Suppressive effect of dengue virus envelope protein domain III on megakaryopoiesis

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ABSTRACT

Dengue virus (DENV) infection can cause severe, life-threatening events, and no specific treatments of DENV infection are currently approved. Although thrombocytopenia is frequently observed in dengue patients, its pathogenesis is still not fully understood. Previous studies have suggested that DENV-induced thrombocytopenia occurs through viral-replication-mediated megakaryopoiesis inhibition in the bone marrow; however, the exact mechanism for megakaryopoiesis suppression remains elusive. In this study, a reductionist approach was applied, in which C57B/6J mice were inoculated with recombinant DENV-envelope protein domain III (DENV-EIII) instead of the full viral particle. Our results demonstrated that DENV-EIII-suppressed megakaryopoiesis is similar to those observed with DENV infection. Furthermore, in agreement with our in vivo analyses, DENV-EIII sufficiently suppressed the megakaryopoiesis of progenitor cells from murine bone marrow and human cord blood in vitro. Additional analyses suggested that autophagy impairment and apoptosis are involved in DENV-EIII-mediated suppression of megakaryopoiesis. These data suggest that, even without viral replication, the binding of DENV-EIII to the cell surface is sufficient to suppress megakaryopoiesis.

KEYWORDS

apoptosis; autophagy; dengue virus; envelope protein domain III; megakaryocytic differentiation; megakaryopoiesis; thrombocytopenia

Introduction

Dengue virus (DENV) is transmitted by mosquitoes, and infection causes dengue and severe dengue (also known as dengue hemorrhagic fever), with nearly 400 million reported cases annually. Although thrombocytopenia is a frequently observed manifestation of DENV infection significantly associated with severe dengue patients and is one of the warning signs in the World Health Organization 2009 Guidelines, its mechanism remains elusive. The increased destruction of platelets in the peripheral blood and the decreased production of platelets in the bone marrow are 2 major causes of thrombocytopenia.

A previous report demonstrated that the monoclonal anti-nonstructural protein 1 (NS1) of DENV could cross-react with human fibrinogen, thrombocytes, and endothelial cells. Anti-DENV NS1 antibodies could recognize protein on platelets and inhibit platelet aggregation, thus causing platelet lysis in the presence of complements. DENV NS1 protein elicited platelet-bound immunoglobulins (Igs) accelerated clearance by phagocytes, complement-mediated lysis, and that activation of platelets, leading to the development of thrombocytopenia. Studies have revealed that platelets were infected by DENV and produced dengue viral-like particles in patients with dengue. All of these studies have demonstrated that thrombocytopenia can be induced by anti-DENV NS1 antibody elicited-platelet destruction or directly through DENV infected platelet induced-platelet dysfunction in the peripheral blood.

Megakaryocytes (MKs) are platelet precursor cells present in the bone marrow. DENV was observed to inhibit megakaryopoiesis by infecting MKs and producing viral particles in the bone marrow. Direct infection of bone marrow MKs by DENV and the production of viral particles in MK–erythrocyte progenitor cells has also been demonstrated. All of
these evidences collectively suggest that DENV-infection-elicited thrombocytopenia is partly induced by megakaryopoiesis inhibition in the bone marrow, but the mechanism remains elusive.

The DENV virion is a spherical, enveloped virus with a diameter of approximately 50 nm. The virion contains 3 structural proteins [capsid (C), membrane (M), and envelope (E)] and the RNA genome. The E protein is the major protein on the virion surface. The E glycoprotein can be divided into 3 functional domains: the central domain (domain I; EI), the dimerization and fusion domain (domain II; EII), and the receptor-binding domain (domain III; EIII).21 In the absence of direct viral infection, human hepatitis C virus envelope glycoprotein 2 can induce an inflammatory response and apoptosis in human umbilical vein endothelial cells.22,23 However, whether the binding of DENV-EIII to the cell-surface receptors can similarly induce cellular signaling and toxicity remains unclear. The differentiation of hematopoietic progenitor cells, including MKs, is extremely sensitive to the perturbation of cellular signaling.24-26 Accordingly, in this study, we hypothesized that DENV-EIII could suppress megakaryopoiesis. To test this hypothesis, the in vivo effect of DENV-EIII on megakaryopoiesis suppression was evaluated in a mouse model. A colony-forming unit (CFU) assay of MKs (CFU-MK assay) and megakaryocytic differentiation were performed in vitro using human cord blood-derived CD34+ cells and mouse bone marrow cells.

Materials and methods

Ethics statement

Cord blood cells were collected from full-term pregnancy cases at the Department of Obstetrics and Gynecology, Mennonite Christian Hospital, Hualien, Taiwan. Written informed consent was provided by participants, and this study followed the protocols approved by the Research Ethics Committee of Hualien Tzu Chi Hospital, Buddhist Tzu Chi Medical Foundation (approval ID: IRB101–76). All experimental protocols related to animal studies were conducted in accordance with the national guidelines of the Animal Protection Act (Taiwan) and were approved by the Institutional Animal Care and Use Committee, Tzu Chi University (approval ID: 98026).

Virus preparation

The DENV-2 PL046 strain was amplified in a mosquito C6/36 cell line (ATCC CRL-1660), and the virus titer was determined through a plaque assay using BHK-21 cells (ATCC CCL-10) as described previously.10,26,27

Construction and protein purification

The plasmid Den2E3/pET21b containing DENV-EIII (amino acids 578–674) was kindly provided by Prof. Yiling Lin (Institute of Biomedical Sciences, Academia Sinica, Taiwan). To achieve a sufficient quantity and easy purification of the induction protein, a DNA fragment encoding DENV-EIII was subcloned into a pET28a expression vector (Novagen, Madison, WI) through EcoRI cutting. The BamHI cutting site was filled in to enable the in-frame construct to translate recombinant DENV-EIII protein fused to a 6-His-Tag. The plasmid Den2E3/pET28a was transformed into the Escherichia coli strain BL21 (DE3), which was induced to express recombinant protein at 37°C for 4 h by adding isopropyl β-D-1-thiogalactopyranoside at a 1 mM final concentration when the culture medium reached the mid-log phase (OD600 between 0.6–0.8). Recombinant DENV-EIII protein was purified using nickel-nitrilotriacetic acid (Ni-NTA) metal-affinity chromatography matrices (Qiagen, TAIGEN Bioscience, Taipei, Taiwan) under denaturing conditions, according to the manufacturer’s instructions with some modifications. Briefly, the inclusion body containing DENV-EIII obtained after sonication in a lysis buffer (50 mM Tris-HCl, 50 mM NaCl, and 1 mM EDTA) was denatured by 8 M urea. The cell lysate and Ni-NTA resin were incubated in a binding buffer (8 M urea, 100 mM NaH2PO4, and 10 mM Tris-HCl; pH = 8) at room temperature for 1 h and were then loaded into a column and washed with a washing buffer (8 M urea, 100 mM NaH2PO4, and 10 mM Tris-HCl; pH = 6.3) containing 1% Triton X-114 (Sigma–Aldrich) to remove endotoxins.27 The recombinant protein was eluted by an elution buffer (8 M urea, 100 mM NaH2PO4, and 10 mM Tris-HCl; pH = 4.5) and refolded using a linear 4–0 M urea gradient in a dialysis buffer (2 mM reduced glutathione, 0.2 mM oxidized glutathione, 80 mM glycine, 1 mM EDTA, 50 mM Tris-HCl, 50 mM NaCl, and 0.1 mM phenylmethylsulfonyl fluoride) at 4°C for 2–3 h. Glutathione-S transferase (GST) recombinant protein was used as the control protein. The expression plasmid pGEX-2KS28,29 was transformed into the E. coli strain BL21 (DE3) and induced to express the protein, which was purified according to the protocol of our previous study.30 Endotoxin contamination was verified through the Limulus Amoebocyte Lysate assay (Lanza), according to the manufacturer’s instructions as described previously.28,31

Antibodies generation and purification

Antibodies against DENV-EIII were generated and purified following the procedure detailed in a previous study

Materials and methods

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with some modifications. Protein G Sepharose 4 Fast Flow (GE Healthcare) was used to purify immunoglobulin G (IgG) from the serum of DENV-EIII immunized rabbits according to the manufacturer’s instructions. Eluted IgG was dialyzed using normal saline (0.9% NaCl) and concentrated using polyethylene glycol 20000. The IgG from preimmunized rabbit serum was purified using the same protocol.

**In vivo assay**

An *in vivo* assay was performed as described in our previous study with some modifications. To test whether DENV-EIII has the ability to suppress megakaryopoiesis *in vivo*, DENV (0.6–1.2 × 10⁵ PFU/mouse, 3–6.8 × 10⁴ PFU/mL) in a range approximately equivalent to the average viral load in dengue patients (3.265 × 10⁴ PFU/mL) was used. C57BL/6J mice (males, 9–12 weeks) with a body weight of 25 g, who had approximately 1750–2000 μL of blood in circulation, were treated with DENV (0.6–1.2 × 10⁵ PFU/mouse) through a retro-orbital injection twice every 24 h or treated with recombinant GST or DENV-EIII protein (3 mg/kg, equal to 37.5–42.9 μg/ml in the circulation and functionally equivalent to 5 × 10⁴ PFU DENV based on the competition analysis as described previously, Fig. S1) through retro-orbital injection every 12 h for 3 cycles. Peripheral blood was collected at 24 and 48 h, and MKs were isolated from the bone marrow and analyzed as described previously at 48 h after the first DENV, GST, or DENV-EIII injection. Mice treated with saline at the same volume for the same time course were used as controls.

**MK differentiation from mouse bone marrow cells**

Mouse bone marrow cell isolation and *in vitro* MK differentiation were performed as described previously, except that 100 ng/mL recombinant mouse thrombopoietin (rmTPO, PeproTech) was added to the differentiation medium. On Day 0 and Day 3, mouse bone marrow cells were treated with recombinant DENV-EIII (25 μg/mL; functionally equivalent to 3 × 10⁴ PFU, approximately the dose that can trigger megakaryopoiesis suppression *in vivo* and block approximately 30% of the infection and replication of DENV in BHK21 cells; Fig. S1) or GST protein (25 μg/mL), and the cell surface marker CD41 and DNA content were analyzed on Day 6 as described previously.

**Binding assay of DENV-EIII protein and MKs**

MKs were isolated from the mouse bone marrow as described previously. A total of 1 × 10⁶ MKs were incubated with 25 μg of purified recombinant DENV-EIII protein at 4°C for 1 h in 250 μL of MK buffer with 3% bovine serum albumin (BSA) [Ca²⁺/Mg²⁺-free phosphate buffered saline (PBS) containing 3% BSA; 2.8 μM prostaglandin E1; 5.5 mM D-glucose; 10.2 mM tridodium citrate, pH 7.3]. After washing with PBS, cells were stained with anti-CD61 antibody conjugated with allophycocyanin, anti-DENV-EIII antibody, and the IgG-purified from preimmunized rabbit serum at 4°C for 30 min in 300 μL of MK buffer with 3% BSA. Cells were washed with PBS again, and then they were incubated with goat anti-rabbit IgG (H+L) F(ab’)_2 fragment conjugated with fluorescein isothiocyanate at 4°C for 30 min in 300 μL of MK buffer with 3% BSA. After washing with PBS, cells were dissolved in 500 μL of PBS and analyzed using flow cytometry as described previously.

**CFU-MK assay**

The experimental protocol was performed according to the manufacturer’s instructions (MegaCult-C, StemCell Technologies, Vancouver, Canada) and the procedures described in our previous publication. Briefly, 1 × 10⁵ mononuclear cells (MNCs) derived from human umbilical cord blood were seeded in a double-chamber slide containing cytokines for MK differentiation. MNCs were treated with DENV-EIII (25 μg/mL) and GST protein (25 μg/mL) and incubated at 37°C for 12 d. MK colonies were identified through anti-GPIIb/IIIa staining and counted through counterstaining with 1% Evans Blue.

**MK differentiation from human cord blood CD34⁺ cells**

The purification of human cord blood CD34⁺ cells and *in vitro* MK differentiation were performed according to our previous study with some modification. To obtain a large number of CD34⁺ cells for performing the 16-day MK differentiation, methylcellulose (3.75 g/L) was added for 4–5 d to slow the cell movement and increase the cell number. CD34⁺ cells were treated with DENV-EIII (25 μg/mL) or GST protein (25 μg/mL) on Days 0, 4, 8, and 12 and were incubated with an MK differentiation medium. Flow cytometry was used to monitor MK differentiation and apoptosis on Day 16, as described previously. Autophagic cells were stained using the Cyto-ID Autophagy Detection Kit (Enzo Life Science, NY, USA) on Day 16 and were analyzed using a flow cytometer (FACSCalibur, Becton–Dickinson, USA).

**Statistics**

The mean, standard deviation (SD), and statistics for the quantifiable data were calculated using Microsoft Office.
Excel 2003. Comparisons between groups were made using the 2-tailed Student t test. P values less than 0.05 were considered significant.

Results

DENV-EIII suppressed megakaryopoiesis in vivo

After treatment with DENV (0.6–1.2 × 10^5 PFU/mouse, a range approximately equivalent to the average viral load in dengue patients), our analysis revealed that DENV could reduce the platelet number in the peripheral blood of mice (Fig. 1A and B), as previously reported. The expression of specific surface markers (CD41, CD61, and CD42b) and polyploid were used to identify MKs. Flow cytometry and propidium iodide (PI) staining revealed that the percentages of differentiated MK (large, CD61 cells in the R1-gated region) and polyploid cell (DNA content ≥ 4N) populations in the R1 region were significantly suppressed in DENV-treated mice compared with saline-treated mice (Fig. 1C, D, and E), suggesting that DENV suppressed megakaryopoiesis in mice. To investigate whether DENV-EIII is sufficient to suppress megakaryopoiesis in mice, experimental mice were treated with DENV-EIII (3 mg/kg) every 12 h for 3 cycles (Fig. 2A). Our data revealed that compared with saline and GST-treated groups, DENV-EIII-treated mice displayed significantly lower counts of R1 cells and a lower percentage of polyploid cells in the R1 region (Fig. 2C, D, and E); a similar response was observed in DENV-treated mice (Fig. 1C, D, and E). These results suggest that DENV-EIII-mediated suppression of megakaryopoiesis plays a crucial role in the DENV-induced negative impact on MKs in mice.

DENV-EIII suppressed megakaryopoiesis from mouse bone marrow cells

To investigate whether DENV-EIII exerted a suppressive effect on murine MKs, we performed in vitro megakaryocytic differentiation by using erythrocyte-depleted mouse bone marrow cells, according to a described previously method. Treatment with DENV-EIII (25 μg/mL) reduced the percentage of the CD41^+ cell population (Fig. 3B and C; R1-gated region). DNA content analysis using PI staining revealed that compared with GST, DENV-EIII reduced the percentage of polyploid cells and increased the percentage of sub-G1 cells significantly (Fig. 3B and D). This suggests that DENV-EIII suppresses megakaryopoiesis in primary cultured mouse haematopoietic progenitor cells present in the bone marrow. To investigate whether DENV-EIII can directly

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**Figure 1.** DENV suppressed megakaryopoiesis in vivo. The experimental outlines are indicated (A). C57BL/6J mice were retro-orbitally injected with DENV (0.6–1.2 × 10^5 PFU/mouse). The peripheral blood cells were collected, and the platelet counts were monitored at 24 h before the experiment and at 24 and 48 h after the first injection (B). Saline-treated groups were used as controls. Flow cytometry analyses were performed on bone marrow cells at 48 h (C) after the first injection. High-FSC (cell size) and CD61^+ cells were gated as the R1 region. PI staining revealed the cellular DNA contents. The percentage of cells in the R1 region (D) and the percentages of polyploid cells (DNA content ≥ 4N) in the R1 region are illustrated (E). Data are reported as the mean ± standard deviation (SD) and represent 3–5 independent experiments. **P < 0.01 compared with the saline-treated groups.
bind MKs, murine MKs purified from the bone marrow were incubated with DENV-EIII at 4°C for 1 h. Then, they were stained with anti-CD61 antibody to identify MKs and stained with anti-DENV-EIII antibody (Western blot analysis revealed that anti-DENV-EIII antibody specifically bound to DENV-EIII, Fig. S2). After flow cytometry analysis, the data revealed that the percentages of CD61 and DENV-EIII double positive cells are significantly increased when compared with controls without adding DENV-EIII or anti-DENV-EIII antibodies (Figs. 3E and F). This indicated that DENV-EIII could bind directly on the surface of MKs.

**DENV-EIII suppressed megakaryopoiesis in a CFU-MK assay**

Compared with murine bone marrow, human umbilical cord blood is a relatively more accessible resource for haematopoietic progenitor cells. A CFU-MK assay was performed to investigate whether DENV-MK has the ability to suppress megakaryopoiesis in human haematopoietic progenitor cells. Our data revealed that compared with GST-treated control groups, DENV-EIII-treated groups showed a significant reduction in the number of MK colonies (Fig. 4). These results suggest that DENV-EIII can suppress megakaryopoiesis in primary human haematopoietic progenitor cells.

**DENV-EIII suppressed megakaryopoiesis in human cord blood CD34+ cells by autophagy impairment and apoptosis**

Next, we investigated the mechanism through which DENV-EIII treatments lead to megakaryopoiesis suppression. Megakaryocytic differentiation was performed using human umbilical cord blood-derived CD34+ cells in a 16-day experiment (Fig. 5A). Consistent with a previous report,24 the cell size (FSC) and cellular granularity (SSC) were increased after the induction of differentiation (Fig. 5B; Day 0 vs. Day 16-Vehicle). Compared with the vehicle and GST treatments, DENV-EIII decreased the cell number in the R1 region and increased the cell number in the R2 region (Fig. 5B, C, and F). Flow cytometry and PI staining revealed that the percentages of CD61+ (premature MK), CD61+/CD42b+ (mature MK), and sub-G1 cells in the R2 region were increased significantly in the DENV-EIII-treated groups compared with the vehicle and GST-treated groups (Fig. 5D, E, and G). These results suggest that DENV-EIII suppresses megakaryopoiesis partly by initiating cell death in premature and mature MKs. Recent studies have revealed that autophagy plays an essential role in megakaryocytic differentiation, and the loss of autophagy leads to failure in megakaryopoiesis.40,41 Our data revealed that the percentage of autophagic cells was decreased in the R1 region and increased in the R2 region in the DENV-EIII-
Figure 3. DENV-EIII killed differentiated MKs derived from mouse bone marrow. Murine bone marrow cells (erythrocyte-depleted fraction) were isolated and triggered to differentiate into MKs in the presence of mouse thrombopoietin. The GST protein was used as a control. DENV-EIII or GST protein was added on Day 0 and Day 3, and data were analyzed on Day 6 through flow cytometry (A). Cells were stained with CD41 antibodies, and CD41+ cells were gated to analyze DNA content through PI staining (B). The percentages of R1 cells among all analyzed cells (C), and the percentages of polyploid cells (DNA content ≥ 4N) and sub-G1 hypoploid cells in the R1 region are shown (D). Murine MKs were isolated from bone marrow, incubated with DENV-EIII proteins for 1 h, and stained with antibodies against DENV-EIII and CD61. IgG isolated from preimmunized rabbit serum served as control Ig (Clg). Cells were divided into 3 groups: anti-CD61 + anti-EIII, EIII + anti-CD61 + Clg, and EIII + anti-CD61 + anti-EIII. The percentages of CD61+/DENV-EIII+ double positive cells among the total cells and total CD61+ cells were analyzed (E) and quantified (F). Data are reported as the mean ± SD and represent 4 (C and D) and 3 (F) independent experiments. *P < 0.05, **P < 0.01 compared with the GST-treated groups (C, D). ***P < 0.01 compared with the anti-CD61 + anti-EIII groups (F).
Figure 4. DENV-EIII suppressed megakaryopoiesis in the CFU-MK assay. Mononuclear cells isolated from human umbilical cord blood were used in the CFU-MK assay. The morphology (A–B) and quantitative numbers (C) of MK colonies after GST (A1–A3 and B1–B3) and DENV-EIII (A4–A6 and B4–B6) treatments are shown. Arrowheads indicating specific colonies in the low magnification images (A1–A6) are highlighted in the high magnification photographs (B1–B6). Scale bar: 2 mm (A), 200 μm (B). Data are presented as the mean ± SD and represent 4 independent experiments. ** P < 0.01 compared with the GST-treated groups.
Figure 5. DENV-EIII suppressed cytokines-triggered megakaryopoiesis by killing differentiated cells in umbilical cord blood-derived CD34<sup>+</sup> cells. A 16-day experiment on megakaryocytic differentiation was performed using expanded umbilical cord blood-derived CD34<sup>+</sup> cells. DENV-EIII was added on Days 0, 4, 8, and 12, and flow cytometry data were analyzed on day 16 (A). The vehicle and GST protein were used as controls. The FSC and SSC are shown (B). The cell numbers in the R1 and R2 regions (C) and the percentages of CD61- and CD61/CD42b-expressing cells in the R2 region (D) are shown. PI staining revealed the cellular DNA contents, and the sub-G1 hypoploid cells were increased in the DENV-EIII-treated groups (E). Summarized events of (C), (D) and (E) are shown in (F) and (G), respectively. Data are reported as the mean ± SD and represent 3–5 independent experiments. *P < 0.05, **P < 0.01 compared with the vehicle-treated groups.
Figure 6. DENV-EIII killed differentiated MKs in umbilical cord blood–derived CD34+ cells through impaired autophagy and apoptosis. The 488-nm-excitable Cyto-ID green fluorescent reagents were used to monitor the autophagic cells in the R1 and R2 regions (A). Annexin V-allophycocyanin (B) and an antibody against activated caspase-3 conjugated with fluorescein isothiocyanate (C) were used to investigate the apoptotic changes in DENV-EIII-treated cells through flow cytometry. Summarized events are shown in (D). Data are reported as the mean ± SD and represent 3–4 independent experiments. **P < 0.01 compared with the vehicle-treated groups.
treated groups compared with the vehicle-treated and GST-treated groups (Fig. 6A and D). In addition, the intensity of Cyto-ID in the R2 region was lower than that in the R1 region in the DENV-EIII treated groups [Fig. 6A, Day 16-EIII (R1) vs. Day 16-EIII (R2)]. These results indicate that DENV-EIII triggers impaired autophagy. Furthermore, to verify whether the cell death of these CD61+ (premature MK), CD61+/CD42b+ (mature MK), and autophagy-impaired cells was associated with apoptosis, annexin V and activated caspase-3 staining was performed in flow cytometry. Our results revealed that compared with the vehicle and GST treatments, DENV-EIII produced significantly more annexin V+ and active caspase-3+ cells in the R2 region (Fig. 6B, C, and D). Taken together, our data indicate that DENV-EIII suppresses megakaryopoiesis in human cord blood CD34+ cell cultures through autophagy impairment and apoptosis.

Discussion

Thrombocytopenia is an important manifestation of DENV infection and is associated with higher mortality; however, the mechanism remains unclear. Bone marrow suppression is one possible mechanism contributing to thrombocytopenia. Studies have reported that DENV can directly infect MKs in the bone marrow of humans and monkeys and interfere with megakaryopoiesis. However, how DENV elicits thrombocytopenia and interferes with megakaryopoiesis and whether viral replication is required for the suppression remain elusive. Our data revealed that DENV-EIII suppresses megakaryopoiesis independent of viral infection, and DENV-EIII-suppressed megakaryopoiesis is associated with autophagy impairment and the apoptosis of differentiated MKs (Fig. 5 and Fig. 6). These findings suggest that DENV-induced thrombocytopenia is partly mediated by the suppressive effect of DENV-EIII on megakaryopoiesis.

Theoretically, the presence of additional mechanisms, such as the infection and replication of DENV in MKs and/or the nearby supporting bone marrow stroma cells interfering with MK differentiation and cell survival, remains possible. However, unlike humans, mice are not native hosts of DENV; therefore, DENV cannot normally replicate in wild type mice unless the mice are deficient in interferon (IFN) α/β and γ receptors or type I IFN receptors. We demonstrated that DENV can’t infect and replicate on mouse MKs (Fig. S3). Our data suggested that DENV-EIII can directly bind MKs (Fig. 3E and F) and that DENV-EIII alone is sufficient to elicit MK suppression and decrease platelet numbers, similarly to the in vivo effect of DENV (Fig. 1 and Fig. 2, 24 hr). The result is consistent with the in vitro data obtained from mouse (Fig. 3) and human cells (Fig. 4 – 6). Our reductionist approach (use of mouse cells/mouse models) supports a simple way to focus on the effect of DENV-EIII on megakaryopoiesis irrespective of any viral replication; however, the approach can’t totally mimic real DENV infections especially in vivo models. DENV cannot replicate in mice, DENV nonetheless induces elicitation of proinflammatory cytokines such as TNF-α, which is well known to elicit thrombocytopenic responses. This is probably the reason why DENV injections have a higher potency than DENV-EIII on the elicitation of thrombocytopenic response (Fig-1B vs. Fig-2B, 48 hr).

Previous studies have demonstrated that impaired autophagy led to apoptotic death in various cell types, including MKs. The deficiency of Atg7 resulted in mitochondrial damage, followed by apoptosis in mouse erythrocytes and mature T lymphocytes. Similar to data from previous publications, our data showed that DENV-EIII-impaired autophagy is associated with apoptosis in differentiated MKs (Fig. 6). Further investigation is warranted to elucidate the detailed mechanism through which DENV-EIII induces impaired autophagy and apoptosis.

Currently, some live attenuated dengue vaccines are being assessed in clinical trials. DENV-EIII is the protein subdomain responsible for DENV binding to target cells; therefore, DENV-EIII is a feasible vaccine candidate to elicit protective antibodies. A DENV-EIII-specific epitope-recognizing antibody that ameliorated the clinical symptoms of infection for all 4 serotypes of DENV was demonstrated in a humanized mouse model. All 4 serotypes of DENV induce thrombocytopenia, and the sequences of DENV-EIII share high homology, with an estimated 68–87% similarity. Therefore, use of the DENV-EIII subunit vaccine to produce neutralizing antibodies or treatment with the small-molecule DENV-EIII antagonist can theoretically not only block DENV infection but also ameliorate DENV-EIII-induced megakaryopoiesis suppression and could be a feasible approach to managing severe dengue.

In conclusion, this study provides a new prospect that DENV-EIII can be a virulence factor for suppressing megakaryopoiesis. DENV-EIII induced the cell death of differentiated MKs through autophagy impairment and apoptosis. DENV-EIII-induced megakaryopoiesis suppression is one of causes of the thrombocytopenia observed in DENV infection. Therefore, DENV-EIII could be considered a drug target to treat MK defects and thrombocytopenia in severe dengue.
Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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Author contributions

GLL, TSL, PKC, HC, and MTS performed the experiments and analyzed the data. HHC and DSS conceived, designed the experiments and wrote the paper. CYL provided human umbilical cord bloods.

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