-mouse IgM (Calbiochem, San Diego, CA) staining, and analyzed by confocal microscopy or flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA). Polyclonal rabbit anti-pan cadherin IgG (Abcam, Cambridge, MA) was used for plasma membrane binding analysis, followed by Alexa 594–conjugated anti-rabbit Ab staining. For the detection of ceramide generation, cells were analyzed using flow cytometry with excitation set at 488 nm. The emission was detected with the FL-1 channel followed by CellQuest Pro 4.0.2 software (BD Biosciences) analysis, and quantification was done using WinMDI 2.8 software (The Scripps Research Institute, La Jolla, CA). The ceramide-positive cells were gated and compared with untreated (mock) cells. For the measurement of NF-κB nuclear translocation, rabbit anti–NF-κB p65 (Cell Signaling Technology, Beverly, MA) was used for FITC-conjugated goat anti-rabbit IgG staining and analyzed by fluorescence microscopy. DAPI (Calbiochem) was used for nuclear staining. All immunostaining studies were performed in at least two independent experiments. ImageJ software (National Institutes of Health, Bethesda, MD) and Image-Pro Plus version 6.0 software (Media Cybernetics, Bethesda, MD) were used for image quantification analysis.

**NO assay**

The production of NO was detected using the ApoAlert NO/Annexin V Dual Sensor Kit (Clontech, Palo Alto, CA) according to the manufacturer’s instructions. In brief, cells were incubated with 5 μM NO Sensor Dye for 30 min and then treated with anti-DENV NS1 or ceramide for indicated periods of time. Cells were washed and resuspended in PBS, then analyzed using flow cytometry with excitation set at 488 nm. The emission was detected with the FL-1 channel. Samples were analyzed using CellQuest Pro 4.0.2 software (BD Biosciences), and quantification was done using WinMDI 2.8 software (The Scripps Research Institute). The NO+ cells were gated and compared with untreated (mock) cells. The NO production was also detected using Griess reagent. In brief, 80 μl of the culture supernatant was reacted with 20 μl enzyme cofactor and nitrate reductase.

**FIGURE 2.** Anti-DENV NS1 Ab treatment induces ceramide generation. (A) HMEC-1 cells were treated with 10 μg control IgG or anti-DENV NS1 at 37°C for 1 h. After treatment, the colocalization of lipid rafts and ceramide generation in nonfixed HMEC-1 cells was determined using Alexa 594–conjugated cholera toxin (Santa Cruz Biotechnology, Santa Cruz, CA), FITC-conjugated cholera toxin subunit B, and Alexa 594–conjugated cholera toxin subunit B (Invitrogen) were used followed by confocal microscopy analysis (Leica TCS SPII, Nussloch, Germany; Olympus FV-1000, Tokyo, Japan). For the detection of acid sphingomyelinase (aSMase) and ceramide generation, fixed or nonfixed HMEC-1 cells were incubated respectively with specific Abs against aSMase (Santa Cruz Biotechnology) and ceramide (GlycoTech Productions and Handels, Kükels, Germany) at 4°C or 37°C for 1 h followed by FITC-conjugated goat anti-rabbit IgG or anti-mouse IgM (Calbiochem, San Diego, CA) staining, and analyzed by confocal microscopy or flow cytometry (FACSCalibur; BD Biosciences, San Jose, CA). Polyclonal rabbit anti-pan cadherin IgG (Abcam, Cambridge, MA) was used for plasma membrane binding analysis, followed by Alexa 594–conjugated anti-rabbit Ab staining. For the detection of ceramide generation, cells were analyzed using flow cytometry with excitation set at 488 nm. The emission was detected with the FL-1 channel followed by CellQuest Pro 4.0.2 software (BD Biosciences) analysis, and quantification was done using WinMDI 2.8 software (The Scripps Research Institute, La Jolla, CA). The ceramide-positive cells were gated and compared with untreated (mock) cells. For the measurement of NF-κB nuclear translocation, rabbit anti–NF-κB p65 (Cell Signaling Technology, Beverly, MA) was used for FITC-conjugated goat anti-rabbit IgG staining and analyzed by fluorescence microscopy. DAPI (Calbiochem) was used for nuclear staining. All immunostaining studies were performed in at least two independent experiments. ImageJ software (National Institutes of Health, Bethesda, MD) and Image-Pro Plus version 6.0 software (Media Cybernetics, Bethesda, MD) were used for image quantification analysis.

**FIGURE 3.** Accumulation of aSMase in anti-DENV NS1 Ab–induced ceramide generation, NO production, and apoptosis. (A) HMEC-1 cells were treated with 10 μg control IgG or anti-DENV NS1 Ab at 37°C for 1 h. The distribution of aSMase was determined by immunostaining using specific Abs and analyzed by confocal microscopy. (B) In the presence or absence of 1 μM of aSMase inhibitor CHL, the generation of ceramide was measured using specific Ab staining followed by flow cytometric analysis. Data are shown as the means ± SD of triplicate cultures. *p < 0.05, **p < 0.01. (C) In the presence or absence of 1 μM CHL, the time-kinetic responses of NO production after 10 μg anti-DENV NS1 Ab treatment were detected by flow cytometry. (D) HMEC-1 cell apoptosis was determined by TUNEL assay followed by cytometric analysis. Data are shown as the means ± SD of triplicate cultures. *p < 0.05 as compared with the groups without CHL treatment.
mixture (Cayman Chemical Company, Ann Arbor, MI) for 30 min at room temperature followed by adding 100 μl Griess reagent (1% sulfanilamide, 0.1% naphthylethylenediamine dihydrochloride, and 2.5% H₃PO₄) and incubation for 10 min at room temperature. The relative concentration of nitrite was measured using a microplate reader (Emax microplate reader; Molecular Devices) at 540 nm.

Cell apoptosis analysis

Cells were fixed with 70% ethanol in PBS for TUNEL reaction using the ApoAlert DNA Fragmentation Assay Kit (Clontech) according to the manufacturer’s instructions, or for propidium iodide (PI, Sigma-Aldrich) staining, and then analyzed by flow cytometry with excitation set at 488 nm. The emission was detected with the FL-1 channel for TUNEL assay or the FL-2 channel for PI staining. Samples were analyzed using CellQuest Pro 4.0.2 software (BD Biosciences), and quantification was done using WinMDI 2.8 software (The Scripps Research Institute). The apoptotic cells were gated and compared with untreated (mock) cells. To detect apoptotic cells in formalin-fixed sections of liver tissues, we performed TUNEL staining (Clontech) according to the manufacturer’s instructions. Apoptotic cells were analyzed by fluorescence microscopy. DAPI (Calbiochem) was used for nuclear staining. To measure cell viability, we used the WST-8 cell proliferation assay kit (Cayman Chemical Company) according to the manufacturer’s instructions.

Immunoblotting analysis

Total cell lysates were extracted using a Triton X-100–based lysis buffer containing 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 10 mM Tris (pH 7.5), 5 mM NaF, 10 mM sodium pyrophosphate, protease inhibitor mixture I, and protein phosphatase inhibitor mixture I (Sigma-Aldrich). Proteins were resolved using SDS-PAGE and then transferred to a polyvinylidene fluoride membrane (Millipore Corporation, Billerica, MA). After blocking, blots were developed with a series of Abs as indicated. Abs specific for GSK-3β, phospho-GSK-3β (S9), iNOS, and NF-κB were purchased from Cell Signaling Technology. Mouse Abs specific for proliferating cell nuclear Ag (PCNA; ZYMED) and GAPDH (Santa Cruz Biotechnology) were used for internal controls. Finally, blots were hybridized with HRP-conjugated goat anti-rabbit IgG or anti-mouse IgG (Cell Signaling Technology) and developed using an ECL Western blot detection kit (Millipore Corporation) according to the manufacturer’s instructions. All immunoblotting studies were performed in at least two independent experiments. The band intensity was measured using ImageJ software.

Statistical analysis

Between-group comparisons were performed using Student t test with SigmaPlot version 8.0 and GraphPad Prism version 5.0. The p values < 0.05 were considered significant.

Results

Lipid raft formation is involved in anti-DENV NS1 Ab-induced NO production and apoptosis

We previously showed that anti-DENV NS1 Abs induced endothelial cell apoptosis, which was mediated through the production of NO, upregulation of p53 and Bax, downregulation of Bcl-2 and...
Bcl-xL, cytochrome c release, and caspase-3 activation (4, 26, 29). However, the upstream signaling pathways of anti-DENV NS1 Ab–induced endothelial cell apoptosis are still unclear. Because anti-DENV NS1 Ab–induced apoptosis was initiated after Ab binding to cells, the involvement of biomembrane changes through lipid raft formation was first investigated. By immunostaining and confocal microscopic observation, the formation of membrane raft-like structures by anti-DENV NS1 clustering with endothelial surface molecules was shown by colocalization with caveolin-1 protein (Fig. 1A). The colocalization coefficients of anti-DENV NS1 Abs and caveolin-1 were analyzed (Fig. 1B) and quantified (Supplemental Fig. 1A, 1B). To confirm the formation of raft-like structures after anti-DENV NS1 stimulation of endothelial cells, we performed cell staining with cholera toxin B, a high-affinity protein for lipid rafts. Results showed that binding of anti-DENV NS1 Abs to HMEC-1 cells could induce lipid raft formation, which was inhibited by pretreatment with the lipid raft–specific inhibitor MβCD (Fig. 1C). The colocalization coefficients of anti-DENV NS1 Abs and cholera toxin B were also analyzed (Fig. 1D) and quantified (Supplemental Fig. 1C, 1D). The binding of anti-pan cadherin Abs to HMEC-1 could not induce raft formation, which indicated that anti-DENV NS1 specifically regulated lipid raft formation.

In our previous studies, we found that anti-DENV NS1 Abs induced NO-mediated endothelial cell apoptosis (26). To further study the effects of raft formation on anti-DENV NS1 Ab–induced endothelial cell injury, we measured the NO production in the presence of MβCD. Pretreatment with MβCD inhibited anti-DENV NS1 Ab–induced NO production (Fig. 1E) and cell apoptosis (Fig. 1F). These results suggest the involvement of lipid rafts in anti-DENV NS1 Ab–induced cell cytotoxicity.

**Anti-DENV NS1 treatment induces ceramide generation**

Lipid raft formation has been observed in response to multiple stimuli (31–33, 43–45). Generally, lipid rafts play an important role in the activation of signal transduction after extracellular stimulation. Moreover, the generation of ceramide, an intracellular lipid with signaling functions, has been demonstrated in lipid rafts (43, 46, 47). To further investigate the involvement of lipid raft formation in anti-DENV NS1 signaling, the accumulation of ceramide was investigated. Using confocal microscopic analysis, we found ceramide-rich raft formation after anti-DENV NS1 treatment in nonfixed HMEC-1 cells (Fig. 2A). The colocalization coefficients of ceramide and cholera toxin B were also analyzed (Fig. 2B) and quantified (Supplemental Fig. 2). Anti-DENV NS1 Ab–induced ceramide generation was further demonstrated using flow cytometric analysis as compared with control IgG treatment (Fig. 2C).

**aSMase regulates anti-DENV NS1 Ab–induced ceramide generation, NO production, and apoptosis**

Ceramide can be generated from sphingomyelin hydrolysis by aSMase (41, 43, 46, 47). Interestingly, after anti-DENV NS1 stimulation, the accumulation of aSMase could be detected by confocal microscopic observation (Fig. 3A). The quantification analysis showed significant increase in cells with aSMase condensation after anti-DENV NS1 Ab stimulation as compared with control IgG treatment (Supplemental Fig. 3). Anti-DENV NS1 Ab–induced ceramide generation was inhibited by pretreatment with CHL, an inhibitor of aSMase (Fig. 3B). Moreover, the production of NO (Fig. 3C) and cell apoptosis (Fig. 3D) by anti-DENV NS1 stimulation were also inhibited with CHL pretreatment. Anti-DENV NS1, but not control IgG, caused NO production and cell death in HMEC-1 cells. Treatment with MβCD or CHL did not cause an effect in control IgG group (Supplemental Fig. 4). For completeness, we also examined the role of neutral sphingomyelinase (nSMase) in these processes. The role of nSMase in the formation of ceramide-rich platforms is less clear than that of aSMase (41). The accumulation of nSMase on plasma membrane was also detectable after anti-DENV NS1 stimulation; however, the nSMase inhibitor sphingolactone-24 (Sph-24) could not block anti-DENV NS1 Ab–induced ceramide generation (data not shown). These results demonstrate that the activation of aSMase may account for ceramide generation, and trigger the subsequent NO production and apoptosis in anti-DENV NS1 Ab–stimulated endothelial cells.

**Exogenous ceramide treatment induces NF-κB–mediated iNOS expression, NO production, and apoptosis**

Because the generation of ceramide seems to play a role in anti-DENV NS1 Ab–induced endothelial cell injury, we treated cells directly with ceramide. Results showed that ceramide treatment could induce iNOS protein expression (Fig. 4A), NO production (Fig. 4B), and cell apoptosis (Fig. 4C). NF-κB can be involved in the cell death pathway mediated by ceramide (48, 49). Our previous study also showed that anti-DENV NS1 Ab–induced endothelial cell activation was regulated by NF-κB activation (27). We therefore investigated the activation of NF-κB by ceramide stimulation. Results showed that ceramide caused NF-κB nuclear translocation in HMEC-1 cells within 2 h (Fig. 4D, 4E). Ceramide-
induced iNOS protein expression was inhibited by pretreatment with NF-κB inhibitors, PDTC and BAY 11-7085 (Fig. 4F). These results demonstrate that ceramide induces NF-κB activation followed by iNOS/NO biogenesis.

**The activation of GSK-3β is involved in regulation of ceramide-induced NF-κB activation and iNOS expression**

We previously showed ceramide-induced apoptosis via GSK-3β activation in various types of cells (50). We further showed that ceramide induced dephosphorylation of GSK-3β at S9, which is the inhibitory phosphorylation site of GSK-3β, in HMEC-1 cells (Fig. 5A). We therefore investigated the possible involvement of GSK-3β in regulating ceramide-mediated NF-κB activation and iNOS expression. Pretreatment with GSK-3β inhibitors, SB415286 and LiCl, inhibited ceramide-induced NF-κB nuclear translocation (Fig. 5B). Furthermore, ceramide-induced iNOS expression was also blocked by GSK-3β inhibition (Fig. 5C).

**Anti-DENV NS1 Ab induces GSK-3β-mediated NF-κB activation**

Because ceramide induces GSK-3β-regulated NF-κB activation and iNOS expression in HMEC-1 cells, we further investigated the involvement of GSK-3β in anti-DENV NS1 stimulation. Results showed the dephosphorylation of GSK-3β at S9 after anti-DENV NS1 Ab treatment (Fig. 6A). Pretreatment with SB415286 inhibited anti-DENV NS1 Ab–induced NF-κB nuclear translocation (Fig. 6B). Anti-DENV NS1 Ab–induced expression of iNOS was also inhibited by GSK-3β inhibition (Fig. 6C). We further tested whether ceramide generation is involved in anti-DENV NS1 Ab–induced GSK-3β activation. Results showed that anti-DENV NS1 Ab–induced dephosphorylation of GSK-3β at S9 was blocked by CHL pretreatment (Fig. 6D). These results suggest that anti-DENV NS1 Ab induces GSK-3β–mediated NF-κB expression through ceramide generation.

**LiCl inhibits anti-DENV NS1 Ab–induced hepatic endothelial cell apoptosis**

We further confirmed the involvement of GSK-3β activation in anti-DENV NS1 Ab–induced endothelial cell injury using a passively immunized mouse model as described previously (28). After i.v. administration with anti-DENV NS1 Abs, mouse liver damage as determined by serum levels of aspartate aminotransferase and alanine aminotransferase was higher than the control IgG group (28) (data not shown). We thus verified whether anti-DENV NS1 Ab–induced endothelial cell apoptosis in vivo was inhibited by GSK-3β inactivation. TUNEL results showed that LiCl treatment reduced anti-DENV NS1 Ab–induced hepatic endothelial cell apoptosis (Fig. 7A), which was further quantified and shown in Fig. 7B. These results suggest that GSK-3β plays a crucial role in regulating anti-DENV NS1 Ab–mediated endothelial cell injury (Fig. 8).

**Discussion**

Dengue has become a globally emerging problem in public health with high incidence and extensive geographic distribution (13–15, 51, 52). Although several candidate vaccines are under clinical trials (8, 53, 54), as yet no effective dengue vaccines are available. A complicating factor in dengue vaccine design is ADE, by which Abs against viral surface proteins can amplify infection. Viral non-structural proteins, including NS1, are potential dengue vaccine candidates to avoid ADE. However, anti-DENV NS1 Abs cross-react with endothelial cells by a mechanism of molecular mimicry and cause their dysfunction (29, 30). The cross-reactive Ab levels that bind to endothelial cells correlate with the disease severity and the disease phase (22). These results are consistent with a dengue pathogenetic model in which cross-reactive Abs play a contributory role. The generation of anti-DENV NS1 Abs may therefore need to be considered in dengue vaccine development.

In our previous studies, we explored the pathogenic effects, including apoptosis and inflammation (26–28), induced by anti-DENV NS1 Abs. In this study, we further elucidate the molecular signaling of the endothelial cell response and apoptosis caused by anti-DENV NS1 Abs. As summarized in Fig. 8, we found that the binding of anti-DENV NS1 Abs to endothelial cells can efficiently cause lipid raft formation followed by aSMase-regulated ceramide generation. The sequential process includes GSK-3β–regulated NF-κB activation followed by NF-κB–regulated iNOS/NO biogenesis.
Lipid rafts have been shown to play important roles in cell responses to multiple extracellular stimuli, including cell–cell contact, cytokine and chemokine stimulation, drug therapy, and Ab-binding reactions (32, 33, 44, 45). For example, anti-CD95 (55, 56) and anti-phospholipid Abs (57) mediate protein clustering in the plasma membrane followed by lipid raft formation. This process generally triggers intracellular signal transduction and cellular activation, including apoptosis and inflammation. In this study, our findings show an essential role of lipid raft formation in anti-DENV NS1 Ab–induced signaling of NO production and apoptosis.

Ceramide is enriched in lipid rafts and acts as the second messenger in response to multiple stimuli (37, 41, 43, 46, 47). We found that ceramide is also increased in anti-DENV NS1 Ab–treated endothelial cells and that aSMase is required. Similar to CD95 signaling via aSMase activation and ceramide-rich lipid raft formation leading to cell death (55), anti-DENV NS1 stimulation also induces apoptotic signaling through aSMase/ceramide. In addition to aSMase, ceramide is also generated by de novo biosynthesis and by nSMase-mediated hydrolysis of sphingomyelin (37, 58). However, inhibiting ceramide synthase and nSMase did not block anti-DENV NS1 Ab–induced ceramide generation (data not shown).

We further showed that treatment with exogenous ceramide caused NO production and apoptosis in endothelial cells. Ceramide-regulated NO production has been reported in vascular smooth muscle cells, astrocytes, microglia, macrophages, and C6 rat glioma cells after TNF-α and LPS/IFN-γ stimulation (59, 60). Expression of iNOS is required for NO production under inflammatory stimulation. We previously demonstrated that anti-DENV NS1 Abs could induce NF-κB activation and iNOS expression in endothelial cells (26, 27); however, the molecular mechanism upstream of NF-κB activation remains unclear. In this study, we demonstrated that lipid raft formation and aSMase-mediated ceramide generation play important roles in NF-κB–regulated iNOS/NO biogenesis after anti-DENV NS1 Ab stimulation.

In this study, we also found GSK-3β may act as an upstream regulator for NF-κB activation in anti-DENV NS1 Ab–induced ceramide signaling. As demonstrated in an in vitro model, both exogenous ceramide and anti-DENV NS1 Ab treatment caused GSK-3β activation followed by NF-κB activation and iNOS expression. Although several critical transcription factors, including NF-κB, are regulated by GSK-3β, the effects of GSK-3β on NF-κB in regulating apoptosis are complex (61–63). GSK-3β–facilitated NF-κB activation and NO biosynthesis were demonstrated in this study and in a previous report (64). In anti-DENV NS1 Ab–stimulated endothelial cells, aSMase effectively regulated GSK-3β activation, suggesting an essential role for ceramide in this pathway. The proapoptotic effect of ceramide through a GSK-3β–regulated manner has been previously demonstrated (50). To further demonstrate the pathogenic role of GSK-3β, we investigated an in vivo model of anti-DENV NS1–induced endothelium injury in liver using GSK-3β inhibitor LiCl. The results showed a GSK-3β–regulated endothelial cell apoptosis in mice passively administrated anti-DENV NS1 Abs. We have therefore demonstrated the crucial regulatory role of GSK-3β in anti-DENV NS1 Ab–stimulated endothelial cell apoptosis not only in vitro but also in vivo. However, LiCl could not significantly decrease the serum...
levels of aspartate aminotransferase and alanine aminotransferase induced by anti-DENV NS1 Abs (data not shown). This is probably due to the fact that, in addition to cell apoptosis, other factors such as complement activation induced by anti-DENV NS1 Abs may also be involved in vivo.

The symptoms of DHF/DSS are multifaceted, including thrombocytopenia, coagulopathy, and vasculopathy, which are caused by viral effects and immunopathological host responses (7–15, 51). Currently, no dengue vaccine is available, although DENV NS1 has been proposed as a possible candidate. Given the pathological effects reported for anti-NS1 Abs, the underlying mechanisms need to be characterized and the relevant epitopes identified. Considerable progress toward these goals has been achieved (30, 65–68), raising the hope that safer NS1 candidate vaccines may be developed with modified or deleted harmful epitopes such as those involved in autoimmune responses.

Disclosures
The authors have no financial conflicts of interest.

References