Vaccine xxx (xxxx) xxx

Contents lists available at ScienceDirect

Vaccine

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

Construction of a novel tetravalent dengue vaccine with a *Salmonella* Typhimurium bacterial ghost and evaluation of its immunogenicity and protective efficacy using a murine model

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ARTICLE INFO

Article history:
Received 25 June 2019
Received in revised form 14 October 2019
Accepted 25 October 2019
Available online xxxx

Keywords:
DENV
Inactivated vaccine
Salmonella delivery vector
EDIII protein

ABSTRACT

Efforts to develop a safe, effective, and affordable dengue vaccine have focused on providing simultaneous immunity against all four serotypes of the dengue virus (DENV). In the current study, *Salmonella* Typhimurium (ST) lysed by gene *E* activation was genetically constructed to deliver the envelope protein domain III (EDIII) of all four serotypes of DENV using a foreign antigen delivery and expression vector, pJHL184. Each DENV-EDIII protein expressed in the constructed strain was validated by immunoblot analysis. To assess the immunogenicity and protective efficacy of the constructs against dengue infection, BALB/c mice were injected once orally with either the individual ST-EDIII constructs or a mix of all four ST-EDIII constructs followed by intramuscular administration of the purified EDIII protein. Significantly elevated titers of EDIII-specific IgG, IgG1, and IgG2a were observed in the immunized mice ($P < 0.01$). Furthermore, lymphocyte proliferative activity and $CD3^+CD4^+$ T-cell subpopulations increased significantly *in vitro* in re-pulsed splenic T cells compared with those from non-immunized mice. In addition, a lower viral load was detected in the BG-EDIII vaccinated group after challenge with DENV-infected K562 cells. Collectively, the results demonstrate that DENV-EDIII expressed in the inactivated ST strain could induce robust humoral and cell-mediated immunity specific to the target antigen and could provide significant protective potential.

1. Introduction

Dengue, a vector-borne viral disease, has been globally recognized in tropical, subtropical, and some temperate areas of the world [1]. An estimated 390 million infections, resulting in 50–100 million cases of dengue fever and 250,000–500,000 cases of life-threatening dengue hemorrhagic fever and dengue shock syndrome, are reported annually worldwide [2,3]. The etiological agent, dengue virus (DENV), belongs to the genus *Flavivirus* of the family *Flaviviridae* and is transmitted to humans primarily by the bites of female Aedes aegypti and Aedes albopictus mosquitoes [4]. Several dengue vaccine candidates containing live-attenuated and inactivated viruses, DNA constructs, vector-based expression factors, virus-like particles, and recombinant subunit antigens are in development [5–7]. However, the creation of an ideal vaccine candidate against this viral infection has been challenging because it should be simultaneously effective against all four antigenically distinct serotypes (DENV-1 to -4) [8,9].

The first dengue vaccine, a recombinant live-attenuated vaccine called the chimeric yellow fever 17D virus–DENV tetravalent dengue vaccine (Dengvaxia®) has been licensed in 19 countries since 2016 [10,11]. The vaccine expresses structural genes that encode the membrane protein (prM) and envelope protein (E) of each DENV serotype [12]. The DNA vaccine also targets the prM and E proteins, but those targets do not yield antigen-specific T cell activity in PBMCs from vaccinated mice [13]. When interferon-γ (IFN-γ) was measured by ELISPOT assay to evaluate antigen-specific T cell responses, the results were similar or moderate compared to the control group, and the World Health Organization has recommended the vaccine for use only in individuals who are seropositive for dengue antibodies due to safety concerns [14]. Dengvaxia® provides marginal protection in individuals who have not been exposed to dengue infection [14,15] and has shown an overall protective efficacy of nearly 60% in clinical trials, especially for DENV-2 [16]. However, as a recombinant, live-attenuated dengue vaccine, Dengvaxia® could pose a risk to children younger than 9 years, and it has shown a tendency of increased risk and safety issues during long-term follow-up [17]. A subunit vaccine, DEN-80E, consists of a recombinant truncated dengue envelope protein

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https://doi.org/10.1016/j.vaccine.2019.10.075
0264-410X/

containing 80% of the N-terminal E protein [18]. However, both those vaccines are difficult to produce in large quantities. Thus, despite recent advances in vaccine development, safe and effective vaccines that provide equivalent and broad protection against all DENV serotypes are still needed.

Bacterial ghosts (BGs), Gram-negative bacteria inactivated by the single lysis gene *E* of bacteriophage ϕX 174, have been applied as a heterologous antigen delivery tool endowed with intrinsic adjuvant activity [19]. Lysis gene *E* consists of a 91-aa polypeptide that inhibits peptidoglycan biosynthesis of the bacterial cell wall, resulting in an empty bacterial cell envelope [20,21]. Because BGs preserve immune-stimulatory surface molecules such as PAMPs, lipopolysaccharide, and adhesins, they mimic certain immunogenic properties of the native bacteria [22,23,19]. The strategy of vaccine development using a BG system is cost effective and can produce large quantities. Additionally, BGs express the antigen stably and do not require an additional adjuvant, making them a fascinating antigen delivery system [19].

In this work, we constructed an inactivated *Salmonella* Typhimurium (ST) BG to deliver the envelope domain III (EDIII) of all four dengue serotypes as a vaccine candidate against DENV infection. The E protein of DENV is a multifunctional glycoprotein that consists of three distinct structural domains: I, II and III [24]. Dengue EDIII is involved in a wide range of biological activities that mediate infection, such viral entry and attachment to the host cellular target receptors [25,26]. Specific neutralizing epitope regions containing residues 307, 333–351, and 383–389 within the EDIII are known to induce the production of neutralizing antibodies against DENV [26,27]. Domain III of glycoprotein E is a major antigenic target for potential vaccine candidates against DENV infection.

Immunogenicity and protective efficacy were assessed in BALB/c mice immunized with the constructed ST strains lysed by gene *E* expression carrying dengue EDIII proteins (ST-EDIIIs). ST-EDIIIs were prepared for each of the four serotypes (monovalent), and all four were mixed (tetravalent). We found that vaccination with either the monovalent or tetravalent form via an oral route followed by intramuscular inoculation with the purified EDIII protein induced significant DENV-EDIII-specific humoral and cell-mediated immune responses. In addition, a lower viral load was detected in the BG-EDIII vaccinated group compared with the non-vaccinated group after challenge with DENV-infected K562 cells.

2. Materials and methods

2.1. Bacterial strains and plasmids

The bacterial strains and plasmid vectors used in this study are shown in supplementary data 1. The ST mutant strain JOL912 (Δ*asd* Δ*lon* Δ*cpxR*) was prepared for to deliver the DENV-specific EDIII gene [28]. The *asd* gene-deleted attenuated ST strain was inoculated with 50 μg/ml of diaminopimelic acid in medium at 37 °C. The *asd* gene-deleted vaccine strain complemented by an *asd*$^+$ lysis plasmid (pJHL184) was incubated in medium supplemented with 0.2% L-arabinose. All bacterial strains were stored at −80 °C in Luria Bertani (LB) broth with 20% glycerol.

2.2. Construction of ST ghosts expressing DENV-EDIII proteins

The EDIII gene sequences of each of the four serotypes of DENV (DENV-1 to -4) were obtained from the NCBI database (NCBI Gene Bank Accession: KU291686.1 for DENV1; KU094070.1 for DENV2; MF682975.1 for DENV3, KX270821.1 for DENV4) and chemically synthesized (Bioneer, Korea). To construct ST ghosts to deliver the target antigenic proteins, the *Eco*RI/*Hin*dIII digested *EDIII* gene was inserted in-frame downstream of the transmembrane domains (TMDs) of the outer membrane protein A (*ompA)* signal sequence (SS) of the lysis plasmid pJHL184 vector carrying the *E* lysis gene cassette and a heterologous antigen delivery site. The four resultant lysis plasmids were individually transformed into a *Δasd E. coli* χ6212 strain (JOL232) to increase plasmid stability. Subsequently, the four constructed plasmids were electrophoretically introduced into the attenuated ST strain JOL912, producing JOL2141, JOL2095, JOL2142, and JOL2143, respectively (Supplementary Data. 1) [65–67]. The EDIII gene fragment was ligated in a pET28a(+) plasmid, followed by transformation into the protein overexpression vector of *E. coli* strain BL21 (DE3) pLysS (Novagen, San Diego, USA) to purify the DENV protein [29].

2.3. Production of ST ghosts expressing DENV EDIII proteins

In the lysis plasmid pJHL184, gene *E* is located between the thermosensitive *λpR* promoter with repressor *cI857* and an antisense *ParaBAD* promoter with the *araC* regulatory system [30]. To generate an ST ghost expressing EDIII protein, a single fresh colony was inoculated into LB broth containing 0.2% L-arabinose at 28 °C. Then, to induce gene *E*-mediated bacterial lysis and the concurrent expression of the heterologous antigen, DENV-EDIII, the arabinose was removed from the cultures, and the growth temperature was shifted from 28 °C to 42 °C with 150 rpm agitation. After a 24-hr incubation, 100 μl of the culture was sampled at the end of the lysis process and plated onto LB agar to confirm the magnitude of cell lysis. The presence of colonies on the agar plate in triplicate indicated cell viability. The LB medium was removed from the cell lysate by centrifugation at 4000*g* for 20 min, and the BG pellets were stored at −20 °C until further processing. The *in vitro* expression of the DENV-EDIII protein in the vaccine candidate strains was validated by a Western blot analysis using anti-His antibody (Life Technologies, USA) [31]. The formation of ST ghosts expressing the target antigenic protein was examined by scanning electron microscopy (SEM), following an established protocol described previously [32].

2.4. Challenge viruses

Virus stocks were titrated by focus-forming assay using the DENV NS1-specific monoclonal antibody DN3 (Abcam, Cambridge, UK) and stored in aliquots at −80 °C until use [33]. The human cell line K562 was used to prepare the challenge DENV strain [24,34]. The cells (1×10^7) were mixed with $4\log_{10}TCID_{50}$/ml of each of the four serotypes suspended in serum-free RPMI medium. After incubation at 37 °C for 2 hr, the infected cells were resuspended in 20 ml of 5% FBS RPMI medium and cultured at 37 °C. Infected-K562 cells (1×10^6) collected 4 days after inoculation were mixed with naïve K562 cells (1×10^7). After 48 hr of incubation, the cells were harvested and injected into mice.

2.5. Ethics statement

All animal experiments involving the use of specific-pathogen-free mice were approved by the Chonbuk National University Animal Ethics Committee [35] in accordance with the guidelines of the Korean Council on Animal Care and Korean Animal Protection Law, 2007; Article 13 (Experiments with animals). The animal research protocols used in this study followed the guidelines set by the nationally recognized Korea Association for Laboratory Animal Sciences. All experimental protocols requiring biosafety were approved by the Institutional Biosafety Committee of Chonbuk National University.

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2.6. Immunization and challenge studies in BALB/c mice

Five-week-old female BALB/c mice were purchased from Samtako (O-san, Korea). The mice (n = 90) were randomly divided into six groups and acclimated for a week before vaccination. The immunization schedule is described in Table. 1. Heterologous prime-boost vaccination strategies were applied to improve the effectiveness of immunization against viral disease [35–39]. Briefly, group A mice (n = 15) were vaccinated once orally with 2×10^9 particles of the DENV serotype-1 BG-EDIII construct (JOL2141) on day 0, followed by intramuscular (IM) inoculation on day 21 with 25 μg of a purified DENV serotype-1 EDIII recombinant protein. Groups B, C, and D mice (n = 15, each) were also vaccinated with ST-BGs expressing an EDIII recombinant protein of serotype 2, 3, and 4 (i.e., JOL2095, JOL2142, or JOL2143), respectively, via IM injection. Group E mice (n = 15) were immunized orally with a cocktail of all four ghost vaccine candidates (total 2×10^9 particles), followed by IM inoculation with 25 μg of a purified mixture of the four EDIII recombinant proteins on day 21. Group F mice (n = 15) were orally immunized once with the vector control of JOL912 alone. At 14, 21, and 28 days post-vaccination (dpv), serum samples were collected from the mice to assess the level of EDIII-specific IgG antibody responses. For the challenge experiment, mice were intraperitoneally injected with 5×10^7 DENV-infected K562 cells suspended in 0.2 ml of serum-free RPMI medium at 28 dpv [24,40]. In a previous study, 100% DENV infection was observed in K562 cells following cell culture incubation at 37 °C for 72 hr [41]. Five hours and 20 hr after the injection, blood samples (n = 5) were drawn via the orbital sinus of the mice and collected into 1.5 ml centrifuge tubes containing 0.02 ml of 1% potassium EDTA. The tubes were placed on ice, and the plasma was subsequently collected to determine the serum viral titers by using the 50% tissue-culture infective–dose–cytopathic effect ($TCID_{50}$-CPE) of the challenge virus in the Vero E6 cell line [33]. For the lymphocyte proliferation assay and fluorescence activated cell sorting (FACS) analysis, additional mice (n = 5 per group) were also immunized, and then splenocytes were aseptically isolated from immunized and non-immunized mice at 28 dpv.

2.7. Measurement of antibody titers and cross-reactivity

Titers of DENV-EDIII-specific total (Ig) G, G1, and G2a were evaluated in the mice using an indirect enzyme-linked immunosorbent assay (ELISA) as previously described [32] with the purified EDIII protein (250 ng per well) as the coating antigen. The cross-reactivity of the antibodies against EDIII protein produced by the mice immunized with each vaccine candidate was also determined with an indirect ELISA assay [32]. The purified EDIII protein of each of the four DENV serotypes (250 ng per well) was used as the coating agent. Sera were diluted 1:50 to examine the cross-reactivity of the antibodies. Titers of serum antibodies are presented as absorbance values at 470 nm.

2.8. Lymphocyte proliferation assay

The cell proliferation response was assessed in pulsed splenocytes isolated from the immunized mice using an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]-based assay [42]. The splenocytes (1×10^6cells per well) obtained from the mice in groups A to F were stimulated *in vitro* with the purified EDIII protein of each DENV serotype (400 ng per well) for 24 hr. Subsequently, the murine splenocytes were incubated with 20$\mu\ell$ of MTT reagent (5.0 mg/ml). The plate was incubated in the dark at 37 °C for 4 hr, and then the culture supernatant was aspirated, followed by the incorporation of 200 μl of dimethyl sulfoxide to dissolve the purple formazan precipitates. The magnitude of cell proliferation was assessed by detecting the absorbance at a test wavelength of 570 nm and a reference wavelength of 650 nm. The stimulation index was calculated as previously described [43].

2.9. FACS analysis

The change in the proportion of $CD3^+CD4^+$ and $CD3^+CD8^+$ splenic T cells in the immunized mice was determined using a FACS analysis. The prepared splenocytes (1×10^6/ml) were stained with a mixture of fluorescently labeled antibodies containing anti-mouse CD3a-PE, anti-mouse CD4-perCP vio700, and anti-mouse CD8a-FITC (Miltenyi Biotec, Bergisch Gladbach, Germany) for 30 min on ice in the dark. The samples were washed twice with FACS buffer (Miltenyi Biotec) and examined using a MACSQuant Analyzer 10 (Miltenyi Biotec, Germany).

2.10. Cytokine measurements

The gene expression levels of immunomodulatory cytokines, including interleukin-4 (IL-4) and IFN- γ, were evaluated by performing reverse transcription real-time PCR (RT-PCR). The prepared splenocytes (1×10^6cells per well) isolated from the mice were re-stimulated *in vitro* with 400 ng of EDIII protein for 24 hr. Subsequently, total RNA was extracted from the pulsed cells with a GeneAll® Hybrid-R kit (GeneAll Biotechnology, Seoul, Korea), and it served as the template for cDNA synthesis using a ReverTra

Table 1
Vaccine groups, doses, routes and immunization schedule.

Group	Days	Vaccine	Dose	Route
A	0	DENV serotype1 BG-EDIII construct (JOL2141)	2×10^9 particles	Oral
	21	Purified DENV serotype1 EDIII recombinant protein	25 μg	IM
B	0	DENV serotype1 BG-EDIII construct (JOL2095)	2×10^9 particles	Oral
	21	Purified DENV serotype2 EDIII recombinant protein	25 μg	IM
C	0	DENV serotype1 BG-EDIII construct (JOL2142)	2×10^9 particles	Oral
	21	Purified DENV serotype1 EDIII recombinant protein	25 μg	IM
D	0	DENV serotype1 BG-EDIII construct (JOL2143)	2×10^9 particles	Oral
	21	Purified DENV serotype1 EDIII recombinant protein	25 μg	IM
E	0	mixed all four serotype BG-EDIII constructs	2×10^9 particles	Oral
	21	Cocktail of the mixture of four EDIII recombinant proteins	25 μg	IM
F	0	PBS	100 μl	IM
	21	PBS		

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Ace® qPCR RT kit (FSQ-101, TOYOBO, Japan) following the manufacturer's instructions. mRNA expression was detected using SYBR Green Realtime PCR Master Mix (QPK-201, TOYOBO, Japan) [44]. The level of expression was quantified using the comparative CT method ($2^{-\Delta\Delta CT}$) based on a fold-change in target gene expression relative to a reference sample (i.e., control sample) normalized to a reference gene (i.e., mouse β-actin) [45].

2.11. Statistical analysis

Groups were compared using two-tailed unpaired Student's t-tests. A one-way analysis of variance (ANOVA) with Tukey's multiple comparison test was used between different groups. P values < 0.05 were considered statistically significant.

3. Results

3.1. Expression of DENV EDIII in the Salmonella ghost vector system

The EDIII gene of each of the four DENV serotypes was introduced *in frame* downstream of the TMD region of the *ompA* SS, which directs the TMD-EDIII fusion protein to the bacterial outer membrane (Supplementary Data. 2A). Lysis plasmids carrying the heterologous antigens were individually transformed into the attenuated ST strain, JOL912, to construct the vaccine candidates JOL2141, JOL2095, JOL2142, and JOL2143. DENV-EDIII proteins expressed *in vitro* in these four vaccine candidates were authenticated by immunoblotting assays using the 6xHis tag® antibody. A distinct immunoreactive band of the recombinant EDIII protein fused to the OmpA signal peptide was detected at ~20 kDa in the pellet of the ST strain (Supplementary Data. 2B). The purified recombinant EDIII protein (~18 kDa) was separated by SDS polyacrylamide electrophoresis (SDS-PAGE) analysis as a positive control (Supplementary data. 3). Furthermore, the production of the ST ghost made to deliver the target antigen was validated by SEM (Supplementary data. 4). The cell surface of the lysed ST strain was partially collapsed (Supplementary data. 4B), compared with the morphology of the normal bacterial cell (Supplementary data. 4A), because the cytoplasmic contents, including DNA, are expelled through the transmembrane tunnel formed by lysis gene *E* expression due to high osmotic pressure. No single cell was observed in the ghost cultures 24 hr after the lysis process (data not shown). Therefore, the vaccine candidate strains were successfully inactivated by the gene *E*-mediated lysis controlled by the dual promoter system.

3.2. Humoral immune responses

The ability of the BG-EDIII to effectively induce humoral antibody responses was evaluated in mice immunized with JOL2141, JOL2095, JOL2142, or JOL2143, as monovalent vaccinations, and a cocktail of all four candidates as a tetravalent vaccination. The immunized mice showed significantly higher levels of IgG, IgG1, and IgG2a responses against BG-EDIII than mice injected with the vector control, the JOL2067 ghost. The peak IgG titers were found 28 dpv in all groups (Fig. 1). In particular, the titers of IgG, IgG1, and IgG2a were efficiently increased in the mice immunized with the JOL2141 ghost expressing DENV serotype 1-EDIII compared with the mice receiving the tetravalent vaccination (Fig. 1A, B, and C). The levels of IgG1 showed a tendency to rise in comparison with the levels of IgG2a in all the immunized mice 21 and 28 dpv, indicating a Th-2 dominant pattern in the BG-EDIII immunized mice (Fig. 1B and C). We also assessed the cross-reactivity among IgG antibodies induced by the monovalent vaccine candidates 28 dpv. The IgG antibodies induced by the monovalent BG-EDIIIs were not highly cross-reactive with antibodies against other serotypes (Fig. 2). Particularly in the case of serotype 2, the cross-reactivity of the IgG antibody had significantly low affinity compared with the other serotypes. Our results demonstrate that the BGs expressing DENV-EDIII induced an antibody response for each serotype of the recombinant DENV-EDIII protein.

3.3. Cellular immune responses

The introduction of foreign antigens into a host elicits the transformation of naïve T-cells into effector T-cells that initiate cell-mediated immunity (CMI) [46]. The magnitude of T-cell proliferation was measured by the MTT assay in splenocytes isolated from vaccinated mice 28 dpv. The stimulation indices of splenocytes from mice receiving monovalent or tetravalent vaccines revealed significantly greater proliferation activity than seen in cells from non-infected mice ($p < 0.05$) (Fig. 3). Furthermore, $CD4^+$T-cells have the capacity to form memory, which provides useful information for the production of antibodies [47]. A FACS analysis was performed to evaluate the differentiation of immune T-cell subsets in the vaccinated mice. Alterations in the proportions in $CD3^+$, $CD4^+$, and $CD8^+$ surface marker expression were assessed in the splenic T-cells of vaccinated mice and compared with those in non-vaccinated mice (Fig. 4A and B). The proportions of $CD3^+$, $CD3^+$-$CD4^+$, and $CD3^+CD8^+$ T cell subsets increased by an average of 7.1%, 5.4%, and 2.8%, respectively, in the monovalent vaccinated mice (Fig. 4A) and by 4.9%, 3.9%, and 1.6%, respectively, in the mice receiving the tetravalent vaccination compared with those of the non-vaccinated mice (Fig. 4B). Therefore, immunization with BG-EDIII could have the potential to elicit CMI in mice.

3.4. Cytokine gene expression

We evaluated immunomodulatory cytokines in the splenic T-cells of immunized mice using qRT-PCR. IFN-γ is produced by $CD4^+$ Th-1 and $CD8^+$ cytotoxic effector T-cells in an antigen-specific manner [48]. Furthermore, it has been demonstrated that up-regulated IFN-γ leads to efficient lysis of DENV-infected cells [48]. The effect of BG-EDIII vaccines on cytokine expression was investigated *in vitro* in re-pulsed splenocytes originating from immunized mice. The up-regulation of IFN-γ and IL-4 mRNA levels was detected in all vaccinated mice (Fig. 5). The mRNA level of IFN-γ detected in mice immunized with the monovalent BG-EDIII was higher than that in re-pulsed splenocytes from mice that received the tetravalent vaccine (Fig. 5A). The level of IL-4 was slightly increased in splenocytes from mice receiving either monovalent or tetravalent immunization (Fig. 5B).

3.5. Protective efficacy of BGs expressing DENV-EDIII against DENV infection

Protective efficacy was evaluated using the magnitude of viremia reduction in vaccinated mice challenged with DENV-infected K562 cells. Viral loads declined in plasma sampled from BG-EDIII vaccinated mice compared with non-vaccinated mice (Fig. 6). All mice immunized with BG-EDIII vaccines had reduced viremia, from approximately 3 to 2 $\log_{10}(TCID_{50}/ml)$, by 20 hr post-challenge compared with non-vaccinated mice. These results indicate that the vaccine candidates could induce functional immune responses to reduce plasma viremia levels.

4. Discussion

In this study, we developed a tetravalent vaccine candidate against DENV by using EDIII, an effective antigen of DENV that

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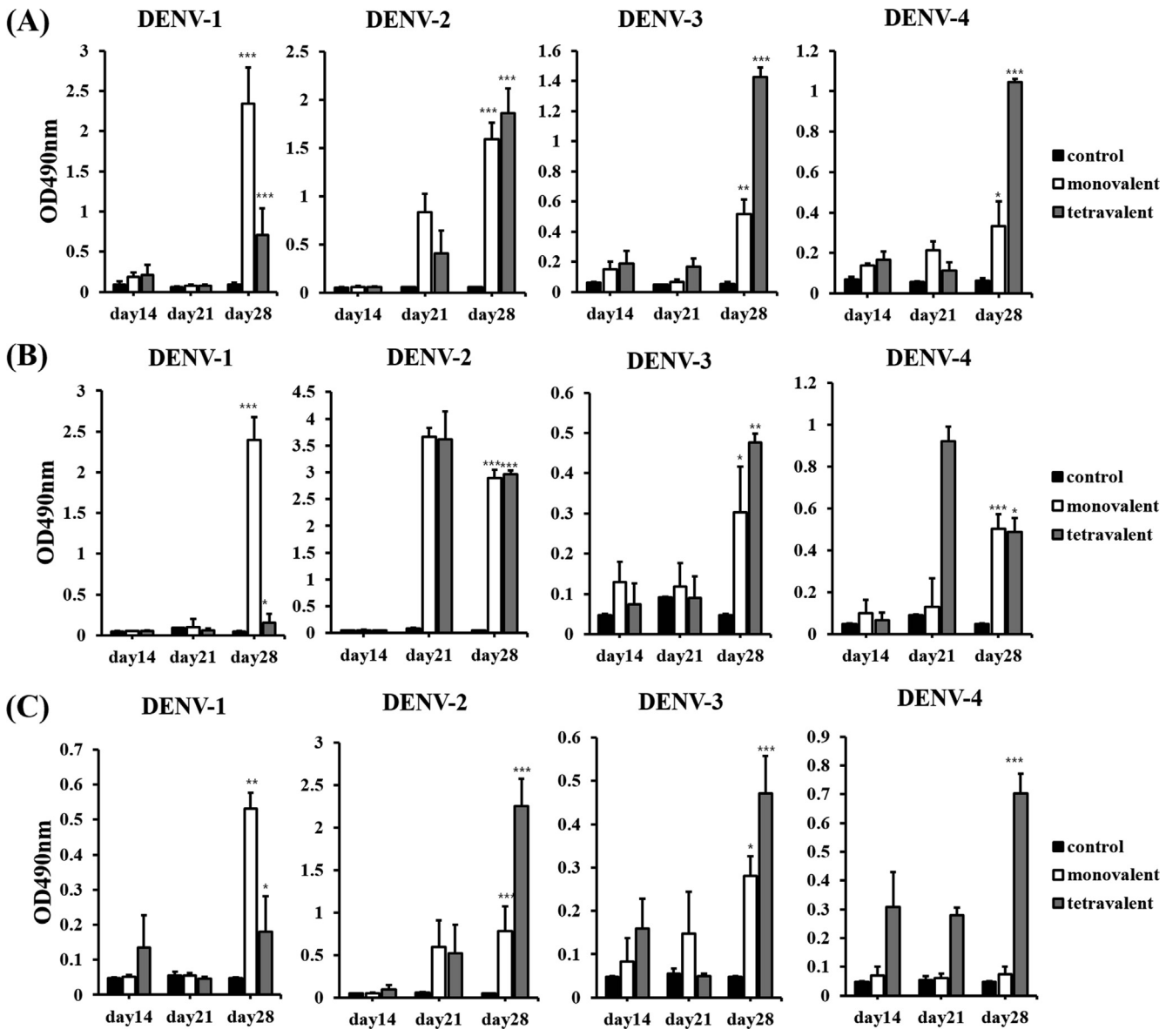

Fig. 1. Analysis of IgG antibody subclasses in mice immunized with monovalent and tetravalent BG-EDIII. Groups of BLAB/c mice were orally immunized with bacterial ghosts expressing monovalent or tetravalent dengue-EDIII, and mice that received BG-EDIII vaccine were intramuscularly boosted with recombinant protein at day 21 post vaccination. Serum samples were collected at 14, 21, and 28 days after the first immunization. (A) total IgG. (B) Subclass IgG1. (C) Subclass IgG2a. Data represent the mean ± standard deviation (sd) of 5 mice from two independent experiments. $^{*}p < 0.05$. $^{**}p < 0.005$. $^{***}p < 0.001$. The *p*-values indicate significant differences compared to the control group.

affects cellular receptor recognition and contains several neutralizing epitopes [25,49–51]. Previous research reported that an EDIII fragment carrying mutated neutralizing epitopes failed to be neutralized by conventional EDIII-specific monoclonal antibodies [52]. Anti-EDIII monoclonal antibodies act as powerful blockers against DENV infection and have a low intrinsic potential to induce cross-reactivity between serotypes [53,54]. Recent studies have shown that immunizing mice with an EDIII vaccine induced significant cellular immune responses upon stimulation with DENV-EDIII antigen [24,42,55]. Taken together, these findings suggest that DENV-EDIII is a promising target to induce both humoral and cellular immunity against all four serotypes of DENV infection. In the present study, we constructed BGs expressing the EDIII of all DENV serotypes (−1, −2, −3 and −4). BG is a non-living cell envelope of Gram-negative bacteria produced by the controlled expression of the lysis gene *E* of bacteriophage phiX174 [21,31]. The expression of envelope-antigen fusion proteins does not interrupt protein folding, so it preserves the immunogenicity of foreign antigenic proteins [30,56]. The expression of DENV-EDIII for all four serotypes in the ST-BGs was confirmed *in vitro* by Western blot analysis. The immunoreactive band of the target protein was detected at ~20 kDa (Supplementary Data. 2B). The formation of a transmembrane on the surface of the BG-EDIII constructed by gene *E* lysis under optimal conditions was confirmed by SEM analysis (Supplementary data. 4).

The DENV-EDIII antigen exhibited effective immunoreactivity, with polyclonal DENV EDIII-specific antibodies (Abs) responsible for providing defense against DENV infection [25,50]. Several subclasses of IgG response, such as IgG, IgG2a, IgG2b, and IgG3, were also detected in sera sampled from mice infected with a live DENV strain [57,58]. We also found that titers of IgG, IgG1, and IgG2a were augmented in mice that received monovalent or tetravalent BG-EDIII vaccination (Fig. 1). In particular, the titers of EDIII-1-specific IgG, IgG1, and IgG2a were significantly higher in mice

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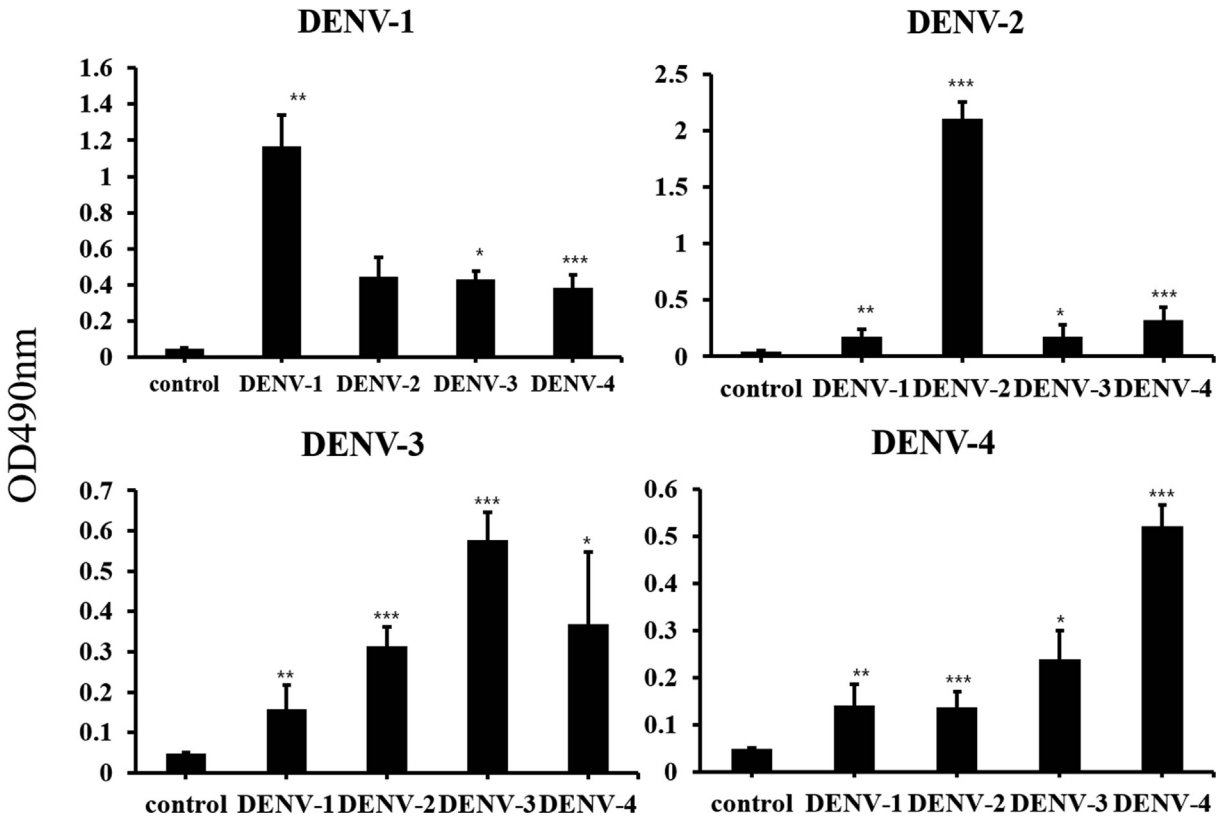

Fig. 2. Cross-reactivity against DENV 1–4 by IgG. Cross-reactivity against DENV1-4 recombinant protein by IgG indirect ELISA performed on sera samples collected at day 28 post-first vaccination. Serum IgG increased for each specific serotype in mice vaccinated with BG-EDIII. Data represent mean ± sd. *$p < 0.05$. **$p < 0.005$. ***$p < 0.001$. The p-values indicate significant differences compared to the control group.

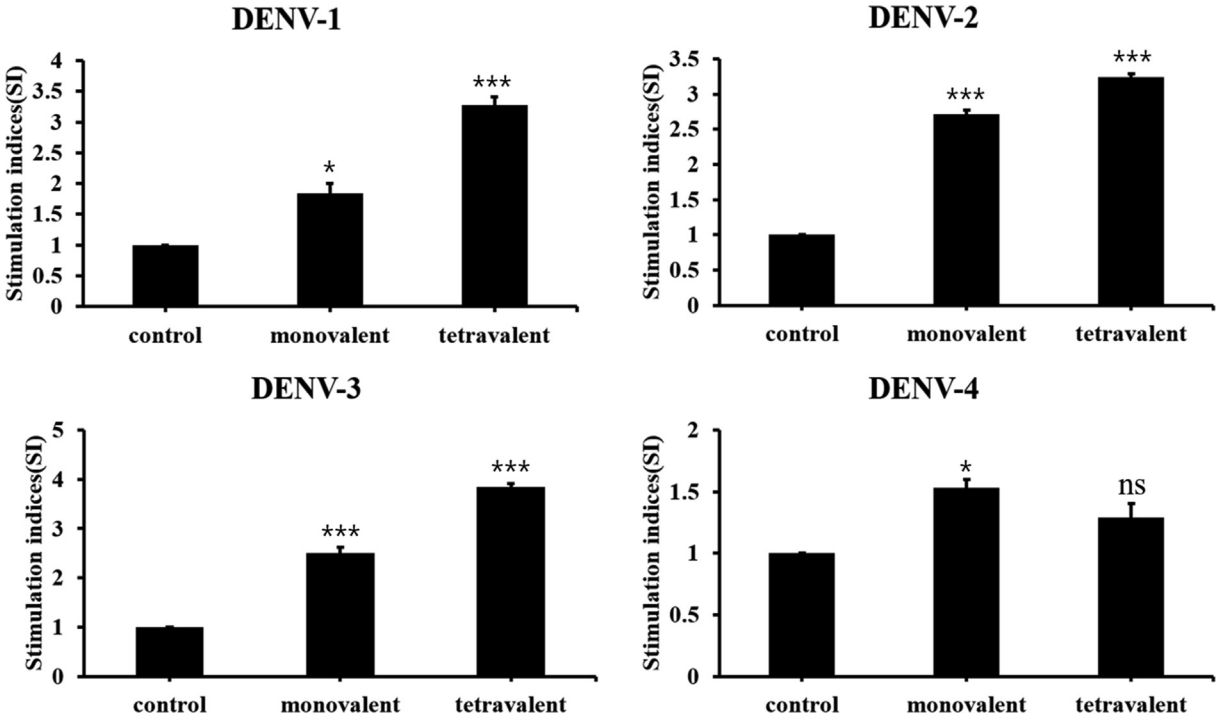

Fig. 3. Analysis of cell-mediated immune responses in vaccinated and non-vaccinated mice. Groups of BALB/c mice were vaccinated once orally with BG-EDIII monovalent and tetravalent vaccine. Cellular immune responses represented by lymphocyte proliferation were determined from an MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]-based assay after *in vitro* stimulation of isolated lymphocytes with recombinant EDIII protein, and results are expressed as stimulation indices (SI). Error bars indicate the sd.

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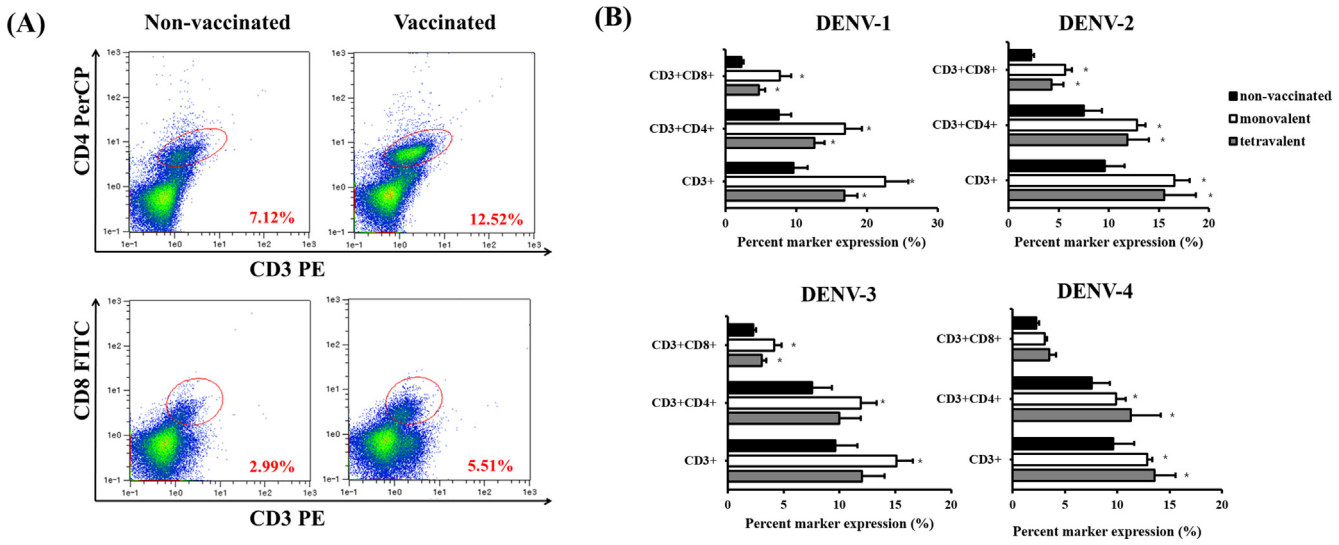

Fig. 4. FACS analysis of splenic T lymphocytes. (A) Representative flow cytometry histogram plots for $CD4^+$ and $CD8^+$ splenic T cell populations. (B) Change in T cell subpopulations in non-vaccinated mice and vaccinated mice. Error bars indicate the sd. *$p < 0.05$. The p-value indicates significant differences compared to the control group.

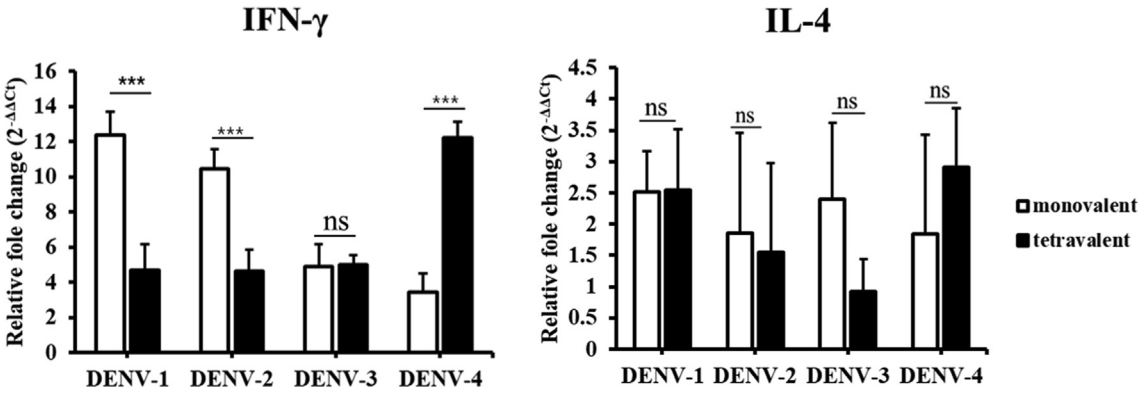

Fig. 5. Cytokine analysis in splenocytes following *in vitro* re-stimulation. mRNA transcript levels of IFN-γ and IL-4 were evaluated by RT-PCR of primed splenocytes pulsed *in vitro* with each antigen. (A) IFN-γ. (B) IL-4. Error bars indicate the sd. *p < 0.05. **p < 0.001. The p-values indicate significant differences compared to the control group.

immunized with BG-DENV-1 than in mice immunized with the tetravalent vaccine candidate. The results suggest that our BG-EDIII vaccine candidates sufficiently induce diverse subclasses of IgGs against the target DENV protein even without the use of an adjuvant formulation.

Antibody-dependent enhancement (ADE) is a phenomenon mediated by pre-existing DENV IgG Abs cross-reacting with different DENV serotypes [59]. The pre-existing non-neutralizing Abs induced by the first infection with a different DENV serotype form immune-complexes that can boost virus production by suppressing intracellular antiviral responses [60]. Given the characteristics of DENV infection, the vaccine-induced antibodies to EDIII needed to have low cross-reactivity to reduce the risk of ADE. In this study, we tested the cross-reactivity of the DENV-EDIII Abs against the other serotypes of recombinant DENV-EDIII delivered by the ST-BGs, and IgG specific to EDIII fused with a his-tag mainly reacted with the specific target protein of its own serotype, not with the other serotypes (Fig. 2). This result shows that the constructs efficiently induce highly specific Abs against each recombinant DENV-EDIII expressed in the ST-BGs, which should minimize the risk of ADE.

An effective vaccine against DENV should trigger the cellular immune response and efficiently lower viral load [61]. In the current study, the BG-EDIII vaccine candidate markedly increased lymphocyte proliferative activity in the immunized mice in response to stimulation with the recombinant DENV-EDIII protein (Fig. 3). The immunization also successfully drove naïve T-cell subsets to become effector $CD4^+$ and $CD8^+$ cells (Fig. 4). Concurrently, cytokines activated by $CD8^+$ T-cells, such as IFN-γ [62], were efficiently up-regulated in splenocytes from the BG-EDIII tetravalent vaccinated mice that were re-stimulated with DENV-EDIII protein *in vitro* (Fig. 5). Previous research reported that a panel of 15-mer peptide-sized epitopes included in the EDIII region were the regions efficiently recognized by $CD4^+$ and $CD8^+$T cells in mice [24]. Those findings clearly showed that tetravalent vaccine candidates sufficiently induced antigen-specific T-cell mediated immune responses, indicating that antigen-specific T-cell responses are critical to protection against DENV by reducing the severity of disease manifestations [63,64]. Furthermore, in the challenge study with DENV-infected K562 cells, the plasma viremia level was reduced in the vaccinated mice compared with the non-vaccinated mice (Fig. 6). The reduction in viral replication in the murine model could be caused by an immunostimulatory effect elicited by the ST-EDIII vaccination.

In conclusion, we demonstrated that each of the four DENV-EDIII serotypes was successfully adapted and expressed in an ST strain lysed by gene *E* activation. The constructs induced not only humoral immunity but also CMI against the target antigen, and

Please cite this article as: E. Kim, G. Won and J. H. Lee, Construction of a novel tetravalent dengue vaccine with a *Salmonella* Typhimurium bacterial ghost and evaluation of its immunogenicity and protective efficacy using a murine model, Vaccine, https://doi.org/10.1016/j.vaccine.2019.10.075

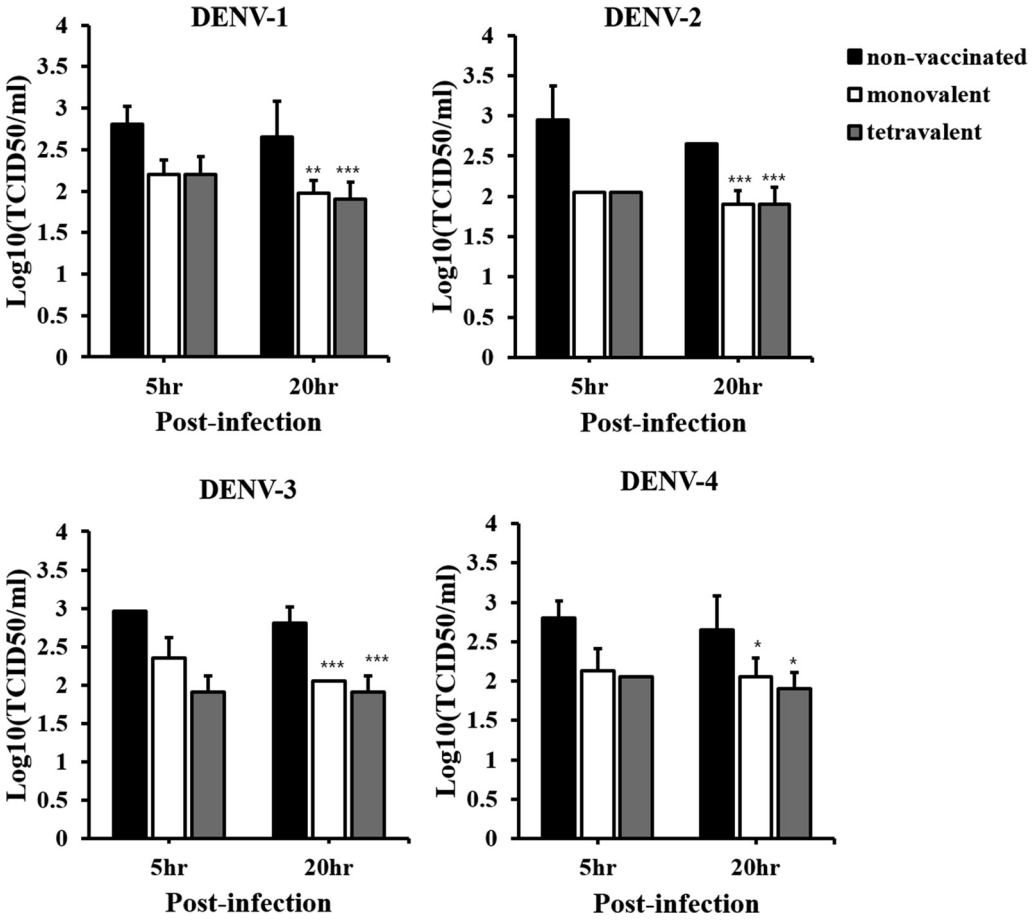

Fig. 6. Analysis of viremia levels in BG-EDIII vaccinated mice. Mice were vaccinated once orally with BG-EDIII and boosted with EDIII recombinant protein at day 21 post-vaccination. The mice were intraperitoneally challenged with DENV-infected K562 cells. The mice were bled via orbital sinus after a 24hr challenge, and plasma was extracted for analysis. Data represent mean ± standard deviation in 5 mice from two independent experiments. $^{*}p < 0.05$. $^{**}p < 0.005$. $^{***}p < 0.001$. The *p*-values indicate significant differences compared to the control group.

they conferred protective potential against a peritoneal-injection of DENV-infected cells in mice immunized with either the monovalent or tetravalent vaccine candidates. Although all the parameters for determining the immunogenicity of the target protein expressed in the inactivated ST were evaluated in a murine model and not the natural host, this preliminary study suggests that a certain level of immunity specific to the target protein could be expected in an infected host.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2019R1A6A1A03033084).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.vaccine.2019.10.075.

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