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Anti-DENV-ED3 antibody cross-talks generate immune interference among the four DENVs

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ABSTRACT

Objective: To evaluate the effects of primary anti-dengue virus envelop protein domain 3 (DENV-ED3) antibodies on secondary heterotypic anti-DENV ED3 antibody responses and the status of anti-DENV antibody responses against multivalent DENV ED3s in mice.

Methods: Four different DENV-ED3s were purified and their biophysical characteristics were confirmed. Swiss albino mice aged 3-4 weeks were immunized with four different DENV-ED3s and the anti-ED3 IgG responses were determined by ELISA.

Results: Firstly, the primary 1ED3-2ED3-3ED3 cross-reactive anti-DENV1 ED3 response boosted the secondary anti-2ED3 and anti-3ED3 antibody responses. In contrast, primary anti-2ED3 and anti-3ED3 antibodies neither had cross-recognition of 1ED3, nor had any effect on secondary anti-1ED3 response. Besides, the strict serospecificity of the anti-4ED3 sera did not affect other secondary anti-DENV ED3 responses. Secondly, 1ED3, 2ED3, and 3ED3 were co-dominantly immunogenic in trivalent ED3 formulations. However, the poorly immunogenic 4ED3 became almost non-immunogenic when injected after or together with 2ED3 and 3ED3, but showed slightly increased immunogenicity when injected with 1ED3, suggesting an adjuvanticity of 1ED3 on 4ED3's immunogenicity.

Conclusions: Although DENV1~4 ED3s share similar sequence homologies and structures, their immune induction potentials differ significantly in terms of immune magnitude, sero-specificity, and sero-cross-reactivity. Such intrinsic features of DENV1~4 ED3s may lead to 'antigen interference', limiting both the understanding of dengue etiology and the success of dengue vaccine development, which needs to neutralize all four DENV serotypes equivalently.

KEYWORDS: Dengue viruses (DENVs); DENV sero-specificity; DENV serotype-cross-talks; Primary DENV infections; Secondary DENV infections; Multivalent DENV-ED3s

Significance

The heterogeneity of DENV1-4's immmunogenicity remains a bottleneck for understanding dengue disease etiology and dengue vaccine success. Primary DENV1-4-ED3 immunization in mice resulted different anti-DENV IgG titers with different DENV1-4 sero-cross-recognition patterns. In multivalent DENV1-4-ED3 immunization, DENV2 and DENV3 were not only immunodominant over DENV4, but also suppressed DENV4's immunogenicity, while DENV1-ED3 boosted DENV4-ED3' immunogenicity. The immune interference of DENV1-4 should be in consideration for rational tetravalent DENV vaccine design.

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1. Introduction

Dengue, a severe recurrent global public health threat in the tropics and subtropics, is caused by four very similar but serologically distinct dengue virus serotypes (DENV1~4)[1–3]. Several dengue epidemics have already been reported in over 100 countries[4], and the global dengue burden has been increasing with the resurgence of new serotypes replacing the existing serotypes[5]. However, to date, little is known about the re-emergence of new serotypes with increased incidence and disease severity[6]. A primary single DENV serotype infection usually results in classical dengue fever, and patients recover within a few days to weeks and develop immunity against the infecting DENV serotype[7,8]. However, a secondary heterotypic DENV infection may increase both the dengue incidence and dengue disease severity, including dengue hemorrhagic fever and dengue shock syndrome, being contributed by cross-reacting sub-neutralizing primary anti-DENV antibodies[9], through antibody-dependent enhancement (ADE) mechanisms[9–13].

The DENV RNA encodes three structural proteins: the capsid (C), pre-membrane (preM), and envelope (E)[14]. The E-protein has three domains: ED1, ED2, and ED3[15], where ED3 is an immunoglobulin-like domain mediating host-virus interaction[15,16]. Furthermore, anti-DENV E-protein monoclonal antibodies could block DENV infections *in vivo* and *in vitro*[14,17,18]. In recent years, both the incidence of recurrent DENV infection and dengue disease severity have been increasing. Besides, the majority of new infection cases might be secondary dengue infections[19–21], being contributed by previous primary DENV infections. However, up to now, little information is available on the effects of a primary infection on secondary and/or recurrent DENV infections, most likely because of the unavailability of a handy model system enabling systematic studies.

In this study, we used ED3s alone instead of the live viruses to examine the primary anti-DENV ED3 IgG responses and their influences on the generation of secondary anti-DENV ED3 antibodies and anti-DENV ED3 antibody responses against concurrent multiple DENV ED3 immunizations in Swiss albino mice. Altogether, we observed that the immunogenicity of four DENV ED3s differed significantly in terms of serospecificity and serotype-cross-reactivity. Besides, each type of primary anti-DENV ED3 sera affected the response against secondary ED3 immunizations in a specific manner. In addition, although 2ED3 and 3ED3 decreased the immunogenicity of 4ED3, 4ED3 showed increased immunogenicity in the presence of 1ED3. In this research, we discussed how these intrinsic features of DENV1~4 ED3s might contribute to the limited understanding of dengue etiology and vaccine development.

2. Materials and methods

2.1. Mutant design, protein expression, and purification

The ED3 sequences of DENV1~4 serotypes were retrieved from

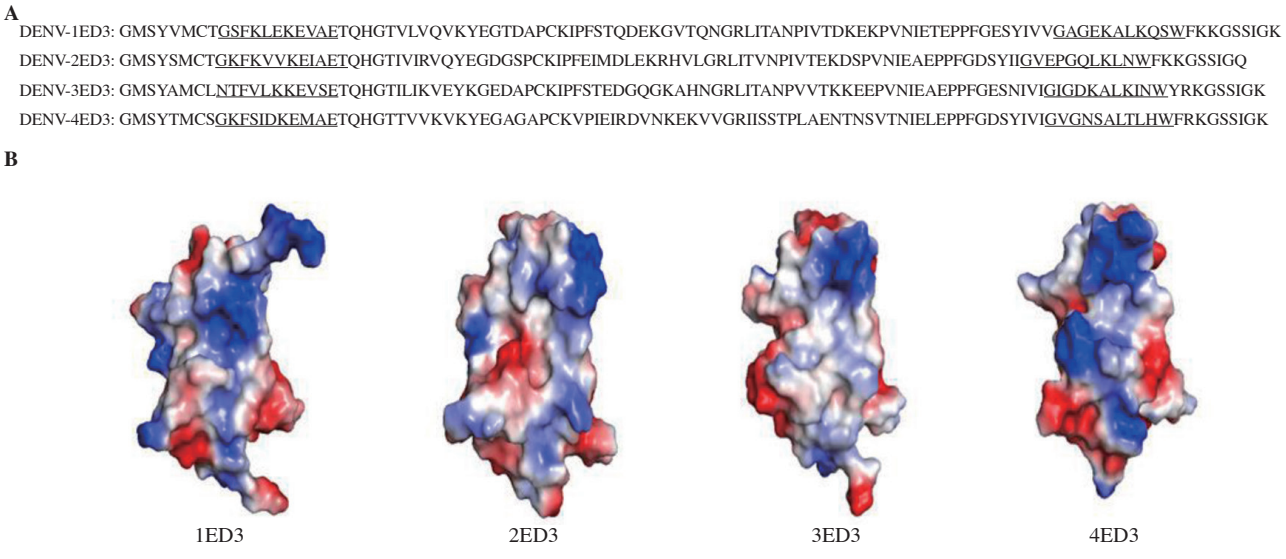

Figure 1. Sequences and structures of DENV1~4 ED3 variants. (A) The sequences of 1ED3, 2ED3, 3ED3, and 4ED3 were retrieved from the UniProt database. The putative epitope residues (epitope 1 and epitope 2) are underlined. (B) The electrostatic potential surface structures of DENV1-4 ED3s were generated by using Pymol graphics. Surface electrostatic negative, neutral, and positive potentials are displayed in red, white, and blue colors, respectively.

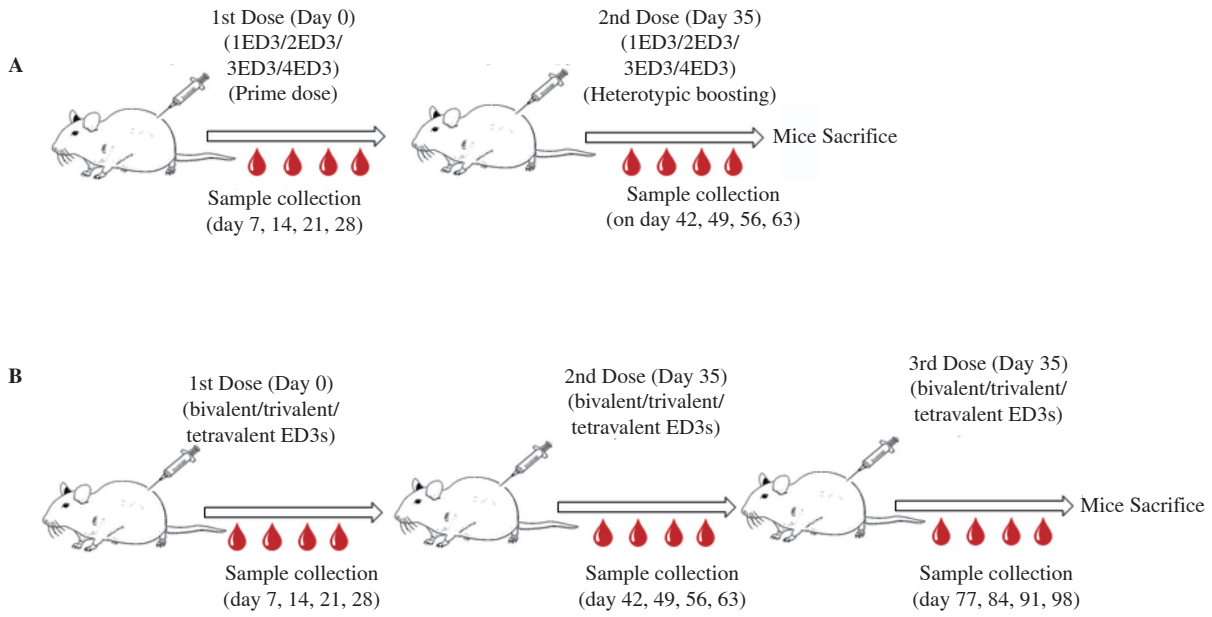

Figure 2. Mouse immunization scheme. (A) Primary and heterotypic secondary ED3 immunization. A total of 13 Swiss albino mouse groups were included. Groups 1-3, 4-6, 7-9, and 10-12 received 1ED3, 2ED3, 3ED3, and 4ED3, respectively, as primary immunogen (Prime dose). After 35 days, all mouse groups were injected with a heterotypic DENV ED3 as a secondary immunogen other than the priming ED3 (heterotypic boosting). The control mouse group (group 13) received PBS only. (B) Multivalent ED3 immunization. A total of 12 Swiss albino mouse groups were used. Mouse groups 14-24 were injected with multivalent ED3s (6 bivalent, 4 trivalent, and 1 tetravalent ED3s) thrice at 35-day intervals. The control group (group 25) received PBS only.

the Uni Prot database (IDs: P17763, P14340, P27915.1, and P09866, respectively), optimized for *Escherichia (E.) coli* expression, and commercially manufactured (Eurofins Genomics, Louisville, KY, USA) (Figure 1). The ED3 sequences were then cloned at the NdeI and BamHI sites into pET15b (Novagen, Darmstadt, Germany) and confirmed by DNA sequencing. The DENV1~4 ED3s were overexpressed as inclusion bodies in *E. coli* JM109 (DE3) pLysS and purified as described previously[22]. Protein identities were validated by analytical HPLC and MALDI-TOF MS, and the purified proteins were stored at -30 ℃ as lyophilized powder until usage[23].

2.2. Physicochemical measurements

The biophysical characteristics of ED3s were evaluated in phosphate-buffered saline (1× PBS, pH 7.4) at a protein concentration of 0.3 mg/mL, the same concentration used in immunogenic investigations.

2.2.1. Structural analysis of ED3 by circular dichroism (CD) and tryptophan–fluorescence spectroscopy

Fluorescence intensity is extremely sensitive to the microenvironment around the tryptophan side-chains and provides useful information on protein stiffness and loose packing[24].

Secondary and tertiary structures of ED3s were monitored through far-UV CD and trp-fluorescence spectroscopy using a JASCO J820 CD spectropolarimeter (JASCO, Tokyo, Japan) and an FP-8500 spectrofluorometer (JASCO, Tokyo, Japan), respectively. The CD spectra of all the ED3s were measured using a 2 mm quartz cuvette with continuous scanning. Three scans were taken from 200 nm to 260 nm wavelengths at temperatures of 25 ℃ and 37 ℃. The secondary structure content was determined with the BeStSel online tool (https://bestsel.elte.hu)[25]. Similarly, trp-fluorescence (λ_{ex} 295 nm) of the proteins was measured at 25 ℃ and 37 ℃. A quartz cuvette with a 3 mm optical path length was used to carry out the fluorescence measurements, and λ_{ex} was recorded between the wavelengths of 300 nm and 500 nm.

2.2.2. Analysis of subvisible protein aggregates by dynamic light scattering (DLS) and static light scattering (SLS)

DLS measurements were taken at temperatures of 25 ℃ and 37 ℃ using a polystyrene cuvette and a Zeta-nanosizer. The hydrodynamic radius [*R*h] was calculated from the size distribution using the Stokes-Einstein equation and averaged over three observations[26]. SLS was measured under the same conditions at a wavelength of 600 nm and temperatures of 25 ℃ and 37 ℃ using an FP-8500 spectrofluorometer and a quartz micro-cuvette with a 3 mm optical

path. Each measurement was accumulated three times, and the values were averaged.

2.3. Immunization studies

The immunization doses were prepared in phosphate-buffered saline (1× PBS, pH 7.4) at 30 μg of protein/dose (100 μL/mice). The first or primary doses were given subcutaneously, and the following secondary doses were prepared in the same way but injected intraperitoneally[22]. A total of twenty-five Swiss albino mouse groups, each group having at least five mice, were used. Groups 1-3, 4-6, 7-9, and 10-12 received 1ED3, 2ED3, 3ED3, and 4ED3, respectively, as primary immunogens. All mouse groups then had a heterotypic DENV ED3 as a secondary immunogen at 35-day intervals (per se, Figure 2A). Mouse groups 14-24 were repeatedly injected with multivalent ED3s (6 bivalent, 4 trivalent, and 1 tetravalent ED3s) as immunogens three times at 35-day intervals (Figure 2B). The control mouse groups (groups 13 and 25) were injected with PBS only following the same protocol followed for the experimental mouse groups.

2.4. Anti-DENV ED3 IgG antibody titers by ELISA

The production of anti-DENV ED3 IgG antibodies was monitored at weekly intervals for five weeks using ELISA as previously described[27]. The 96-well microtiter plates (Dynatech Laboratories, EI Paso, TX, USA) were coated with purified ED3s in PBS overnight at 1 μg/mL (100 μL/well). Unbound proteins were washed out with PBS, and free spaces were blocked with 1% BSA in PBS for 45 minutes at 37 ℃. After washing the microtiter plates once with PBS, dose-specific mouse anti-DENV ED3 sera was added at a 1: 50 dilution in 0.1% BSA in PBS, followed by a 3-fold serial dilution, and incubated at 37 ℃ for 2 hours. Plates were then thoroughly washed three times with PBS supplemented with 0.05% Tween-20 (PBS-Tween-20) and once with PBS. Anti-mouse-IgG-HRP conjugate (Thermo Fisher Scientific, Waltham, MA, USA; 1: 3 000 dilution in 0.1% BSA-PBS-Tween-20, 100 μL/well) were added and incubated at 37 ℃ for 90 minutes. The unbound conjugates were removed by 3-times washing with PBS-Tween-20 and once with PBS. Then, the o-phenylenediamine (OPD) substrate at 0.4 mg/mL supplemented with 4 mM H_2O_2 in 50 mM citrate buffer, pH 5.0 was added (100 μL/well) and incubated for 20 minutes at room temperature. The color intensity was measured at 450 nm using a microplate reader (Thermo Scientific Multiscan®EX Primary EIA V2.3, Waltham, MA, USA). Antibody titers were calculated from the power fitting of $OD_{450\ nm}$ *versus* the reciprocal of antisera dilution using a threshold of $OD_{450\ nm}$ 0.1 above the background values using an MS Excel, Microsoft Office Professional Plus 2019 (Microsoft, Redmond, WA, USA).

2.5. Structure modeling of four different ED3s

The structure models of 1ED3, 2ED3, 3ED3, and 4ED3 were generated from PDB IDs: 3G7T.pdb[28], 4UTC.pdb[29], 3VTT.pdb[30], and 3WE1.pdb[31], respectively, as previously described[22]. In short, the sequences were aligned using the online tool CLUSTAL W (www.ebi.ac.uk/Tools/msa/clustalo), considering the default settings[32]. Then, all four ED3s were superimposed, and surface model structures were generated using Pymol graphics 2.0 software (Schrödinger Inc., New York, USA).

2.6. Statistical analysis

MS Excel (Microsoft Office Professional Plus 2019; Microsoft, Redmond, WA, USA) was used to conduct the statistical analysis, which included a single-factor analysis of variance (ANOVA) and a two-tailed student's *t*-test.

2.7. Ethical approval

The animal study was reviewed by the Animal Ethics Review Board, Faculty of Biological Sciences, University of Chittagong, Bangladesh, and approved on May 12, 2022 [approval No. : AERB-FEBSCU-202000512(1)].

3. Results

3.1. Sequence and structural analysis of ED3s

The amino acid sequence alignment of DENV1~4 ED3s revealed considerable sequence similarity (70%-88%) among the ED3s (Figure 1A; Supplementary Table 1). In addition, the surface structures were found to be quite comparable, as evidenced by minimal root mean square deviation (C-RMSD) between C-alpha (Cα) atoms in each structure using Pymol graphics (Figure 1; Supplementary Table 1).

3.2. Biophysical characterization of the ED3s

We analyzed the biophysical characteristics of the ED3 variants under similar conditions used in the immune response experiment (0.3 mg/mL protein concentration in PBS at 25 ℃ and 37 ℃). The far-UV CD spectra indicated that the secondary structure contents of ED3s were essentially native and contained around 40% β-sheet structures compared to those of protein structures from the PDB files as calculated by the BeStSel online server (Figure 3A, Supplementary Figure 1A).

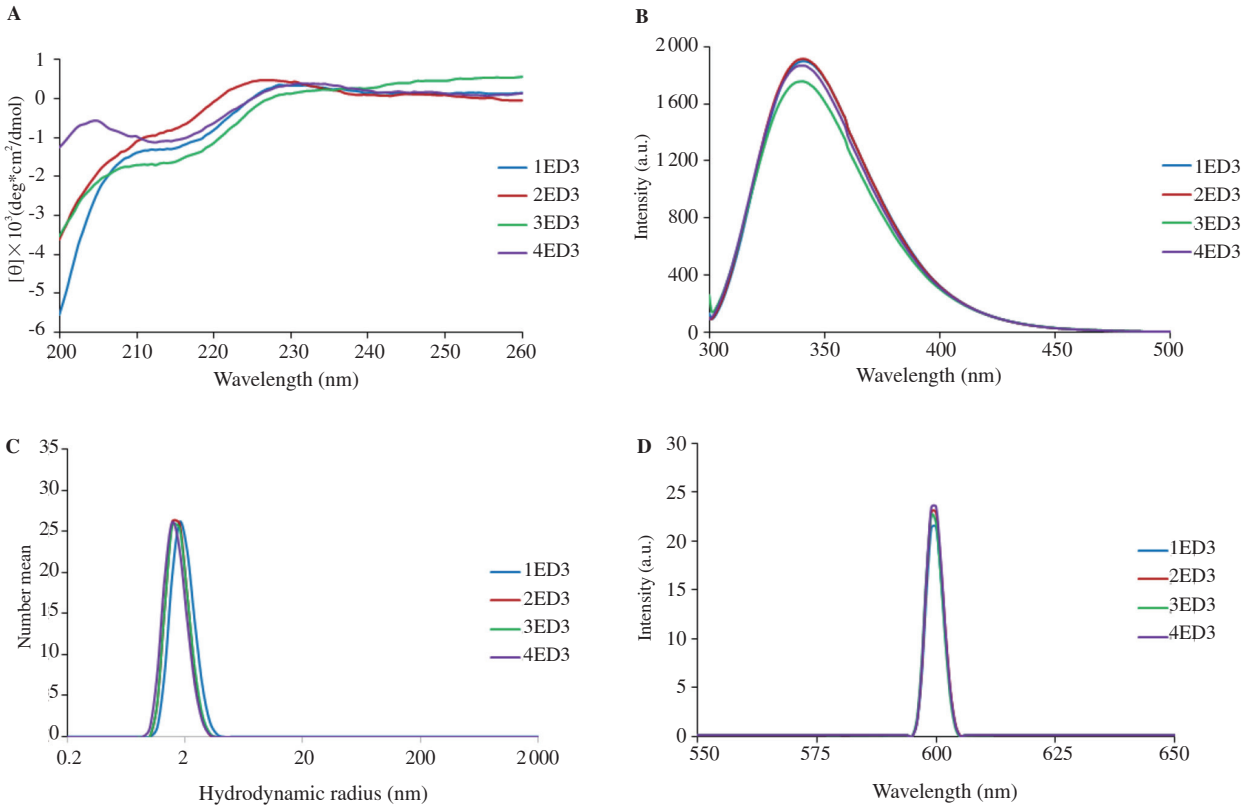

Figure 3. The biophysical properties of DENV1~4 ED3 variants. (A) Far-UV CD spectroscopy, (B) Trp-fluorescence spectroscopy, (C) DLS, and (D) SLS spectra were measured at 25 ℃. All protein samples were formulated in 1XPBS, pH 7.4, at a concentration of 0.3 mg/mL. Three accumulations were taken for each measurement, and the data were averaged. Line symbols are explained within the panels and are the same for each.

The native-like property of the tertiary structure was determined using tryptophan fluorescence spectroscopy. All ED3 variants exhibited a significant tryptophan fluorescence (an emission maximum of 341_{nm})(Figure 3B, Supplementary Figure 1B).

The sizes of the ED3 variants were systematically examined by DLS and SLS. DLS results showed all the ED3 proteins were monomeric in 1XPBS and at both temperatures of 25 ℃ and 37 ℃ (Figure 3C, Supplementary Figure 1C), which were corroborated by the low scattering intensities as measured by SLS (Figure 3D, Supplementary Figure 1D).

3.3. Primary anti–ED3 IgG responses in mice

The serotype-specificity and serotype-cross-reactivity of primary anti-DENV1~4 ED3 IgGs were evaluated at weekly intervals following immunization in Swiss albino mice by ELISA. The anti-DENV1 ED3 sera (hereafter anti-1ED3 sera) were 1ED3-2ED3-3ED3 serotype-cross-reactive (Figure 4A). Such sero-cross-recognition of DENV1~3 ED3s by anti-1ED3 sera could be attributed to their very high sequence and surface structure similarities (Figure 1). The anti-2ED3 and anti-3ED3 sera were 2ED3 and 3ED3-specific, with moderate cross-recognition of 3ED3 and 2ED3, respectively (Figure 4B-4C). However, both the anti-2ED3 and anti-3ED3 did not show any cross-recognition of 1ED3. In contrast, anti-4ED3 sera were solely serotype-specific; neither anti-4ED3 showed any cross-recognition of other ED3s, nor other anti-ED3 sera cross-recognized 4ED3 (Figure 4D).

3.4. Impacts of primary anti–DENV ED3 antibodies on secondary anti–DENV ED3 responses

The primarily ED3-injected mice were secondarily immunized using ED3s other than the primarily injected ED3s. To be precise, primarily 1ED3, 2ED3, 3ED3, and 4ED3-injected mice were secondarily injected with (2ED3, 3ED3, and 4ED3), (1ED3, 3ED3, and 4ED3), (1ED3, 2ED3, and 4ED3), and (1ED3, 2ED3, and 3ED3), respectively, in separate mouse groups. The effects of primary anti-DENV ED3 IgG on the serospecificity and serotype-cross-reactivity of heterotypic secondary anti-DENV ED3 antibodies were investigated using ELISA (Figure 2A).

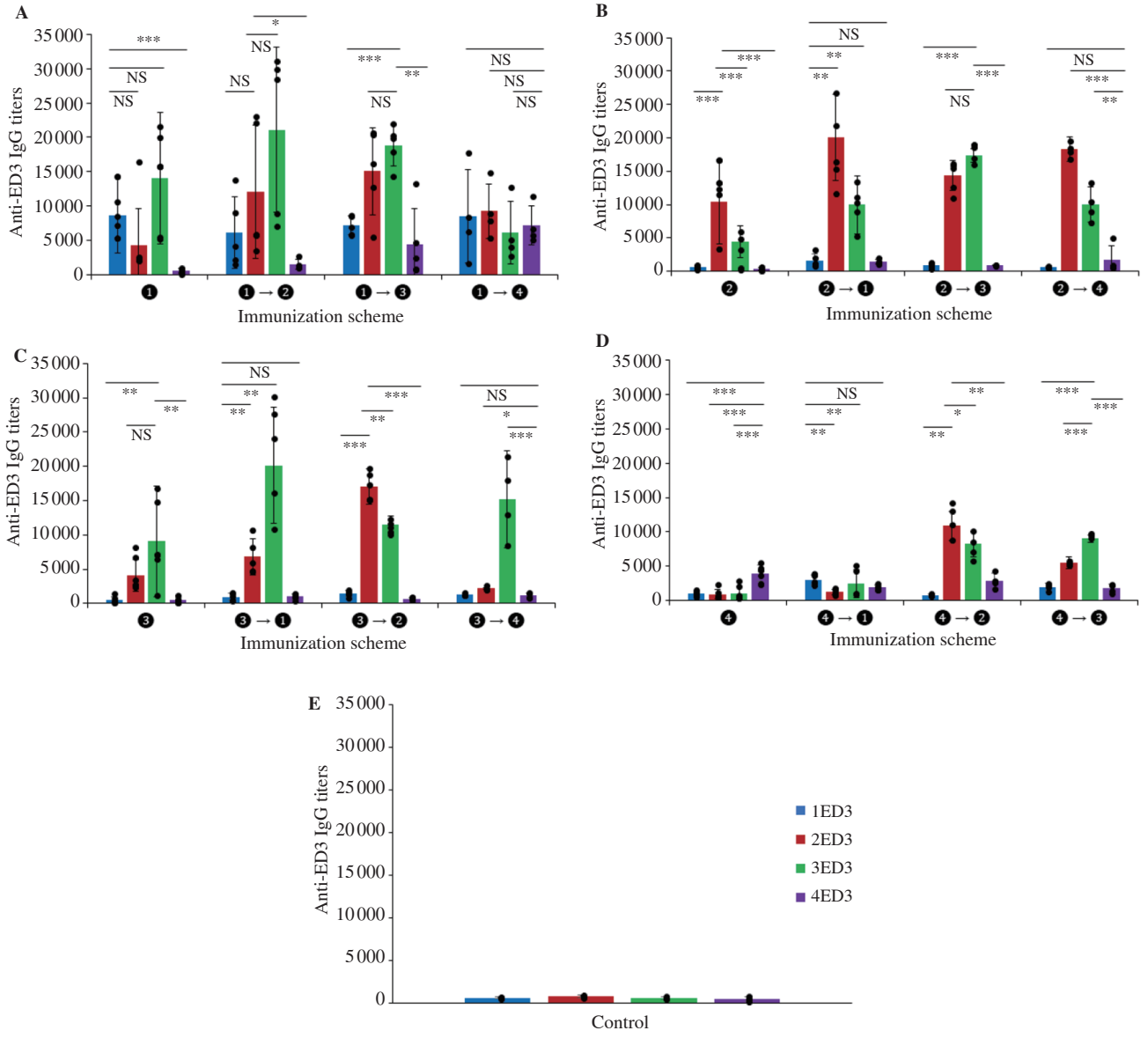

Figure 4. Effects of primary anti-ED3 IgG on secondary anti-ED3 IgG responses in Swiss albino mice. The first dose was the priming dose and the second dose was heterotypic boosting. Effects of primary anti-1ED3 (A), anti-2ED3 (B), anti-3ED3 (C), and anti-4ED3 (D) IgG responses on secondary heterotypic anti-DENV ED3 IgG responses on day 28 are shown. (E) The anti-ED3 responses in control mouse group 13. Antibody titers determined against 1ED3, 2ED3, 3ED3, and 4ED3 are shown by blue, red, green and purple bars, respectively, and same for each. The scattered points stand for individual mice data. Numbers before and after arrow sign stand for primary and secondary ED3, respectively. Asterisks represent the statistical comparison within the groups analyzed by two-tailed student *t*-test. Here, '*', '**', and '***' stand for *P* values < 0.05, < 0.01, and < 0.001, respectively. NS denotes 'no statistical significance'.

3.4.1. Effects of primary anti-1ED3 on secondary anti-ED3 IgG responses

As we expected, a secondarily injected 2ED3 and 3ED3 after primary 1ED3 injection further increased the 2ED3-3ED3 cross-reacting anti-ED3 IgG antibodies. The 2ED3-3ED3 cross-recognition by anti-1ED3 IgG was much higher than the cross-recognitions observed following a primary 2ED3 and/or 3ED3 immunization, mostly being contributed from cross-anti-2ED3/3ED3 memory generated following a primary 1ED3 immunization (Figure 4A). Anti-1ED3 sera did not show any cross-recognition of 4ED3, and accordingly, it did not have any effects on secondary anti-4ED3 responses (Figure 4A).

3.4.2. Effects of primary anti-2ED3 and anti-3ED3 responses on secondary anti-ED3 antibodies

As expected from the sero-cross-recognition pattern of 2ED3 and 3ED3, a secondary 1ED3 immunization following primary 2ED3

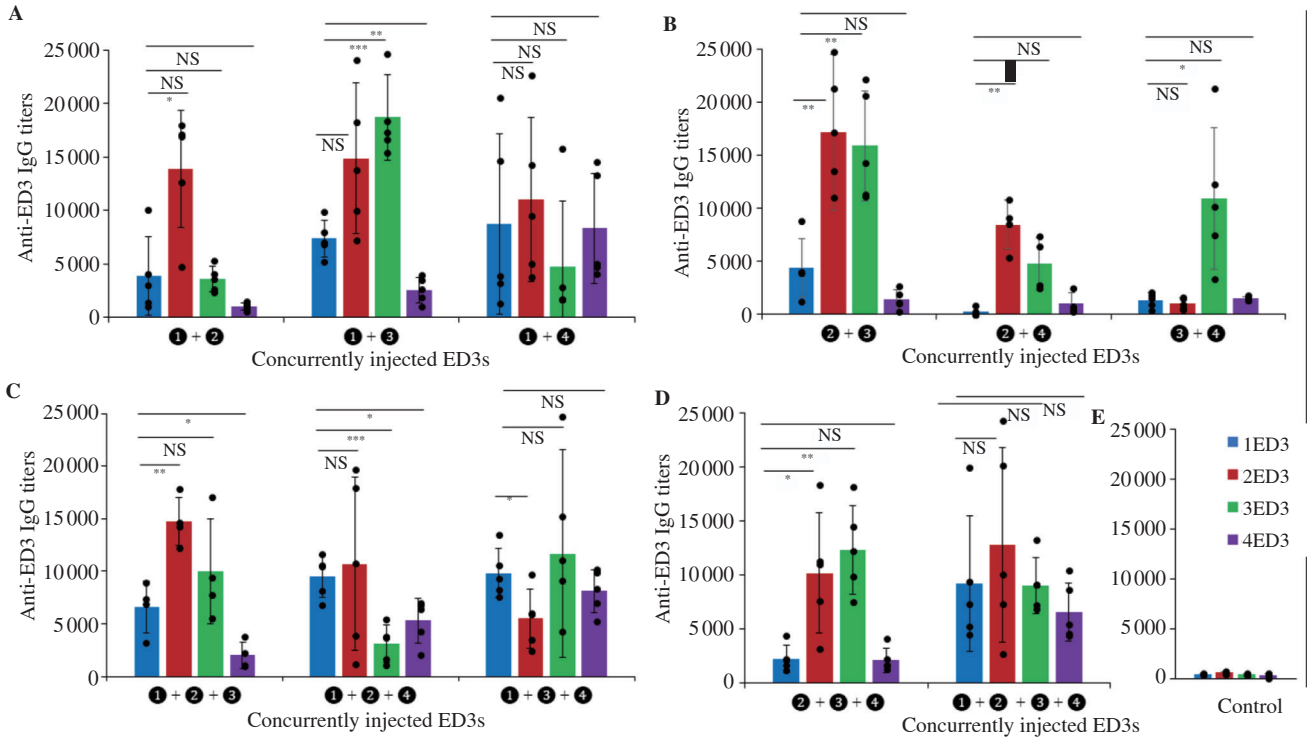

Figure 5. Anti-ED3 IgG responses against concurrently injected multivalent ED3s. Anti-ED3 IgG titers against bivalent ED3s (having 1ED3 in common), bivalent ED3s (avoiding 1ED3), and trivalent ED3s (having 1ED3 in common) are shown in panels (A), (B), and (C), respectively, 7 days after 3rd dose. The adjuvant effects of 1ED3 on anti-4ED3 IgG responses against tetravalent ED3 formulation were shown in panel D. (E) The anti-ED3 responses in control mouse group 25. Antibody titers determined against 1ED3, 2ED3, 3ED3, and 4ED3 are shown by blue, red, green and purple bars, respectively, and same for each. The scattered points stand for individual mice data. Asterisks represent the statistical comparison within the groups analyzed by two-tailed student *t*-test. Here, '*', '**', and '***' stand for *P* values < 0.05, < 0.01, and < 0.001, respectively. NS denotes 'no statistical significance'.

and/or 3ED3 immunization further slightly increased anti-2ED3 and/or anti-3ED3-specific IgG levels (Figure 4B-4C). Similarly, secondary 3ED3 and 2ED3 immunizations in primarily 2ED3 and 3ED3-injected mouse groups, respectively, significantly improved 2ED3 and 3ED3 cross-reacting IgG, as anticipated (Figure 4B). However, the poorly immunogenic 4ED3 became almost non-immunogenic when injected as a secondary immunogen following primary 2ED3 and 3ED3 immunizations (Figure 4B-4C).

3.4.3. Effects of primary anti–4ED3 responses on secondary anti–ED3 antibodies

The 4ED3 was very poorly immunogenic and the anti-4ED3 sera were solely serotype-specific. Therefore, secondarily injected ED3s in primarily 4ED3-injected mice did not show any cross-recognition of other ED3s; rather, secondary 1ED3, 2ED3, and 3ED3 immunization resulted in anti-ED3 IgG responses very similar to what had been observed with primary 1ED3, 2ED3, and 3ED3 immunization, respectively (Figure 4D).

3.5. Anti–ED3 IgG responses against multivalent ED3s

To investigate the anti-DENV1~4 ED3 IgG responses mimicking the anti-ED3 IgG generation by natural concurrent multiple DENV infections, 11 new mouse groups were injected with all possible multivalent compositions of DENV1~4 ED3s (6 bivalent, 3 trivalent, and 1 tetravalent ED3s at equivalent concentration, 30 μg/dose). Anti-ED3 IgG titers were determined at weekly intervals for four weeks. The generation of long-term anti-ED3 responses was monitored through repeated doses at 35-day intervals (Figure 2B).

3.5.1. The immune dominance, interference, and tolerance of DENV1~4 ED3s

Firstly, anti-ED3 IgG responses against six bivalent ED3s clearly suggested that 1ED3, 2ED3, and 3ED3 were co-dominantly immunogenic. However, the immunogenicity of 2ED3 and 3ED3 was higher than that of 1ED3 (Figure 5). Quite unexpectedly, in all combinations of 2ED3, 3ED3, and 4ED3 (2ED3+4ED3, 3ED3+4ED3, and 2ED3+3ED3+4ED3), 4ED3 was completely

non-immunogenic; both 2ED3 and 3ED3 were immunodominant over 4ED3 (Figures 5B and 5D). In precise terms, 4ED3 was non-immunogenic in bivalent (2ED3+4ED3, 3ED3+4ED3) and trivalent (2ED3+3ED3+4ED3) ED3 formulations but retained moderate immunogenicity when the same ED3 formulations had 1ED3 added (*i.e.*, 2ED3+4ED3+1ED3, 3ED3+4ED3+1ED3, 2ED3+3ED3+4ED3+1ED3) (Figure 5). Therefore, it is evident that 1ED3 had an adjuvant effect on the immunogenicity of 4ED3, which is new and very important information. Finally, the immunogenicity of 4ED3 was quite low compared to that of other ED3s in all multivalent ED3 formulations. However, the differential immunogenicity of DENV1~4 ED3s, immune-dominant and -codominant nature, and the adjuvanticity of 1ED3 on the immunogenicity of 4ED3 may pave the way for designing a tetravalent DENV vaccine design with effective and functional immune responses against all four DENV serotypes.

3.5.2. Long-term anti-ED3 IgG responses against multivalent ED3s

The sustainable anti-ED3 memory status was observed following repeated multivalent DENV ED3 immunizations (Figure 5; Supplementary Figure 2). The first two doses of 1ED3 and 4ED3 increased anti-ED3 IgG levels (Supplementary Figure 2). However, a third dose after 35 days of the second dose did not increase anti-ED3 IgG levels; rather, anti-ED3 IgG levels decreased noticeably. On the other hand, anti-2ED3 and anti-3ED3 IgG responses increased with successive doses (Supplementary Figure 2).

4. Discussion

In this study, we expressed fragmented 11 kDa-sized DENV1~4 ED3s in the *E. coli* system and confirmed their biophysical properties after purification. Then, we extensively examined their immunogenicity simulating the single primary, secondary and concurrent heterotypic anti-dengue responses in mouse model. There were a few peculiar observations.

The first one was that the anti-1ED3 sera had the highest recognition (IgG titers) against 3ED3, followed by 1ED3 and 2ED3, though the sera were raised against 1ED3. These observations suggested that the putative epitope residues might have shared and/or similar configurations on 1ED3, 2ED3, and 3ED3, as depicted in their cross-recognition (Figure 1B). This interpretation is furthermore in line with the observations that primary anti-2ED3 and anti-3ED3 responses did not show any cross-recognition of 1ED3. Moreover, the low immunogenicity and absolute serospecificity of 4ED3 indicated that the epitope residues on 4ED3 might have distinct configurations and might primarily be hindered in the 4ED3 scaffold[33]. This interpretation is almost corroborated by their sequences and structures. A detailed comparison of the ED3 sequences and structure similarities indicated that DENV1~3 ED3s are very similar to each other and the 4ED3 sequence is distal from other ED3s, which could have evolved separately from ancestral sequences[22,34]. Furthermore, the distribution of electric charges on the surfaces of 2ED3 and 3ED3 was very similar to each other but different from that of 4ED3 (Figures 1B; Supplementary Figure 3).

The second one observed with secondary 4ED3 immunization was the immune interference and/or immune tolerance of 4ED3 after primary 2ED3 and 3ED3 immunization. The immunological response to 4ED3 was reduced and was not cross-recognized by other anti-ED3 sera. The cause might be the weak immunogenicity of the 4ED3 and the existence of cross-reactive antibodies at levels below detection. However, the immunogenicity of the 4ED3 was further reduced when both 2ED3 and 3ED3 were priming immunogen and/or present with 4ED3 in the formulations. This phenomenon is supported by the 'antigenic sin' idea well-documented for a variant of concerns of influenza virus and SARS-CoV-2[35]. Besides, these observations also corroborate the classical observations on antigen interference, immune dominance, and immune tolerance observed upon multivalent vaccination[36]. Furthermore, such heterogeneity of anti-ED3 responses against four different DENVs might be hindering the induction of immune responses against four DENV serotypes and limiting the efficacy of dengue vaccines[37].

Finally, 1ED3 and 4ED3 were co-dominantly immunogenic in bivalent 1ED3+4ED3 formulations (Figure 5A). In addition, the 4ED3 showed some immunogenicity only when 4ED3 was secondarily injected after a primary 1ED3 immunization and if 1ED3 was present in the concurrent heterotypic immunization. When 1ED3 was injected in the vaccination design, the increase immunogenicity of 4ED3 may result in 1ED3 cross-reactive memory B cells that detect 4ED3 in a subsequent response[38].

In summary, despite having very high sequence homologies of up to 88% and almost identical structures, the immunogenicity of the four DENV serotypes differs significantly. Such heterogeneous immunogenicity and immune interference could be the origin of the limited success of several tetravalent vaccines in development and vaccines in conditional deployment[39,40]. Furthermore, the 'immune interference', namely, co-dominant immunogenicity, immune dominance and immune suppression, and immune potentiation of DENV1~4 ED3s observed in multiple ED3 immunizations in mouse model, could be the origin of both limited successes in understanding dengue disease etiology and the success of dengue vaccines. Although the accommodation of the serotype-cross-talks of anti-DENV antibodies and immune interference of four DENVs in a single vaccine formulation is very challenging, it is inevitable

to achieve equivalent neutralizing anti-DENV antibodies against all four DENV serotypes. The observations that we report here, namely, the immune-dominance nature of DENV2 (2ED3) and DENV3 (3ED3) over DENV1 (1ED3) and DENV4 (4ED3) and the adjuvanticity of 1ED3 on the immunogenicity of 4ED3, may provide a platform for the rational design of a potent dengue vaccine.

The DENV serotype specific ED3s induced DENV-specific and DENV sero-cross-reactive antibodies in mouse models. However, we could not investigate the DENV neutralization activities of anti-DENV ED3 sera which is inevitable for proposing ED3s as future vaccine candidates. Furthermore, antibody-ED3 structure and interaction analyses may pave a way rationalizing future dengue vaccine design.

Conflict of interest statement

We declare that we have no conflict of interest.

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Authors' contributions

MMI designed and supervised the project, and wrote the manuscript; MDI, SY, TS, and MAK performed the experiments; MDI wrote the manuscript; YK provided the ED3 variants and wrote the manuscript. All the authors read and approved the manuscript.

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