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Short communication

Inhibition of dengue virus replication in monocyte-derived dendritic cells by vivo-morpholino oligomers

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ABSTRACT

Skin dendritic cells (DCs) are primary target cells of dengue virus (DENV) infection and they play an important role in its immunopathogenesis. Monocyte-derived dendritic cells (MDDCs) represent dermal and bloodstream DCs that serve as human primary cells for *ex vivo* studies of DENV infection. Improved understanding of the mechanisms that effectuate the inhibition of DENV replication in MDDCs will accelerate the development of antiviral drugs to treat DENV infection. In this study, we investigated whether or not vivo-morpholino oligomer (vivo-MO), which was designed to target the top of the 3′ stem-loop (3′ SL) at the 3′ UTR of the DENV genome, could inhibit DENV infection and replication in MDDCs. The findings of this study revealed that vivo-MO-1 could inhibit DENV-2 infection in MDDCs, and that it could significantly reduce DENV RNA, protein, and viral production in a dose-dependent manner. Treatment of MDDCs with 4 μM of vivo-MO-1 decreased DENV production by more than 1,000-fold, when compared to that of the vivo-MO-NC control. Thus, vivo-MO-1 targeting of DENV RNA demonstrates potential for further development into an anti-DENV agent.

Dengue virus (DENV) infection is a mosquito-borne disease that has spread throughout the world's tropical and subtropical regions. Each year, approximately 390 million people are at risk for contracting DENV infection (Bhatt et al., 2013). Clinical manifestation of DENV infection ranges from mild febrile symptom to fatal disease. Severity of DENV infection in affected patients correlates with high viral load, which was observed in many studies (Guilarde et al., 2008; Tricou et al., 2011; Vaughn et al., 2000, 1997). The severe forms of DENV infection, which are dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), are characterized by increased proinflammatory cytokines, immunosuppressive cytokines, and chemokines (Martina et al., 2009; Rathakrishnan et al., 2012). This condition of massive cytokine production is referred to as *cytokine storm*. A licensed vaccine for DENV (Dengvaxia or CYD-TDV) is currently available (Capeding et al., 2014; Sabchareon et al., 2012); however, it was reported that the vaccine efficacy was incomplete. This vaccine is not recommended for the children under 10 years of age and offered sub-optimal protection in other age groups (http://www.who.int/immunization/research/development/dengue_vaccines/). Moreover, there is currently no specific antiviral drug for the treatment of DENV infection. The development of anti-DENV drugs is, therefore, urgently needed.

An important and promising strategy for the treatment of DENV infection is the inhibition of both DENV replication and cytokine storm (Rattanaburee et al., 2015). An accumulation of significant findings during recent years has enhanced our understanding of DENV interactions with target cells, especially dendritic cells (DCs), macrophages, endothelial cells, and liver cells (Schmid et al., 2014). DENV infection in these cells results in the production of immune mediators that then regulate adaptive humoral and cellular immune responses. Remarkably, DENV-infected DCs and monocytes induce an immune response that causes cytokine overproduction and antigen presentation to natural killer (NK) cells, memory B cells, CD-4, and CD-8 T cells (Schmid et al., 2014). Moreover, activated memory T cells are reported to produce too much proinflammatory cytokines (IFN-γ and TNF-α) that have the potential to develop of plasma leakage in severity of DENV infection (Whitehorn and Simmons, 2011). Accordingly, inhibition of DENV

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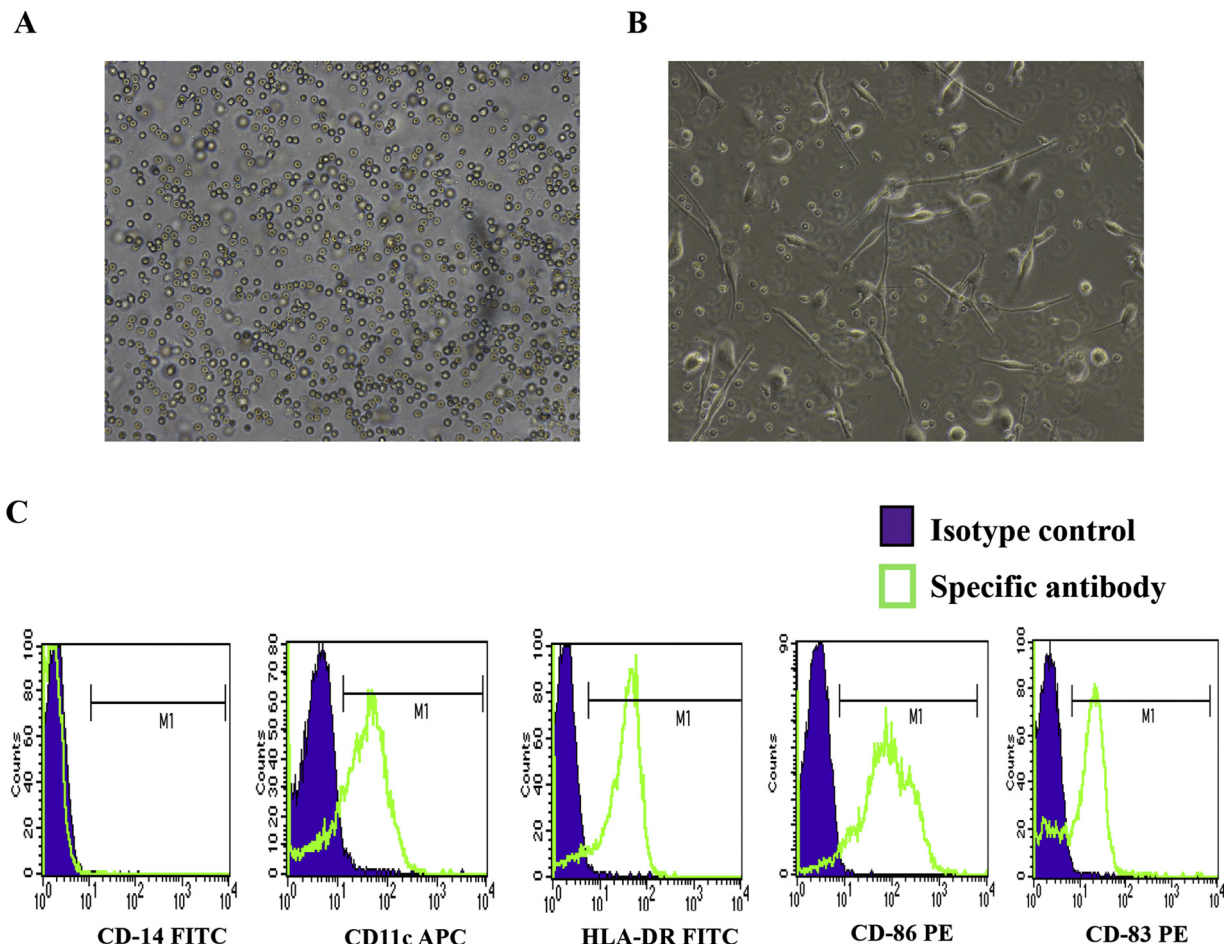

Fig. 1. MDDC phenotypes. PBMCs were collected from healthy Thai adult volunteers to generate MDDCs. The blood samples were collected after the volunteers were given information and signed informed consent no. 198/2560 (EC4). The procedure of MDDC generation was approved by the Siriraj Institutional Review Board. PBMCs were prepared by gradient density centrifugation using Ficoll-Paque PLUS, according to the manufacturer's instructions. (A) Adherent cells were subjected to the generation of dendritic cells (DCs). (B) Adherent PBMCs were cultured in RPMI medium containing GM-CSF (50 ng/mL) and IL-4 (25 ng/mL) on day 5. (C) MDDC marker expression profiles were examined by flow cytometry. The area under the purple curve represents isotype control antibodies, and the area under the green curve represents specific staining antibody. The data represents the results of experiments using blood samples from 5 healthy donors. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

infection in DCs could be expected to attenuate disease severity.

Human skin DCs or Langerhans cells are targets of DENV infection (Wu et al., 2000). Immature DCs (imDCs) and Langerhans cells play important roles in immune response, as well as acting as vehicles for DENV dissemination. DENV infects DCs *via* binding to a specific DC receptor [*i.e.*, the dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN or CD209)], which explains the susceptibility of DCs to DENV infection (Tassaneetrithep et al., 2003; Wu et al., 2000). Moreover, DCs play a key role in the immunopathogenesis of DENV infection (Luplertlop et al., 2006). Previous reports showed that monocyte-derived dendritic cells (MDDCs) that were isolated from human donor blood may not represent all *in vivo* DC subsets. MDDCs are imDCs, and they express both mannose receptor (MR) and DC-SIGN, which makes MDDCs susceptible to DENV infection (Navarro-Sanchez et al., 2003). MDDCs serve as human primary cells for *ex vivo* studies of DENV infection. Other study used different inhibitor to inhibit DENV replication in MDDCs, which was carbohydrate-binding agents (CBAs), and antiviral effects were observed in all four serotypes of DENV (Alen et al., 2011). In addition, monocyte-derived macrophages (MDM) also represent a key target of DENV infection *in vivo*. It is the main cell that produced TNF-α for relation with the severity of DENV patients (Wati et al., 2007).

Antisense oligonucleotides (ASOs) are an efficient therapeutic agent in viral infection. Many antisense molecules have been reported as being inhibitors of DENV (Holden et al., 2006; Kinney et al., 2005; Raviprakash et al., 1995; Stein et al., 2008). A previous study reported that phosphorothioate oligomer targeting 5′ UTR of the DENV genome potentially inhibited DENV replication (Raviprakash et al., 1995). Moreover, other studies reported that DENV-2 was inhibited by arginine-rich peptide-conjugated phosphorodiamidate morpholino oligomers (PPMOs) in DENV-infected BHK cells and mouse model (Holden et al., 2006; Kinney et al., 2005; Stein et al., 2008). A new class of ASOs was recently introduced, namely vivo-morpholinos (vivo-MOs). Vivo-morpholino oligomers (vivo-MOs) are ASOs that contain morpholinos that complement with RNA target conjugated with octaguanidine dendrimer to deliver these molecules into the cells (Moulton and Jiang, 2009). Vivo-MOs were shown to resist endonuclease, to be non-immunogenic, to be easy to enter the cultured cells, and to spread in a wide variety of tissues in transgenic mice, including the small intestine, liver, kidney, and other tissues (Morcos et al., 2008). Vivo-MOs targeting viral RNA were reported as effective against Enterovirus-71 and Japanese encephalitis virus (JEV) infections (Nazmi et al., 2010; Tan et al., 2014). Our group recently reported the use of vivo-MOs in DENV-infected cells (Phumesin et al., 2017). The vivo-MO-1 or vivo-MO-2 targets to the 3′ SL, where the pentanucleotide loop of DENV-2 (10615–10639) or DENV-4 (10615–10639) locates. Vivo-MO-1 could effectively inhibit DENV-1 (strain Hawaii), DENV-2 (strain 16681), and DENV-3 (strain H87) because its sequence is completely

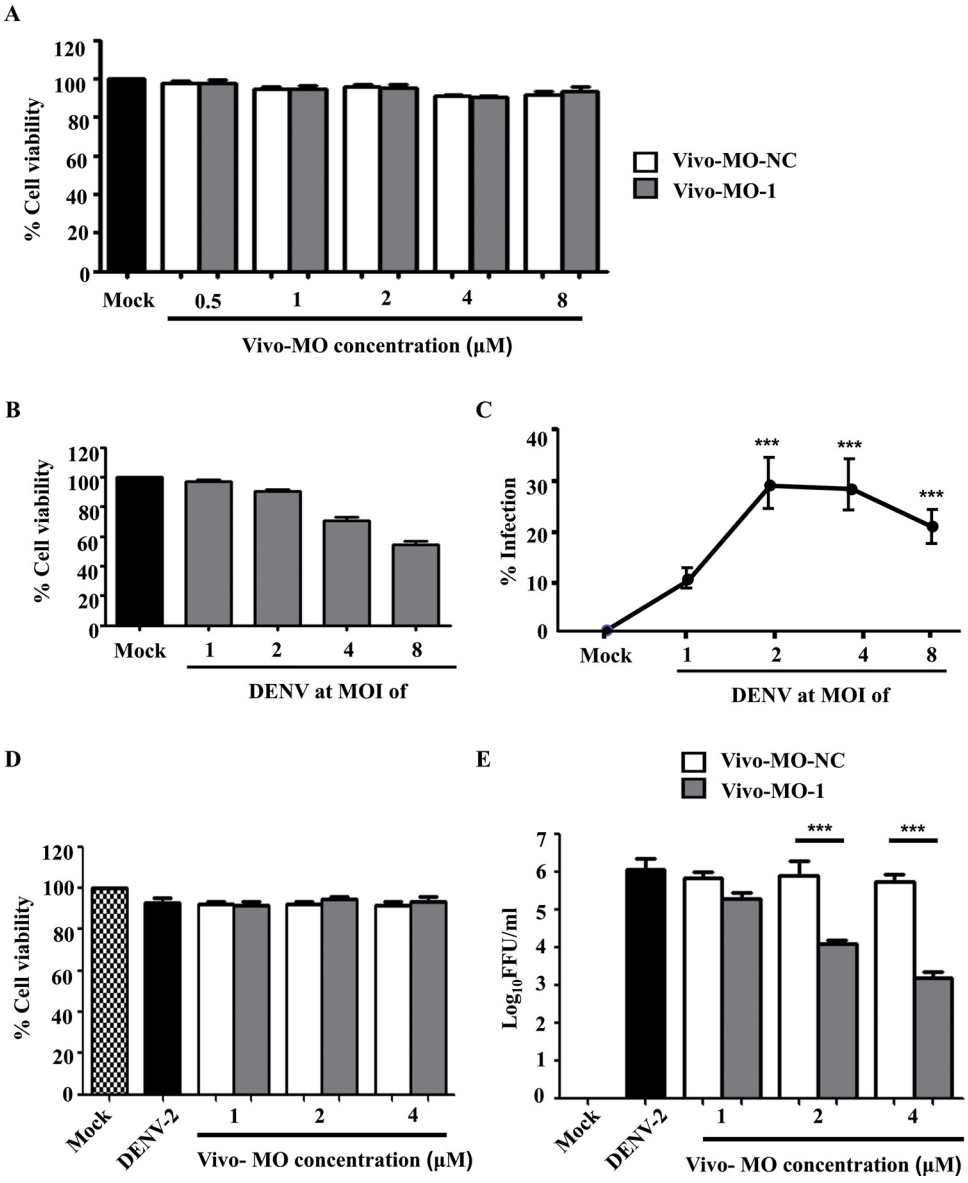

Fig. 2. Determination of infection rate and optimization of vivo-MO concentrations in DENV-2 infected MDDCs. (A) Toxicity test of vivo-MOs on MDDCs. MDDCs were treated with vivo-MOs at concentrations of 0.5, 1, 2, 4, and 8 μM for 24 h. Cell viability was measured by PrestoBLUE™ Cell Viability Assay (Thermo Fisher Scientific). (B) MDDCs were infected with different MOIs of DENV-2 for 24 h. Cells were collected for cell viability measurement by trypan blue dye assay. (C) Infected cells were stained with mouse 4G2 antibody, followed by staining with Alexa-Fluor- 488-conjugated goat anti-mouse IgG antibody to determine the percentage of infection by flow cytometry. (D) MDCCs were infected with DENV-2 for 4 h and treated with different concentrations of vivo-MOs. At 24-h post-infection, PrestoBLUE™ reagent was added to measure of cell viability. (E) Supernatants were then collected for measurement of FFU assay. The error bars indicate the standard error of the mean (SEM) from at least three independent experiments (***$p < 0.0001$).

complementary to 3′ SL in the 3′ UTR of these DENV serotypes. However, vivo-MO-1 contained 5-nucleotide mismatches to the 3′ SL of DENV-4 sequence, which might explain why vivo-MO-1 less effectively inhibited DENV-4 (strain H241). Vivo-MO-1 demonstrated potential antiviral activity by efficiently inhibiting DENV in infected cell lines, including Vero, A549, and U937 cell lines, and in primary monocytes. Since human DCs play an important role in DENV infection and pathogenesis, we conducted an *ex vivo* study to investigate whether or not vivo-MO could inhibit DENV in human MDDCs.

In this study, MDDCs were generated from monocytes isolated as adherent cells from peripheral blood mononuclear cells (PBMC) using GM-CSF and IL-4. Adherent cells (Fig. 1A) were cultured in Roswell Park Memorial Institute (RPMI) medium containing GM-CSF (50 ng/mL) and IL-4 (25 ng/mL) for five days (Fig. 1B). A monocyte marker (CD14) was used to monitor monocyte differentiation from DCs. The expression of MDDC surface markers, including CD11c, HLA-DR, CD86, and CD83, was then analyzed by flow cytometry. The results showed that MDDCs did not express CD14, but that they did express CD11c, HLA-DR, CD83, and CD86 on the cell surface (Fig. 1C).

To evaluate the toxicity of vivo-MOs on MDDCs, MDDCs were treated with different concentrations of vivo-MOs (*i.e.*, 0.5, 1, 2, 4, and 8 μM). After 24-hours of incubation, PrestoBLUE™ reagent (Thermo Fisher Scientific, Waltham, MA, USA) was added to the culture medium to assay cell viability. At all concentrations, more than 90% cell viability was observed, which indicated that vivo-MO-1 and vivo-MO-NC (negative control) had no toxic effect on MDDCs (Fig. 2A).

To determine the optimal infection rate of DENV, MDDCs in a 6-well plate were infected with different MOIs of DENV (*i.e.*, MOIs 1, 2, 4, and 8). At 24-hours post-infection, cells were collected for cell viability determination by trypan blue assay, and DENV infection rates were determined using flow cytometry. The results showed that cell viability decreased as DENV MOIs were increased (Fig. 2B). The DENV infection rates were increased at MOIs 1 and 2, stable at MOIs 2 and 4, and decreased at MOI 8 (Fig. 2C). Since The relative band intensities DENV at MOIs 2–8 induced cell death, MOI 2, which had highest infection rate, but induced the least cell death, was selected for use in the subsequent experiments.

To optimize the concentration of vivo-MO in DENV-2 infected MDDCs, MDDCs were infected with DENV-2 at MOI 2 in a 96-well plate. At 4 h after infection, the DENV-2 containing medium was removed and infected cells were treated with vivo-MO at concentrations of 1, 2, and 4 μM for 24 h. Then the infected cells were collected to determine the cell viability by using PrestoBLUE™ reagent (Thermo Fisher Scientific). To determine DENV production, MDDCs were infected with DENV-2 at

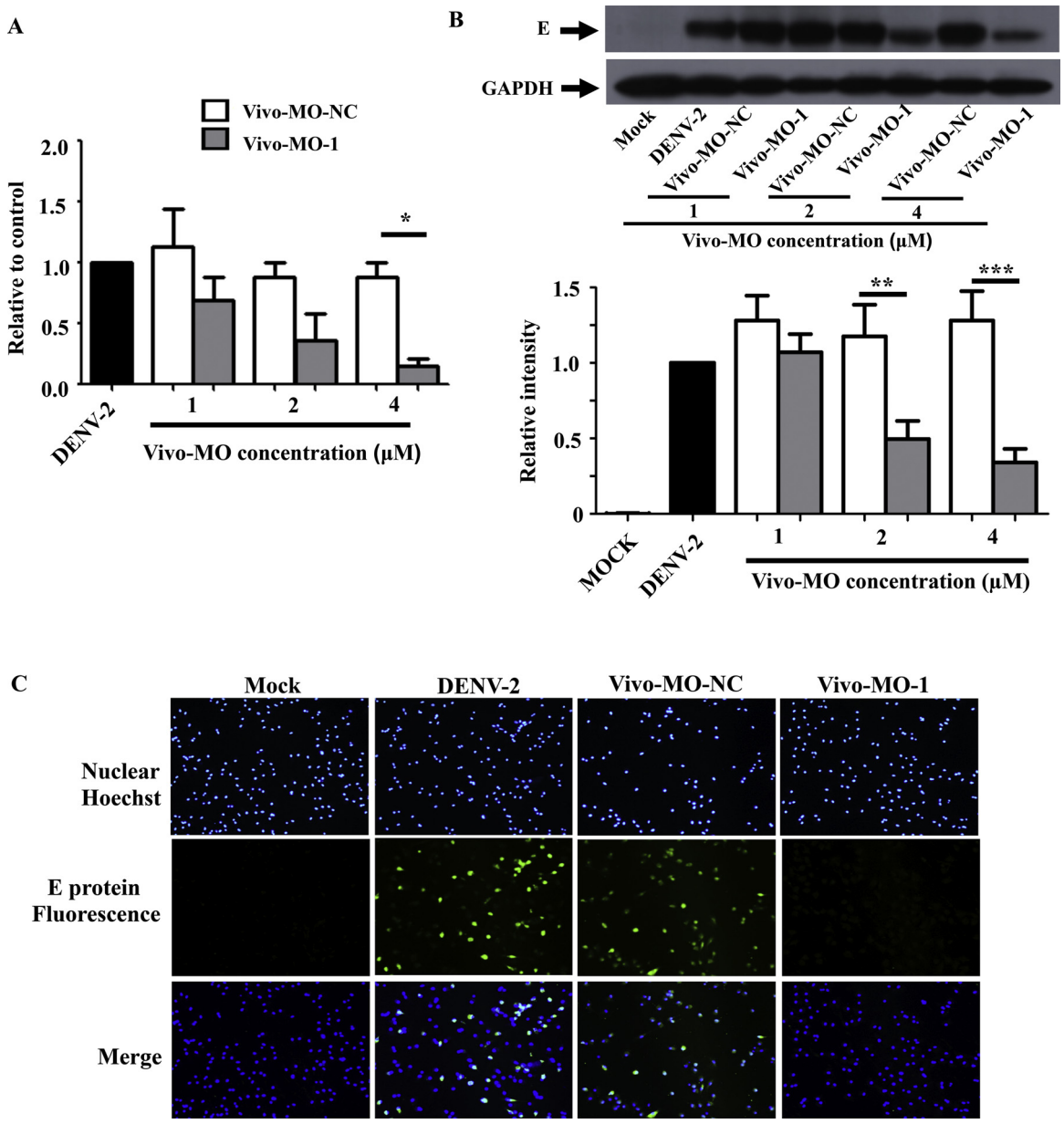

Fig. 3. Effects of vivo-MO-1 on the level of DENV RNA, protein, and infectivity. MDCCs were infected with DENV-2 for 4 h and treated with a high concentration of vivo-MO. At 24-h post-infection, (A) cells were collected for measurement of DENV RNA level by qRT-PCR, and (B) for measurement of protein expression by Western blot analysis. The upper panel represents protein expression by Western blot analysis. Protein band intensities were analyzed by Image J software. In the lower panel, values represent the average fold change of DENV-E protein expression, normalized to GAPDH, relative to DENV-2 infected cells alone. (C) Viral infectivity was determined in DENV-2-infected MDDCs treated with 4 μM of vivo-MO-1 as compared to vivo-MO-NC. Intracellular DENV-2 E antigen was detected by immunofluorescence assay (IFA). The error bars indicate the standard error of the mean (SEM) from at least three independent experiments (*$p < 0.05$, **$p < 0.001$, and ***$p < 0.0001$).

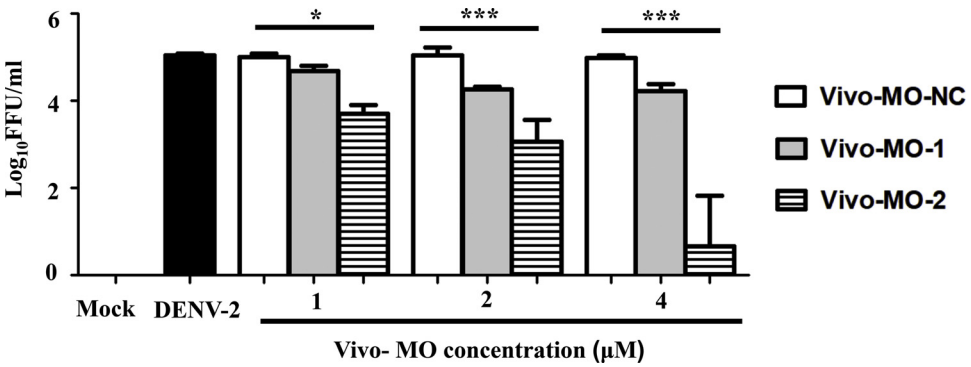

Fig. 4. Inhibitory effect of vivo-MO-1 and vivo-MO-2 treatment on DENV-4 production. MDDCs were infected with DENV-4 for 4 h, then treated with vivo-MO-NC, vivo-MO-1, and vivo-MO-2 for 24 h. Virus production in the supernatants was measured by FFU assay. The error bars indicate the standard error of mean (SEM) from three-difference donor (*$p < 0.05$, **$p < 0.001$, ***$p < 0.0001$).

MOI 2 in a 24-well plate. Supernatants were collected for analysis of DENV production by foci forming unit (FFU) assay (Baer and Kehn-Hall, 2014). All concentrations of vivo-MO-1 showed cell viability of more than 90%, compared to that of mock control (Fig. 2D). Low DENV production was observed in DENV-infected MDDCs treated with vivo-MO-1 (50-fold decrease at 1 μM, 100-fold decrease at 2 μM, and 1,000-fold decrease at 4 μM), compared to those treated with vivo-MO-NC. Therefore, vivo-MO-1 reduced DENV production in MDDCs in a dose-dependent manner (Fig. 2E). It should be pointed out that infection of MDDCs with DENV-2 at MOI 2 produced titers of 10^6 log10 FFU/ml at 24 h post-infection, which indicates a more rapid DENV growth in MDDCs than that in other types of cultured cells at 24 h post-infection. This may be explained by the presence of a specific DC receptor (DC-SIGN or CD209) on MDDCs making them more susceptible to DENV infection, and also MDDCs supporting an efficient DENV replication.

To determine the effect of vivo-MO-1 on the level of viral RNA and viral protein in DENV-2-infected MDDCs, MDDCs in a 24-well plate were infected with DENV-2 at MOI 2, and then treated with vivo-MO-1 or vivo-MO-NC at different concentrations. Cells that were collected at 24-h post-infection were tested for DENV RNA level using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) method. Vivo-MO-1-treated DENV-infected MDDCs had reduced DENV RNA, with fold changes of 0.7 at 1 μM, 0.3 at 2 μM, and 0.15 at 4 μM, when compared to DENV-infected MDDCs treated with vivo-MO-NC (Fig. 3A). Substantial reductions in DENV-E protein were observed in DENV-infected MDDCs treated with vivo-MO-1. Expression levels of DENV-E protein were then investigated by Western blot analysis (Fig. 3B, upper panel). Quantification of DENV-E protein was conducted by using Image J software (Fig. 3B, lower panel). Values represent the average fold changes of DENV-E protein expression, normalized to GAPDH. The results showed that vivo-MO- 1 at 2 and 4 μM decreased DENV-E protein to 0.51 $\pm$ 0.12 and 0.34 $\pm$ 0.09 fold, respectively, relative to DENV-2 infected cells alone (Fig. 3B). Therefore, vivo-MO-1 reduced DENV RNA in a dose-dependent manner, and it markedly reduced DENV-E protein. vivo-MO-1 at 4 μM was further used to evaluate DENV infection in MDDCs by immunofluorescence assay (IFA). The results showed that intracellular DENV-2-E antigen was reduced in cells treated with vivo-MO-1 at 4 μM, when compared with cells that received vivo-MO-NC treatment (Fig. 3C).

The previous study showed that vivo-MO-1 had different impacts on inhibition of different serotypes of DENV in Vero cells. Vivo-MO-1 was insensitive to inhibit in DENV-4. However, vivo-MO-2 contains sequences that perfectly match to that of DENV-4 and efficiently inhibit DENV-4 (Phumesin, et al., 2018). To determine inhibitions of vivo-MO-1 and vivo-MO-2 in DENV-4 infected MDDCs, MDDCs were infected with DENV-4 at MOI 2 in a 24-well plate. Supernatants were collected for analysis of DENV production by foci forming unit (FFU) assay. The results showed that DENV production was reduced in DENV-4-infected MDDCs treated with vivo-MO-2 (10-fold decrease at 1 μM, 100-fold decrease at 2 μM, and 1,000-fold decrease at 4 μM), compared to those treated with vivo-MO-NC. Therefore, vivo-MO-2 reduced DENV-4 production in MDDCs in a dose-dependent manner. Moreover, no statistically significant difference of DENV production when compared between vivo-MO-1 and vivo-MO-NC treatments in MDDCs infected with DENV-4 (Fig. 4).

DCs play an important role in the pathogenesis of DENV infection, since they are the first target cells of DENV infection that replicate and spread the virus to other tissues and organs in the human body. DENV-infected DCs also play a key role in the immunopathogenesis of DHF/DSS, because they can release proinflammatory cytokines and soluble factors that mediate plasma leakage (Green and Rothman, 2006). DENV-infected DCs may also be the cells that transmit DENV when the mosquito bites and draws blood through human skin. Moreover, DCs can produce chemokines, metalloproteinases, and proinflammatory cytokines (Blanco et al., 2008; Luplertlop et al., 2006; Rothman and Ennis, 1999). A high viral load and cytokine storm were found to be associated with DHF/DSS, the most severe forms of dengue infection. Thus, inhibition of DENV replication and cytokine storm was proposed as a foundational strategy relative to the development of drugs treat DENV infection (Rattanaburee et al., 2015). MDDCs isolated from human donor blood may not represent all *in vivo* DC subsets, but they are susceptible to DENV infection (Navarro-Sanchez et al., 2003), which makes them a good *ex vivo* model for studies in DENV infection.

In this study, we successfully generated MDDCs from human monocytes by using GM-CSF and IL-4, which their phenotypes as shown by cell surface markers were characteristics of MDDCs. These MDDCs were infected with DENV and then tested for the inhibitory effects of vivo-MOs at non-toxic concentrations. We found after receiving a 4 μM treatment of vivo-MO-1, MDDCs demonstrated a more than 1,000-fold decrease in DENV production. DENV RNA, protein, and intracellular protein were also greatly reduced. Thus, vivo-MO-1 is a strong inhibitor of DENV, and it should be further tested in *in vivo* studies. Regarding the clinical use of vivo-MO, an *in vivo* study reported that vivo-MO could be delivered into cells after intravenous injection and distributed to many tissues, which suggests the high stability and effectiveness of vivo-MO against the inhibitory effects of serum and other cellular components (Morcos et al., 2008). Although systemic administration of vivo-MO is possible, it should be noted that it was found to be toxic at high dose (10 μg/g) when it was administered to severe spinal muscular atrophy transgenic mice (Zhou et al., 2013). However, the 4 μM concentration of vivo-MO that was used in this study is lower than the toxic concentration dose used in the immediately aforementioned study. The optimal dose to achieve both effectiveness and safety during DENV infection needs to be determined in future study.

Conflict of interest declaration

All authors declare no personal or professional conflicts of interest, and no financial support from the companies that produce and/or distribute the drugs, devices, or materials described in this report.

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