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Recombinant lipidated dengue-4 envelope protein domain III elicits protective immunity

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ABSTRACT

The combination of recombinant protein antigens with an immunostimulator has the potential to greatly increase the immunogenicity of recombinant protein antigens. In the present study, we selected the dengue-4 envelope protein domain III as a dengue vaccine candidate and expressed the protein in lipidated form using an *Escherichia coli*-based system. The recombinant lipidated dengue-4 envelope protein domain III folded into the proper conformation and competed with the dengue-4 virus for cellular binding sites. Mice immunized with lipidated dengue-4 envelope protein domain III without exogenous adjuvant had higher frequencies of dengue-4 envelope protein domain III-specific B cells secreting antibodies than mice immunized with the nonlipidated form. Importantly, lipidated dengue-4 envelope protein domain III-immunized mice demonstrated a durable neutralizing antibody response and had reduced viremia levels after challenge. The study demonstrates that lipidated dengue-4 envelope protein domain III is immunogenic and may be a potential dengue vaccine candidate. Furthermore, the lipidation strategy can be applied to other serotypes of dengue virus.

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1. Introduction

Dengue virus is transmitted by mosquitoes, and infection causes dengue fever or severe dengue hemorrhagic fever and dengue shock syndrome. These diseases are important arthropod-borne viral diseases [1]. Dengue occurs in more than 120 countries throughout tropical and subtropical areas [2]. The growing public health threat of dengue is supported by its wide-spread presence and increasing number of cases. According to estimates, there are 390 million dengue infections per year [3]. It is generally accepted that vaccination is a cost-effective strategy to fight infectious diseases. However, the complex interaction of 4

serotypes of dengue virus with the immune system has complicated the development of an effective vaccine. Consequently, a licensed dengue vaccine is not currently available. Several vaccine candidates using different approaches are being assessed in clinical studies [4]. These approaches include using live attenuated virus [5,6], live chimeric virus [7,8], subunit vaccines [9], and DNA vaccines [10,11]. The most advanced dengue vaccine candidate is Sanofi Pasteur's live chimeric virus vaccine [12]. A phase 2b study of this tetravalent dengue vaccine in Thai schoolchildren has been completed [8]. The results obtained from this study were disappointing because the overall efficacy of the vaccine candidate was low, at 30.2%. Thus, further efforts are required to develop dengue vaccines.

Dengue envelope protein domain III (ED III) folds independently and is accessible and exposed on the virion surface. It has been demonstrated that ED III is the critical region for viral attachment to cellular receptors [13,14]. Several neutralizing epitopes have been mapped to ED III [15–17], indicating that ED III is a suitable target for dengue vaccine development [18]. Recently, ED III subunit vaccine candidates have been evaluated in mice [19–24] and nonhuman primates [25–29]. In general, the immunogenicities of recombinant subunit vaccines are poor, and adjuvants are necessary to enhance immune responses. Unfortunately, aluminum-containing adjuvants, which are the most widely used adjuvants in human vaccines, may not be suitable for complete protection against dengue viral infection [22,27,29].

Abbreviations: D4ED III, dengue-4 envelope protein domain III; ED III, envelope protein domain III; FFUs, focus-forming units; FRNT, focus reduction neutralization tests; LD4ED III, lipidated D4ED III.

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To overcome the obstacles associated with the low immunogenicity of recombinant proteins, we expressed high levels of recombinant protein in lipidated form to enhance the immunogenicity of the recombinant protein [30]. We found that the lipid moiety of the recombinant lipoprotein provided a danger signal that triggered antigen-presenting cell activation via toll-like receptor 2 [31]. Such activation further enhanced immune responses in the absence of exogenous adjuvants [23,24,32]. Herein, we describe the production of the recombinant lipidated dengue-4 envelope protein domain III (LD4ED III) and demonstrate its vaccine potential.

2. Materials and methods

2.1. Virus

Dengue-4/H241 was used for this study. Virus propagation was performed in C6/36 cells, and viral titers were determined by focus-forming assays with BHK-21 cells [32,33].

2.2. Preparation of recombinant proteins

The amino acid sequence of the dengue-4 envelope protein domain III (D4ED III) was described previously [33]. Based on the amino acid sequence of D4ED III, the DNA sequence was determined via *Escherichia coli* codon usage and was fully synthesized by a biotechnology company (Purigo Biotechnology Co., Taipei, Taiwan). The synthesized DNA was then amplified by PCR to generate pD4DE III and pLD4DE III plasmids. *E. coli* BL21 (DE3) (Invitrogen, Carlsbad, CA) and C43(DE3) (Lucigen, Middleton, WI) cells were transformed with pD4DE III and pLD4DE III cells to express D4ED III and LD4ED III, respectively.

After isopropylthiogalactoside (IPTG) induction, recombinant protein was purified by immobilized metal affinity chromatography (IMAC) columns (QIAGEN, Hilden, Germany). The fractions from each critical step were analyzed by SDS-PAGE and immunoblotted with anti-His-tag antibodies. The lipid moiety in LD4ED III was further identified by a MALDI micro MX mass spectrometer (Waters, Manchester, UK). All the details of preparation of recombinant antigens were in Supplementary data.

2.3. Inhibition of dengue virus infection in BHK-21 cells by D4ED III and LD4ED III

To test whether D4ED III and LD4ED III blocked dengue virus infection of BHK-21 cells, the virus was pre-mixed with different amounts of D4ED III, heat-denatured D4ED III, LD4ED III, heat-denatured LD4ED III, or control bovine serum albumin (BSA) as indicated for 10 min at 4 °C. The viral titer prior to pre-mixing was approximately 20–40 FFUs per well. Viral adsorption was allowed to proceed for 3 h at 37 °C. The FFUs were determined by focus-forming assays.

2.4. Experimental mice and immunization

Female BALB/c mice were purchased from the National Laboratory Animal Breeding and Research Center (Taipei, Taiwan). The mice were maintained at the Laboratory Animal Center of the National Health Research Institutes (NHRI). All animal studies were approved and performed in compliance with the guidelines of the Animal Committee of the NHRI. Groups of mice (6–8 weeks of age) were immunized subcutaneously with recombinant D4ED III or LD4ED III. The lyophilized D4ED III and LD4ED III were reconstituted with PBS. Each mouse received a 10 µg/0.2-mL dose. Mice were given 2 immunizations at a 4-week interval with the same regimen. This immunization protocol was used throughout the present

study. Blood was collected by tail bleeding for 0.1–0.2 mL from each mouse at different time points as indicated. Sera were prepared and stored at –20 °C until use.

2.5. Flow cytometry

To determine the number of D4ED III-specific B cells, bone marrow cells were collected at 3–4 weeks after the second immunization. Single-cell suspensions were prepared for flow cytometry. Nonspecific staining was blocked by incubation with a rat anti-mouse CD16/CD32 antibody (93, eBioscience) in PBS for 10 min at 4 °C. Cells were stained with phycoerythrin-cyanine 7-conjugated anti-B220 (RA3-6B2, eBioscience), allophycocyanin-conjugated anti-CD19 (1D3, BD Biosciences), and D4ED III. After washing, cells were incubated with biotin-conjugated anti-His-tag antibodies, and the D4ED III-bound cells were stained with phycoerythrin-conjugated avidin (Sigma-Aldrich). The results were acquired using the CellQuest Pro software on a BD FACSCalibur and were analyzed using FACS 3 software.

2.6. Enzyme-linked immunospot (ELISPOT) assays

To detect and quantify individual anti-D4ED III antibody-secreting B cells, bone marrow cells were analyzed by ELISPOT. All the details were in Supplementary data.

2.7. Measurement of antibody titers

The levels of anti-D4ED III IgG in serum samples were determined by titration. All the details were in Supplementary data.

2.8. Focus reduction neutralization tests (FRNT)

Sera were diluted using a 2-fold serial dilution (starting at 1:8), and the sera were heat-inactivated prior to testing. A monolayer of BHK-21 cells in 24-well plates was inoculated with dengue-4 virus that had been incubated at 4 °C overnight with pre-immunization or post-immunization sera in a final volume of 0.5 mL. The FFUs were determined by focus-forming assays. The neutralizing antibody titer FRNT₇₀ was calculated as the highest dilution that produced a 70% reduction in FFUs compared with control samples containing the virus alone. For calculation purposes, the neutralizing antibody titer was designated as 4 when the neutralizing antibody titer was less than 8.

2.9. Challenge

Sixteen weeks after the second immunization, mice were intraperitoneally injected with 5×10^7 dengue-4-infected K562 cells suspended in 0.5 mL of serum-free RPMI medium [34]. Blood samples were collected 8 h after K562 injection. The blood (0.2 mL) was mixed with 0.02 mL of 3.8% sodium citrate pre-chilled on ice. Plasma was isolated, and the viral titer was determined by focus-forming assays with BHK-21 cells. The detection limit of the assay was $2.3 \log_{10}$ FFU/mL. Any infective titers below the limit of detection were assigned a value of 2.0.

2.10. Statistical analyses

Statistical analyses were performed with ANOVA and a Bonferroni post test using GraphPad Prism software version 5.02 (GraphPad Software, Inc.). Differences with a *p*-value of less than 0.05 were considered statistically significant.

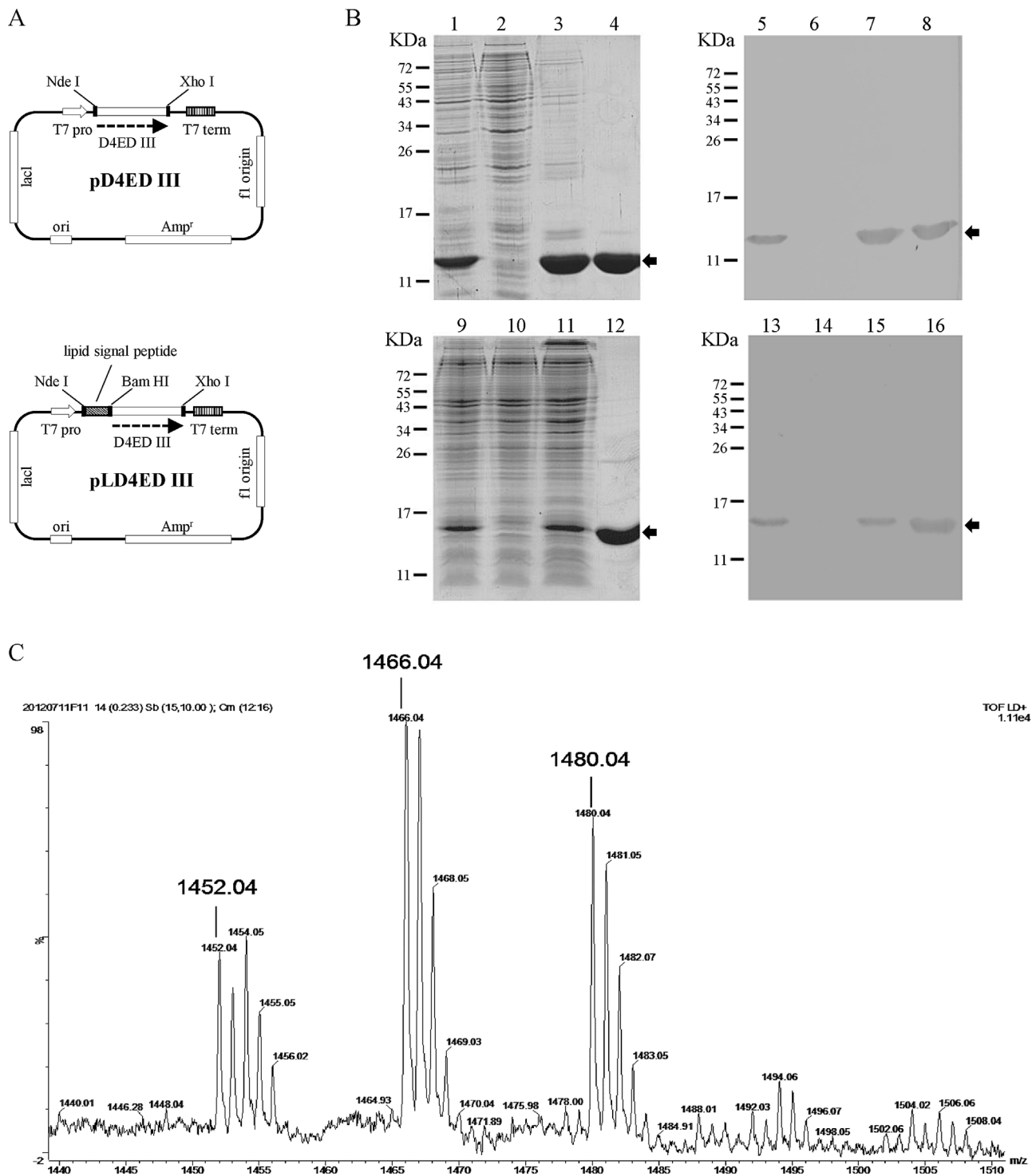


Fig. 1. Cloning, production, and identification of recombinant D4ED III and LD4ED III. (A) The amino acid sequence of D4ED III is a consensus sequence of dengue-4 [33]. The DNA sequence of D4ED III was derived by codon usage of *E. coli* and was fully synthesized by a biotechnology company (see Section 2). The PCR product was cloned into the pET-22b(+) vector to generate the pD4ED III expression plasmid for the production of D4ED III. To produce lipidated D4ED III (LD4ED III), the pD4ED III construct was cloned into the pET-22b(+) vector with a lipid signal peptide in front of the D4ED III gene to generate the pLD4ED III plasmid. Both recombinant proteins contained an additional HHHHHH sequence (His-tag) at their C-termini and were expressed under the control of the T7 promoter. (B) The purification processes of the D4ED III protein were monitored using 15% reducing SDS-PAGE followed by Coomassie Blue staining and immunoblotting using anti-His-tag antibodies (lanes 1 to 8). D4ED III was expressed in *E. coli* strain BL21 (DE3). Lane 1, D4ED III expression after IPTG induction; lane 2, protein expression in the absence of IPTG induction; lane 3, soluble fraction of D4ED III; lane 4, purified D4ED III. Lanes 5–8 show immunoblotting to monitor D4ED III induction and the purification process; the samples in these lanes are the same as those in lanes 1–4. The arrows indicate the electrophoretic positions of D4ED III in the gels or blots. The LD4ED III protein purification process was monitored using 15% reducing SDS-PAGE followed by Coomassie Blue staining and immunoblotting using anti-His-tag antibodies (lanes 9 to 16). LD4ED III was expressed in *E. coli* strain C43 (DE3). Lane 9, LD4ED III expression after IPTG induction; lane 10, protein expression in the absence of IPTG induction; lane 11, soluble fraction of LD4ED III; lane 12, purified LD4ED III. Lanes 13–16 show immunoblotting to monitor the LD4ED III induction and purification processes. The samples in these lanes are the same as those in lanes 9–12. The arrows indicate the electrophoretic positions of LD4ED III in the gels or blots. (C) N-terminal LD4ED III fragments were obtained and identified after digestion of LD4ED III with trypsin. The tryptic fragments of LD4ED III were analyzed on a Waters® MALDI micro MX™ mass spectrometer. The mass spectra revealed the existence of 3 major peaks with m/z values of 1452, 1466, and 1480.

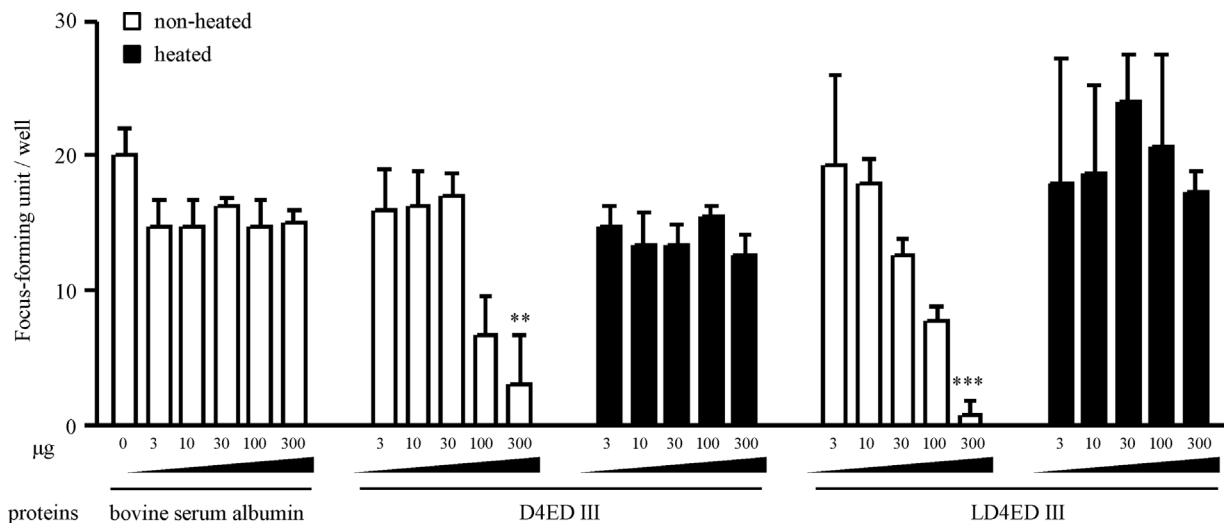


Fig. 2. Infection of dengue-4 virus is blocked by D4ED III and LD4ED III. Dengue-4 virus was pre-mixed with different amounts of bovine serum albumin, D4ED III, or LD4ED III as indicated for 10 min at 4 °C. The heat-denatured protein was used in parallel experiments as controls. Viral adsorption on a monolayer of BHK-21 cells in 24-well plates proceeded for 3 h at 37 °C. The focus-forming units were determined 3 days after the viral infection. Data represent the mean \pm SD of triplicate wells. The results shown are representative of 2 independent experiments. Statistical significance was determined by ANOVA with Bonferroni post test at the same dosage of bovine serum albumin. ** $p < 0.01$; *** $p < 0.0001$.

3. Results

3.1. Preparation and characterization of dengue-4 envelope protein domain III recombinant antigens

The D4ED III gene was cloned into the pET-22b(+) expression vector to produce the plasmids, pD4ED III and pLD4ED III, which were used for the production of the recombinant antigens, D4ED III and LD4ED III, respectively. Both antigens contained an additional hexahistidine sequence (His-tag) at their C-termini and were expressed under the control of the T7 promoter (Fig. 1A). The purification of D4ED III and LD4ED III was monitored and analyzed by SDS-PAGE and immunoblotting (Fig. 1B). After removing LPS, the residual LPS in D4ED III and LD4ED III were less than 0.03 EU/ μ g and 0.04 EU/ μ g, respectively. The yields of D4ED III and LD4ED III were 75 mg/L and 10 mg/L, respectively. First, the exact mass of trypsin-digested N-terminal fragments of LD4ED III was measured. Three major peaks with m/z values of 1452, 1466, and 1480 were identified (Fig. 1C). These peaks have been previously identified as a lipidation signature in other lipidated proteins [23,30,32].

It has been demonstrated that dengue envelope protein domain III is the critical site that is associated with binding to host cell receptors [13,14,22,23]. We evaluated the ability of D4ED III and LD4ED III to hinder dengue viral infection. As shown in Fig. 2, the infection of BHK-21 cells with dengue-4 virus was blocked in the presence of D4ED III in a dose-dependent manner compared with control bovine serum albumin. The ability of LD4ED III to inhibit dengue-4 viral infection was comparable to non-lipidated D4ED III. In contrast, heat-denatured D4ED III and LD4ED III lost the capacity to block dengue-4 viral infection. These results suggest that recombinant D4ED III and LD4ED III maintain the proper conformation and can compete with the dengue-4 virus for cellular binding sites.

3.2. Evaluation of the humoral immune response against dengue-4 envelope protein domain III in mice

To determine the ability of the recombinant antigens to induce D4ED III-specific B cells, bone marrow cells were analyzed by flow cytometry (Fig. 3A). The percentage of D4ED III-specific B cells in B220⁺ CD19⁺ bone marrow cells of D4ED III-immunized mice was $0.05 \pm 0.02\%$, which was equivalent to PBS-immunized mice

($0.03 \pm 0.01\%$). In contrast, the percentage of D4ED III-specific B cells in B220⁺ CD19⁺ bone marrow cells of LD4ED III-immunized mice was $0.43 \pm 0.11\%$, which was significantly higher than that in D4ED III- ($p < 0.0001$) and PBS-immunized mice ($p < 0.0001$). A representative flow cytometry staining panel gated on B220⁺CD19⁺ cells is shown in Fig. 3A (upper panel).

To quantify the frequencies of antibody-secreting cells, total bone marrow cells were analyzed by ELISPOT (Fig. 3B). The frequency of D4ED III-specific antibody-secreting cells in PBS-immunized mice was 3.7 ± 2.7 cells per 10^5 cells. D4ED III-immunized mice showed no increase in the frequency of D4ED III-specific antibody-secreting cells (2.3 ± 1.6 cells per 10^5 cells). However, a significantly increased frequency of D4ED III-specific antibody-secreting cells was detected in LD4ED III-immunized mice (24.6 ± 13.8 cells per 10^5 cells, $p < 0.01$). Representative images of the B cell ELISPOT are shown in the upper panel of Fig. 3B.

We then evaluated the levels of D4ED III-specific antibodies in the sera at different time points after immunization. As shown in Fig. 4, the levels of D4ED III-specific antibodies in mice immunized with D4ED III were comparable to those in control mice (PBS-immunized). Importantly, the titers of D4ED III-specific antibodies in LD4ED III-immunized mice were increased to $10^{3.0}$ – $10^{3.7}$ after the priming vaccination. D4ED III-specific antibody titers were further elevated after the boosting vaccination (range from $10^{5.0}$ to $10^{6.0}$) and were maintained for 20 weeks after the priming vaccination. These results indicate that LD4ED III induces higher antibody responses than the non-lipidated D4ED III ($p < 0.001$).

3.3. Induction of durable protective immunity against dengue-4 virus in mice

Because LD4ED III elicited D4ED III-specific antibody responses, we next assessed the capacity of the antibodies to neutralize dengue-4 virus. As shown in Fig. 5, no neutralizing antibody activities were observed in mice immunized with D4ED III or PBS (<1:8). Interestingly, sera obtained from mice immunized with LD4ED III induced low but significant neutralizing antibody responses. The neutralizing antibody titers peaked at 12 weeks after the priming vaccination and were within the 1:8–1:16 dilutions.

The major objective of this study was to explore whether LD4ED III could induce protective immunity. Mice are not the natural host

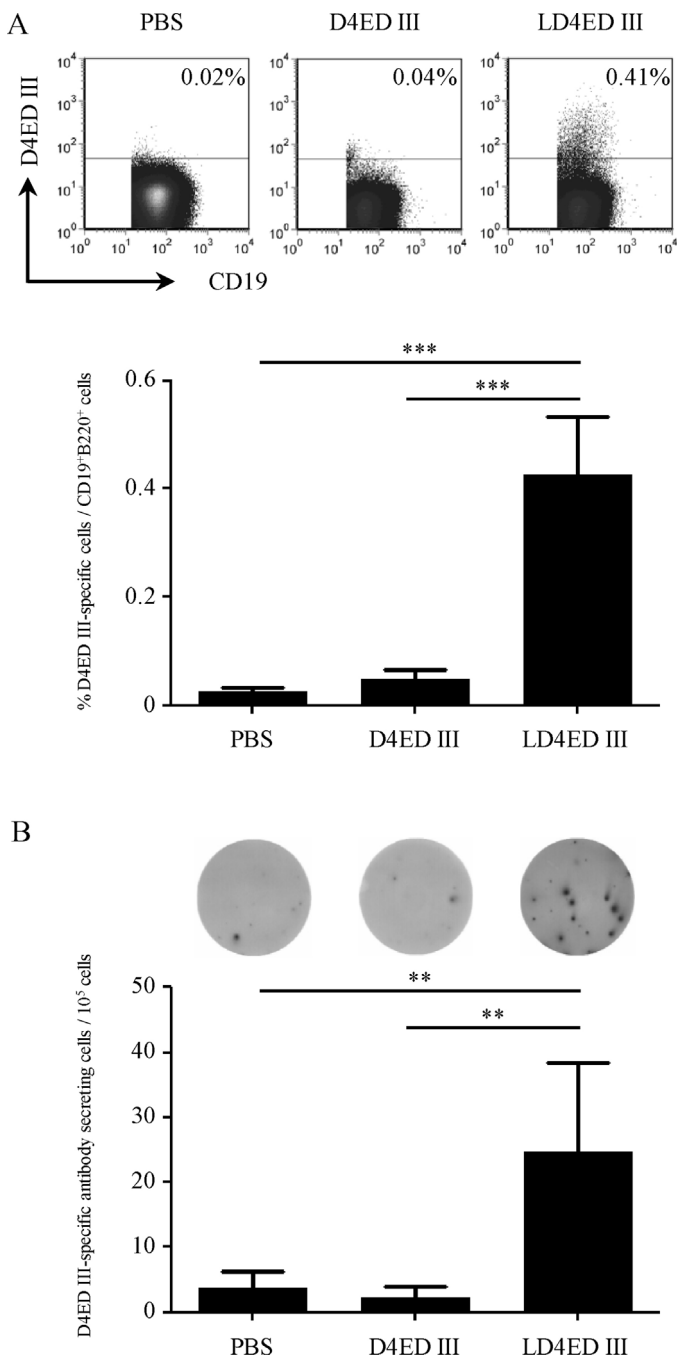


Fig. 3. Identification of D4ED III-specific B cells in the bone marrow of immunized mice. BALB/c mice were immunized subcutaneously twice with 10 μ g of D4ED III ($n=5$) or LD4ED III ($n=5$) in PBS at a 4-week interval. Mice immunized with PBS ($n=2-5$) alone (without antigens) served as controls. Bone marrow cells were harvested 7 weeks after the first immunization. (A) CD19⁺B220⁺ B cells were gated, and the expression of the D4ED III-specific antibody was analyzed. The numbers indicate the percentages of cells expressing D4ED III-specific antibody. A representative result from each group is shown in the upper panel. The means and standard deviations obtained from different mice are shown in the lower panel. (B) D4ED III-specific antibody-secreting cells were evaluated by ELISPOT. A representative well from each group is shown in the upper panel. The means and standard deviations obtained from different mice are shown in the lower panel. One of 2 representative experiments is shown. Statistical significance was determined by ANOVA with Bonferroni post test. ** $p < 0.01$; *** $p < 0.0001$.

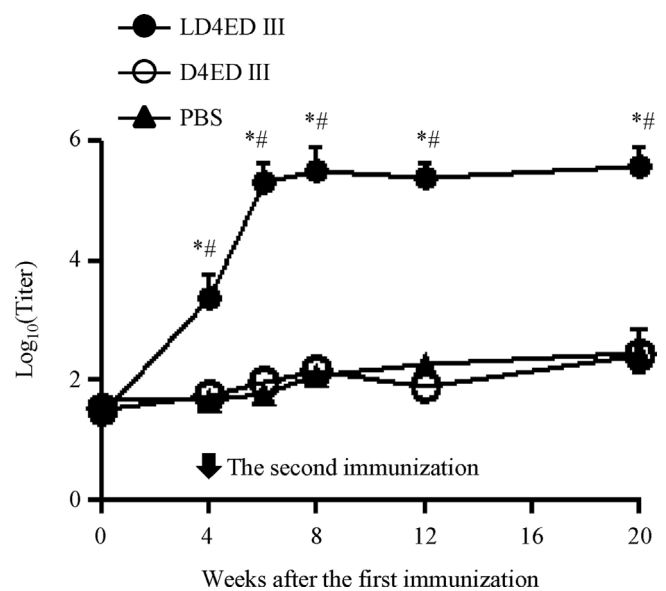


Fig. 4. Humoral immune responses in mice immunized with vaccine candidates. BALB/c mice were immunized subcutaneously twice with 10 μ g of D4ED III ($n=5$) or LD4ED III ($n=5$) in PBS at a 4-week interval. Mice immunized with PBS ($n=3$) alone (without antigens) served as controls. Sera were collected from mice at the indicated time points after the first immunization. IgG antibodies against D4ED III were evaluated by ELISA. Pre-immune sera were collected and used to determine basal levels for comparison. One of 2 representative experiments is shown. All antibody titers were logarithmically transformed before statistical analyses. Statistical significance was determined by ANOVA with Bonferroni post test. * $p < 0.001$ compared with PBS. # $p < 0.001$ compared with D4ED III.

of dengue virus. Dengue fever symptoms are not shown in mice after dengue viral infection. Some immunodeficiency or immunocompromised mice are susceptible to dengue viral infection and produce viremia. However, these animals are lack of a normal immune response, making them inadequate for vaccine evaluation. Yamanaka and Konishi [34] established a simple method for evaluating dengue vaccine effectiveness in laboratory strains of immunocompetent mice. To satisfy the requirement of validating vaccine candidates, we adopted this method. Groups of BALB/c mice were immunized with PBS, D4ED III or LD4ED III twice at a 4-week interval. Twenty weeks after the first immunization, the animals were challenged with dengue-4-infected K562 cells. The protective efficacy results of LD4ED III as determined by the level of viremia are shown in Fig. 6. The viral loads in the blood of D4ED III-immunized mice were $10^{3.4}$ – $10^{3.8}$ FFU/mL, which were comparable to those in PBS-immunized mice ($10^{3.0}$ – $10^{3.6}$ FFU/mL). However, the viral loads in the blood of LD4ED III-immunized mice were lower than the detection limit, $10^{2.3}$ FFU/mL. These results suggest that LD4ED III elicits neutralizing antibody responses and inhibits viremia.

4. Discussion

In the present study, we prepared recombinant LD4ED III using a novel *E. coli*-based system (Fig. 1) and evaluated the vaccine potential of LD4ED III. An important characteristic of adaptive immune responses is the induction of durable protection after initial exposure to a pathogen. Antibodies play a critical role in mediating humoral immune protection. Upon antigen stimulation, naïve B cells differentiate into antigen-specific B cells. Antibodies are then produced by plasma cells. It is believed that long-lived plasma cells migrate to the bone marrow. These bone marrow-resident long-lived plasma cells are responsible for long-term humoral immunity [35]. We demonstrated that D4ED III-specific antibodies were expressed on the surface of B cells in the bone marrow at higher frequencies in LD4ED III-immunized mice than in D4ED

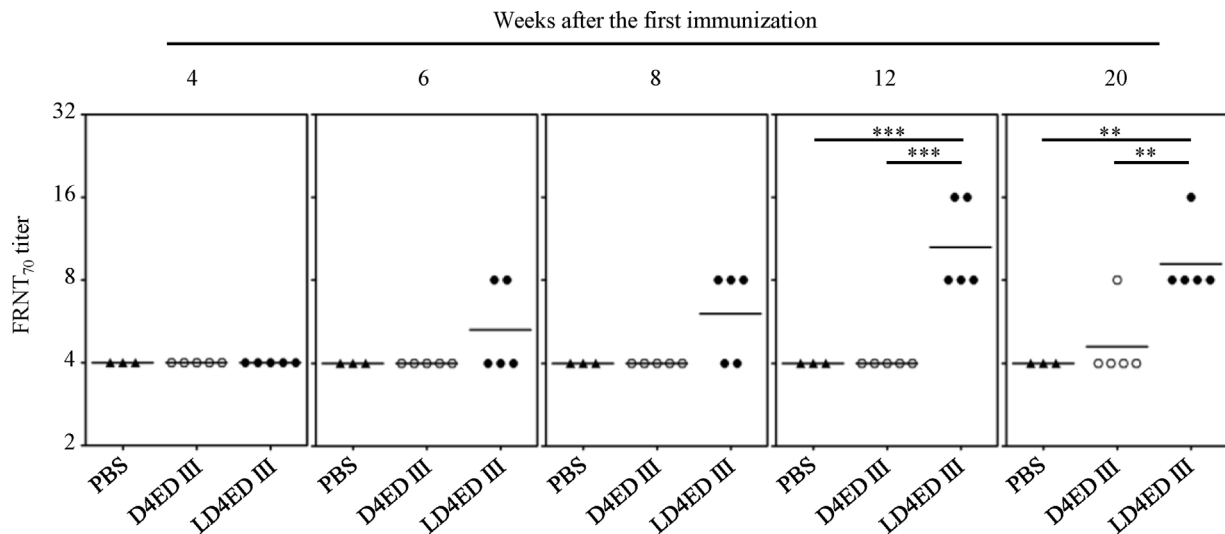


Fig. 5. Neutralizing antibody titers in mice immunized with vaccine candidates. BALB/c mice were immunized subcutaneously twice with 10 μ g of D4ED III ($n=5$) or LD4ED III ($n=5$) in PBS at a 4-week interval. Mice immunized with PBS ($n=3$) alone (without antigens) served as controls. Sera were collected from mice at the indicated time points after the first immunization. The dengue-4 virus neutralizing capacity was determined by FRNT. The neutralizing antibody titer was calculated as the highest dilution that resulted in a 70% reduction in FFU compared with control samples containing the virus alone. One of 2 representative experiments is shown. The neutralizing antibody titers were logarithmically transformed before statistical analyses. Statistical significance was determined by ANOVA with Bonferroni post test. ** $p < 0.01$; *** $p < 0.0001$.

III- or PBS-immunized mice (Fig. 3A). In addition, frequencies of D4ED III-specific antibody-secreting cells in the bone marrow of LD4ED III-immunized mice were higher than those in the bone marrow of D4ED III- or PBS-immunized mice (Fig. 3B). These results indicate that B cell clones bearing D4ED III-specific antibodies with antibody secreting capacity resided in the bone marrow. Consistent with these findings, we also showed that the D4ED III-specific antibody titers in the blood of LD4ED III-immunized mice were maintained at high levels throughout the study (Fig. 4). These

results suggest that D4ED III-specific long-lived plasma cells are generated in LD4ED III-immunized mice.

Recombinant LD4ED III stimulates neutralizing antibodies without exogenous adjuvant formulations (Fig. 5). These results are in agreement with previous studies using recombinant lipidated protein based on the envelope protein domain III. These studies showed that lipidated protein induced memory [24] or long-lasting neutralizing antibody [23,32] responses independently of exogenous adjuvants. Although we observed that the neutralizing antibody titers in mice immunized with LD4ED III were modest (Fig. 5), LD4ED III-immunized mice were efficiently able to suppress viremia (Fig. 6). Viremia is an important factor in dengue disease severity and transmission. Increased dengue disease severity correlates with high viremia [36]. These results suggest that LD4ED III elicits protective immunity.

In our previous studies, we demonstrated that the performance of lipidated antigens is superior or equivalent to non-lipidated antigens formulated with alum [30] or PELC (a multiphase emulsion system) [23]. To further illustrate the merit of lipidated antigens, groups of mice were immunized subcutaneously with D4ED III, D4ED III plus aluminum phosphate, or LD4ED III. Mice were given 3 immunizations at a 2-week interval. Serum samples were collected at week 12. D4ED III specific IgG titers and dengue-4 virus neutralizing capacities were shown in the Supplementary figure. The D4ED III specific IgG titers were significantly enhanced in mice immunized with the D4ED III plus aluminum phosphate. Importantly, mice immunized with LD4ED III induced the highest antibody responses among the other groups (Supplementary Fig. A). Notably, the neutralizing antibody titers were detected in LD4ED III immunized mice but not in mice immunized with D4ED III or D4ED III plus aluminum phosphate (Supplementary Fig. B). Altogether, these results demonstrate that the LD4ED III is better than that by the D4ED III without or with aluminum phosphate.

It is very important for the LD4ED III to fold into the correct conformation because some of the neutralizing epitopes in the envelope protein domain III are conformation-dependent [37,38]. Notably, LD4ED III that retains the proper conformation will elicit antibodies that recognize both linear and conformation-specific epitopes. For this reason, an ideal subunit vaccine using envelope protein domain III must be appropriately folded. In the present

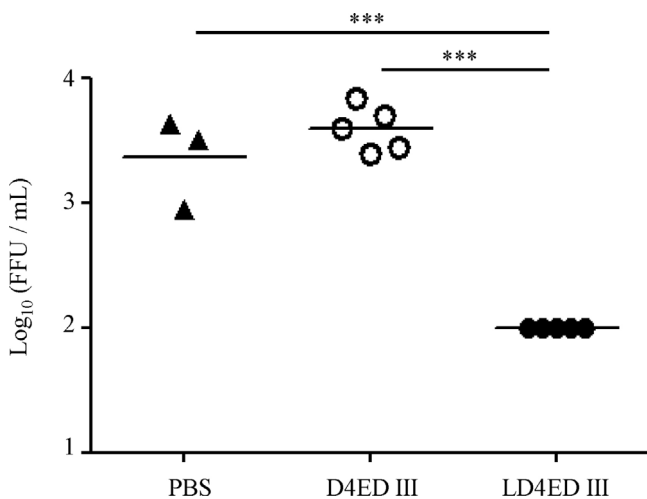


Fig. 6. Viremia in vaccinated mice after challenge with dengue-4 virus. BALB/c mice were immunized subcutaneously twice with 10 μ g of D4ED III ($n=5$) or LD4ED III ($n=5$) in PBS at a 4-week interval. Mice immunized with PBS alone (without antigens, $n=3$) served as controls. Sixteen weeks after the second immunization, mice were intraperitoneally challenged with dengue-4-infected K562 cells. The mice were bled 8 h after challenge. Plasma viral titers were determined by focus-forming assays using BHK-21 cells. The detection limit of the assay was 2.3 \log_{10} FFU/mL. The infective titers below the detection limit were assigned a value of 2.0. Symbols represent individual mice, and horizontal lines are the mean of experiments. One of 2 representative experiments is shown. Viremia levels were logarithmically transformed before statistical analyses. Statistical significance was determined by ANOVA with Bonferroni post test. *** $p < 0.0001$.

study, both D4ED III and LD4ED III formed the proper conformation necessary to block the cellular binding sites of dengue virus. However, the heat-denatured D4ED III and LD4ED III did not (Fig. 2). These results confirm that lipidation of the dengue envelope protein domain III did not interfere with proper folding of the dengue envelope protein domain III [23].

The recombinant subunit dengue vaccine approach is different than the live attenuated virus approach. Both of the vaccine approaches have advantages and disadvantages. In general, recombinant subunit dengue vaccine candidates are not very immunogenic. The administration of recombinant subunit dengue vaccine candidates with modern adjuvants is required to obtain a robust immune response [9]. However, the combination of adjuvant and vaccine will increase the cost of the vaccination. The price of a dengue vaccine is a key factor affecting the demand for dengue vaccine [39,40]. The results of the current study provide tangible evidence that LD4ED III induces protective immunity without the need for adjuvants in mice. Therefore, the cost of LD4ED III vaccination will be reduced, and the potential barrier of including dengue vaccines in national immunization programs will be reduced.

5. Conclusions

We demonstrated that LD4ED III expressed in *E. coli* retains the proper conformation to compete with dengue virus for cellular binding sites. We showed that LD4ED III is more immunogenic than its nonlipidated counterpart, D4ED III. Most importantly, LD4ED III alone triggered a neutralizing antibody response and inhibited viremia in immunized mice. These results provide important information for future clinical studies of the LD4ED III subunit vaccine.

Conflict of interest statement

HWC, CHL, SJL, and PC are named on patents relating to the lipidated vaccine against dengue virus infection. No other authors have conflicts of interest.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vaccine.2014.01.041>.

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