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## Investigating the efficacy of monovalent and tetravalent dengue vaccine formulations against DENV-4 challenge in AG129 mice

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### Abstract

Dengue (DEN) is the most important mosquito-borne viral disease, with a major impact on global health and economics, caused by four serologically and distinct viruses termed DENV-1 to DENV-4. Currently, there is no licensed vaccine to prevent DEN. We have developed a live attenuated tetravalent DENV vaccine candidate (TDV) (formally known as DENVax) that has shown promise in preclinical and clinical studies and elicits neutralizing antibody responses to all four DENVs. As these responses are lowest to DENV-4 we have used the AG129 mouse model to investigate the immunogenicity of monovalent TDV-4 or tetravalent TDV vaccines, and their efficacy against lethal DENV-4 challenge. Since the common backbone of TDV is based on an attenuated DENV-2 strain (TDV-2) we also tested the efficacy of TDV-2 against DENV-4 challenge. Single doses of the tetravalent or monovalent vaccines elicited neutralizing antibodies, anti-NS1 antibodies, and cellular responses to both envelope and nonstructural proteins. All vaccinated animals were protected against challenge at 60 days post-immunization, whereas all control animals died. Investigation of DENV-4 viremias post-challenge showed that only the control animals had high viremias on day 3 post-challenge, whereas vaccinated mice had no detectable viremia. Overall, these data highlight the excellent immunogenicity and efficacy profile of our candidate dengue vaccine in AG129 mice.

### Keywords

Dengue; Vaccines; Immune protection; AG129 mice; Dengue 4

## 1. Introduction

Dengue disease (DEN) is the most important mosquito-borne viral disease, and a major worldwide public health problem [1]. It is caused by four serologically and distinct viruses

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or serotypes, termed DENV-1 to DENV-4. The four DENVs are endemic throughout the world's subtropical and tropical regions, especially in Asia and Latin America. In humans, DENV may cause acute febrile illness dengue fever (DF) that is not life threatening. However, DF can progress to a potentially life-threatening dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) [2,3]. It is estimated that these viruses cause approximately 390 million clinical DEN infections annually [1].

The humoral immune response to DENV primarily targets the premembrane (prM) and envelope (E) structural proteins, and is predominately composed of serotype-cross-reactive antibodies [4–6]. In addition, the secreted nonstructural protein 1 (NS1) is highly immunogenic eliciting antibodies that exhibit complement-fixing activity that can trigger the lysis of virus-infected cells, and induces protective immunity in mice against DENV challenge [7–11]. In contrast, cellular immune responses to DENV, predominantly target epitopes located in the NS proteins [12]. Primary infection with any of the four serotypes is believed to confer lifelong protection to the homologous serotype; however, secondary infection with a different DENV serotype is the major risk factor for severe disease. This may be due to “antibody-dependent enhancement” (ADE) of disease where cross-reactive anti-DENV antibodies facilitate entry of DENV into Fc $\gamma$  receptor-bearing cells [13].

Current vaccine development efforts for DEN are based on inducing simultaneous immune responses to all four DENV serotypes using different technologies. Several of these candidate vaccines are at various stages of preclinical or clinical development [14]. Following successful Phase 1 clinical trials, our candidate live-attenuated tetravalent dengue vaccine (TDV) (formally known as DENVax) is currently being tested in Phase II trials at various endemic geographical locations. This vaccine consists of a molecularly characterized attenuated DENV-2 strain (TDV-2) and three chimeric viruses containing the prM and E protein genes of DENV-1, -3 and -4 expressed in the context of the attenuated TDV-2 genome backbone (TDV-1, TDV-3, and TDV-4, respectively) [15–19]. In preclinical animal models, and phase I clinical trials this vaccine was shown to elicit tetravalent neutralizing antibody responses, with responses highest to DENV-2, and lowest to DENV-4 ([20,21], Osorio et al., submitted for publication). Type-I & II interferon receptor-deficient (AG129) mice allow efficient replication of DENVs and represent a well-characterized animal model of DENV infection [22]. We used this model to investigate the protective efficacy of TDV-4 vaccine and compared it with that elicited by TDV against a lethal DENV-4 challenge. Since the common backbone of TDV is based on the attenuated TDV-2 virus we also tested the efficacy of this monovalent vaccine against DENV-4 challenge.

## 2. Materials and methods

### 2.1. Viruses

The TDV vaccine strains have been previously reported [19]. The human Thai DENV-4 strain 703 was used for the mouse challenge studies (Bourne et al., in preparation). In addition, a number of DENV-4 isolates were used, referred to as; Colombia 2006 (CI/DB039/2006; Indonesian geno-type), Ecuador 1999 (EC/DB041/1999; Indonesian genotype), Micronesia 1995 (MC/DB043/1995; S.E Asian genotype), Mexico 2006 (MX/

DB042/2006; Indonesian genotype), Virgin Islands 1994 (SC/DB045/1994; Indonesian genotype, and Thailand 2006 (TH/DB060/2006; S.E. Asian genotype). The viruses were obtained from the Division of Vector-Borne Diseases, Dengue Branch, CDC, San Juan, Puerto Rico.

## 2.2. Immunizations and virus challenge

On day 0 of the study, groups of 4 week-old AG129 mice ( $n = 15$ ) were immunized subcutaneously (SC) with 0.2 ml of the tetravalent-TDV ( $2 \times 10^4$ ,  $5 \times 10^4$ ,  $1 \times 10^5$  and  $3 \times 10^5$  pfu/ml for TDV 1–4 vaccines, respectively), monovalent TDV-4 ( $3 \times 10^5$  pfu/ml) or monovalent TDV-2 ( $5 \times 10^4$  pfu/ml) vaccines. In addition, a group of mice were injected with  $\times$  FTA (virus diluent) as a negative control. Mice were bled on day 56 post-immunization to measure neutralizing antibodies against each DENV serotype. On day 60 all animals were challenged intraperitoneally (IP) with  $\sim 1.5 \times 10^7$  pfu of DENV-4 strain 703 in a 0.1 ml volume. Following challenge mice were bled on days 1, 2 and 3 (5 mice from each group/bled day) to measure viremia levels by qRT-PCR. In addition, mice were weighed and monitored for morbidity daily to day 28 post-challenge. Animals exhibiting signs of severe disease or with weight loss greater than 20% of initial body weight were euthanized and counted as being dead on the following day for analysis. At the end of the monitoring period a terminal bleed was collected from all surviving animals to quantify the neutralizing antibody response against DENV-4.

To measure cellular responses, two groups of 4–6 week-old AG129 mice were immunized SC with 0.2 ml TDV ( $n = 3$ ) or TDV-4 vaccines ( $n = 2$ ) using the same vaccine doses as described above. Six and seven weeks post-priming, respectively, mice from each group were euthanized and individual spleens were collected for further analysis.

## 2.3. Measurement of anti-NS1 antibodies by ELISA

Purified NS1 antigen from DENV-2 and DENV-4 (abcam, Cambridge, MA) was resuspended in carbonate coating buffer pH 9.6 and coated at 1 ng/ $\mu$ l (50  $\mu$ l/well) onto 96-well ELISA plates (Corning Polystyrene). Plates were washed with PBS/0.1% Tween 20 (PBST) and blocked with 10% milk in PBST. Sera were serially diluted and incubated at 37  $^{\circ}$ C for 1 h. Following washing with PBST, goat anti-mouse HRP (Jackson Immuno, West Grove, PA) at 1:10,000 in 10% milk/PBST was added, and plates were incubated at 37  $^{\circ}$ C for 1hr. Color reaction was developed by adding 100  $\mu$ l TMB solution and incubating plates at room temperature in the dark for 6 min. Reaction was stopped by adding 1 N HCl. Absorbance was recorded at 450 nm and 630 nm using a Biotek plate reader. To account for optical interference the A630 was then subtracted from the A450.

## 2.4. Neutralization test

Vero cells ( $1.5 \times 10^4$  cells/100  $\mu$ l) were plated into 96-well tissue culture plates in DMEM/10% FBS/1% penicillin/streptomycin and incubated at 37  $^{\circ}$ C with 5% CO<sub>2</sub> for 48 h. Heat-inactivated sera were two-fold serially diluted in BA-1 medium, mixed with 2  $\times$  virus in an equal volume and incubated at 4  $^{\circ}$ C, overnight. Dengue viruses used are the parent strains to the vaccine viruses (DENV-1; 16007, DENV-2; 16681, DENV-3; 16562, DENV-4; 1036). In addition, we tested the breadth of neutralizing antibody responses

elicited by TDV or TDV-4 vaccines against several DENV-4 isolates collected from different geographical locations (see Section 2.1). Next, 30  $\mu\text{l}$  of the serum-virus mixture was added to Vero cell monolayers in triplicate and adsorbed at 37  $^{\circ}\text{C}$  for 2 h. Both positive and negative control sera samples were included. At the end of the incubation period, 100  $\mu\text{l}$ /well of 1.2% carboxy-methyl cellulose overlay was added and plates were incubated at 37  $^{\circ}\text{C}$ , 5%  $\text{CO}_2$  for a previously determined time period (plus or minus 3 h) to allow for the formation of detectable foci (DENV-1; 53 h, DENV-2; 72 h, DENV-3; 53 h, DENV4; 48 h). Cells were fixed with 85% ice cold acetone at ambient temperature for 20 min and stored at  $-20^{\circ}\text{C}$ . Plates were equilibrated to ambient temperature and washed 3 times with PBS-T (PBS/0.1% Tween 20) to remove residual overlay and then incubated with primary rabbit anti-DENV polyclonal antibody (1:1000 dilution in PBS-T/2.5% milk) at 37  $^{\circ}\text{C}$  for 2 h. Plates were washed as before and then incubated with secondary HRP-conjugated anti-rabbit antibody at 37  $^{\circ}\text{C}$  for 2 h. Finally, plates were incubated with 100  $\mu\text{l}$ /well of the HRP substrate 3-amino-9-ethylcarbazole until foci were visible. Following washing with water plates were air-dried and foci were quantified on an ELISpot reader. Titers were defined as the reciprocal of the highest serum dilution that reduced the average virus input in the negative control serum by at least 50%.

## 2.5. Virus quantitation by qRT-PCR

RNA was extracted from sera using the Aurum total RNA isolation kit (Bio-Rad, Hercules, CA) as previously described [23]. Reverse transcription was accomplished using an iScript<sup>TM</sup> synthesis kit (Bio-Rad) using the following protocol: 1) 1.5 min, 25  $^{\circ}\text{C}$ , 2) 42  $^{\circ}\text{C}$ , 30 min, 3) 85  $^{\circ}\text{C}$ , 5 min, 4) infinite hold at 4  $^{\circ}\text{C}$ . Samples were evaluated using a DENV-4 serotype-specific qRT-PCR [24] utilizing a TaqMan probe (Sigma–Aldrich, St. Louis, MO) to quantify the specific amplification in each reaction. Each 25  $\mu\text{l}$  qRT-PCR reaction contained: 12.5  $\mu\text{l}$  iQ supermix<sup>TM</sup> (Bio-Rad) and 1  $\mu\text{l}$  (5  $\mu\text{M}$ ) of forward and reverse primer and 1.5  $\mu\text{l}$  of (5.0  $\mu\text{M}$ ) of the TaqMan probe [24] and 3  $\mu\text{l}$  of cDNA template or nuclease-free water (no template controls). The qPCR was completed in a C1000 thermocycler equipped with a CFX<sup>TM</sup> optical module (Bio-Rad) using the following parameters: Cycle 1), 95  $^{\circ}\text{C}$ , 1.5 min; Cycle 2), Step 1. 95  $^{\circ}\text{C}$ , 10 s, Step 2. 62  $^{\circ}\text{C}$ , 30 s, repeat 40 $\times$ . Fluorescent signal data was collected at the end of each annealing/extension step. Starting quantity values were extrapolated from standard curves of plasmids harboring the PCR targets generated in parallel for each run.

## 2.6. Flow cytometry

Spleens from individual animals were processed into single splenocyte suspension for each vaccine group. After lysis of red blood cells with 1x BD Pharm Lyse<sup>TM</sup> buffer, splenocytes were suspended in RPMI1640 (10% FCS, 100 I.U./ml penicillin, 100  $\mu\text{g}/\text{mL}$  streptomycin and 0.14 mM  $\beta$ -mecaptoethanol), and  $1 \times 10^6$  cells/well were plated onto a 96-well U-bottom plate. Cells were stimulated with pools of peptides encompassing the entire sequence of DENV-1–4 at a final concentration of 20  $\mu\text{g}/\text{ml}$  for 16 h in the presence of 1  $\mu\text{g}/\text{ml}$  BD GolgiPlug (Bredfeldin A). Cells were stained for surface markers with anti-mouse CD4 FITC (RM4-5), anti-mouse CD8 $\alpha$  PerCP (53-6.7) and for intracellular cytokines with monoclonal antibodies for IFN- $\gamma$  APC (XMG1.2), IL-2 PE (JES6-5H4), TNF- $\alpha$  PE (MP6-XT22). The samples were acquired using a BD FACS Calibur and analyzed with FlowJo

v7.6.5 (Tree Star). Background values from medium-treated groups were subtracted from each sample. The frequency of cytokine-positive T cells was presented as the percentage of gated CD4<sup>+</sup> or CD8<sup>+</sup> T cells.

### 3. Results

#### 3.1. Immunogenicity of TDV formulations in AG129 mice

A single immunization with TDV-4 or TDV-2 vaccines was sufficient to elicit neutralizing antibody responses to the homologous viruses (Fig. 1a and b) and antibodies to DENV-2 NS1 protein (Fig. 1d). Further, antibodies elicited by TDV-2 were shown to cross-neutralize DENV-4 (Fig. 1b), and to cross-react with the DENV-4 NS1 protein (Fig. 1d). Animals that received a single immunization of TDV developed neutralizing antibody responses to all four DENV serotypes (Fig. 1c). However, the titers to DENV-4 were the lowest while those induced against DENV-1, DENV-2 and DENV-3 were indistinguishable.

In addition to measuring humoral immune responses we assessed the capacity of TDV to elicit cellular immunity. In vitro restimulation of TDV immune splenocytes with pools of peptides (each pool consisted of the entire peptide array for a given E protein) led to the detection of CD4<sup>+</sup> and CD8<sup>+</sup> T cells predominantly producing IFN- $\gamma$  and TNF $\alpha$  (Fig. 2a and b, respectively). Further, since immunized animals were challenged with DENV-4, we also tested whether the TDV-2 backbone elicits cross-reactive T cells recognizing the DENV-4 NS proteins. To this end, TDV-4 immune splenocytes were restimulated in vitro with pools of peptides encompassing the entire sequence of DENV-4 NS1, NS3 or NS5 protein. Although no responses were observed against the NS1 or NS5 proteins (data not shown), pool 4 (representing residues 174–240) from NS3 protein elicited a strong CD4<sup>+</sup> IFN- $\gamma$  producing T cell response (Fig. 2c) but not CD8<sup>+</sup> T cells (Fig. 2d).

#### 3.2. Neutralizing antibody responses against different DENV-4 isolates

Given the fact that the monovalent DENV-4 vaccine induces the weakest neutralizing antibody responses of the four valences in TDV, we further characterized the antibody responses elicited by TDV or TDV-4 vaccines for cross-neutralizing activity against various DENV-4 isolates collected from different geographical locations. Neutralization titers were determined in pooled sera collected on day 56 just prior to DENV-4 challenge. As shown in Table 1, anti-DENV-4 antibody titers elicited by TDV or TDV-4 vaccines exhibited broad neutralizing activity against all DENV-4 isolates tested.

#### 3.3. Protective efficacy against DENV-4 challenge

Following challenge with DENV-4 703 strain vaccinated animals were monitored for morbidity and mortality and bled to measure DENV-4 viral RNA. Fig. 3 shows the survival curve at day 28 post-virus challenge and the summarized outcome. Briefly, 100% of the FTA control treated mice succumbed to infection by day 8 post-virus challenge. In contrast, there was no mortality among animals vaccinated with TDV or TDV-4 vaccines, and only a single animal immunized with the TDV-2 vaccine succumbed to infection on day 27 post-virus challenge. Furthermore, among the vaccinated animals only 1 in the tetravalent vaccine group showed >10% weight loss (Fig. 3). Overall, all of the vaccines provided

significant protection against mortality and clinical signs as compared with controls ( $p < 0.0001$  each; Fisher's exact test).

When the anti-DENV-4 neutralizing titers were measured four weeks post-DENV-4 challenge they were found to increase compared with those seen prior to virus challenge (Fig. 4). In particular, anti-DENV-4 titers in groups of animals vaccinated with TDV or monovalent TDV-4 or TDV-2 vaccines increased by 6.6, 13, and 4.6-fold, respectively (Fig. 4).

Viral RNA in the serum of challenged mice was quantified by qRT-PCR on days 1, 2 and 3 post-challenge. As shown in Fig. 5, virus was detected in all of the FTA control animals in this study with a high titer viremia ranging from 5 log<sub>10</sub> genomes/ml on day 1 increasing to 7 log<sub>10</sub> genomes/ml on day 3 post-challenge. In comparison, for the TDV formulation viremia was detected in only 3/15 animals in total ( $p < 0.001$ ) with 2/5 being viremic on day 1, 1/5 mice on day 2, and 0/5 on day 3 post-challenge. A similar picture was seen for animals immunized with TDV-4 with again a total of 3/15 animals having detectable viremia ( $p < 0.001$  vs FTA controls). Again 2/5 animals had detectable viremia on day 1, 1/5 on day 2, and 0/5 on day 3 post-challenge. Immunization with TDV-2 also significantly reduced the number of animals with detectable viremia ( $p < 0.001$  vs FTA control) although the reduction was not as great as that seen with the other two vaccine formulations containing a DENVax-4 component. Specifically, 4/5 animals were viremic on day 1 and 1/5 mice on day 2, and 0/5 on day 3 post-challenge.

#### 4. Discussion

In this study a candidate tetravalent dengue vaccine (TDV) and two of its monovalent constituents were evaluated for immunogenicity and efficacy against challenge with DENV-4 in the AG129 mouse model. Although this mouse model is IFN  $\alpha\beta\gamma$  receptor-deficient it exhibits a normal adaptive immune response to DENVs when compared with the wild-type mice, and has been extensively used for immunogenicity and efficacy studies against DENV-1 and DENV-2 challenge [20,22,25]. However, this is the first use of a lethal DENV-4 challenge mouse model to evaluate candidate vaccines. The findings presented in this study demonstrate that the monovalent TDV-4 and TDV-2 vaccine formulations are immunogenic eliciting homotypic and heterotypic anti-DENV-4 neutralizing antibody responses, respectively, after a single immunization. This is consistent with our previously published observations on the immunogenicity of each monovalent vaccine [20]. In addition, antibodies to these monovalent vaccine formulations recognized the DENV-2 NS1 protein and cross-reacted with the NS1 protein of DENV-4. The NS1 is an important target protein of the humoral immune response to DENVs and anti-NS1 antibodies are cross-reactive due to the close sequence homology between the NS1 proteins of all four serotypes [7–11]. Moreover, TDV was immunogenic inducing tetravalent neutralizing antibody responses. Neutralizing antibodies elicited by TDV-4 or TDV vaccines exhibited broad cross-neutralizing activity against a number of recent DENV-4 isolates from different geographical locations, from the same genotype 2 or against a genotype 3 strain suggesting potential effectiveness in protecting against contemporary DENV-4 strains circulating in endemic areas.

Recent studies have acknowledged the role that T cells play in protection against dengue. In particular, T cells have been shown to contribute toward faster clearance of the virus in the AG129 mouse model ([25,26], Das et al., in preparation), and more recently the association of T cells with protection from dengue virus disease has been highlighted [12]. Therefore, in our studies we tested the capacity of our vaccine to elicit cellular responses. Indeed, following immunization with TDV we observed CD4<sup>+</sup> and CD8<sup>+</sup> T cells targeting the E protein of each serotype and producing IL-2 and IFN- $\gamma$ , respectively. In addition, we have observed that the common TDV-2 backbone elicited CD4<sup>+</sup> and CD8<sup>+</sup> T responses to the homologous NS1, NS3 and NS5 proteins (Das et al.; in preparation) and cross-reactive CD4<sup>+</sup> IFN- $\gamma$  producing T cells upon restimulation with a pool of NS3 peptides from DENV-4. This pool encompasses amino acids that falls within a highly conserved (78%) region of the NS3 protein for all four DENV serotypes containing motifs and charged residues that are essential for helicase activity and virus replication [27,28]. Therefore, one would predict that responses to conserved epitopes would be seen in humans and should be a highly desirable feature of vaccines for broad coverage.

When mice were challenged with DENV-4, all vaccinated animals survived except for 1 of 15 TDV-2 immunized mice. This is consistent with our previous observations demonstrating the short-term protective efficacy of each of the monovalent and TDV vaccines against challenge with mouse adapted DENV-1 and DENV-2 viruses [20]. It is unfortunate that this model cannot be used for long-term immunogenicity and protective efficacy studies since mice become more resistant to challenge virus as they age (data not shown). All protected animals developed an anamnestic neutralizing (Fig. 4), and anti-DENV-4 NS1 (Fig. 1d) antibody response suggesting that some virus replication has occurred sufficient to stimulate memory B cells for rapid activation and differentiation into antibody-secreting plasmoblasts. Indeed, following DENV-4 challenge viral RNA was detectable in the serum of some of the vaccinated animals on day 1 and 2. However, by day 3 no viral RNA was detectable, with levels comparable with control animals. Therefore, it can be argued that under our experimental conditions the TDV vaccine formulations did not prevent infection (sterile immunity) but only disease. Despite the attractive attributes of the AG129 mouse model for vaccine testing [20,22,25] these mice are immunodeficient and permit the rapid replication of DENVs. Given the fact that the virus inoculum used for challenge does not reflect the dose that humans normally get by a mosquito bite we cannot extrapolate these findings to human vaccination. Although the scope of this study was not to address the mechanism(s) involved in protection, neutralizing antibodies are considered to be the main mediators, and recently the role of T cells has been highlighted as being important both in preclinical and clinical studies ([12,25,26], Das et al., in preparation). In addition, anti-NS1 antibodies might have contributed to protection as they have been shown to be efficacious against infection by several Flaviviruses including DENV [7,9,10,29–31]. In future studies we will be addressing the role of anti-TDV-2 NS1 antibodies in protection against homologous or heterologous DENVs by conducting passive transfer studies in AG129 mice. In conclusion, we have shown that our TDV vaccine formulations are immunogenic and confer protection against DENV-4 challenge.

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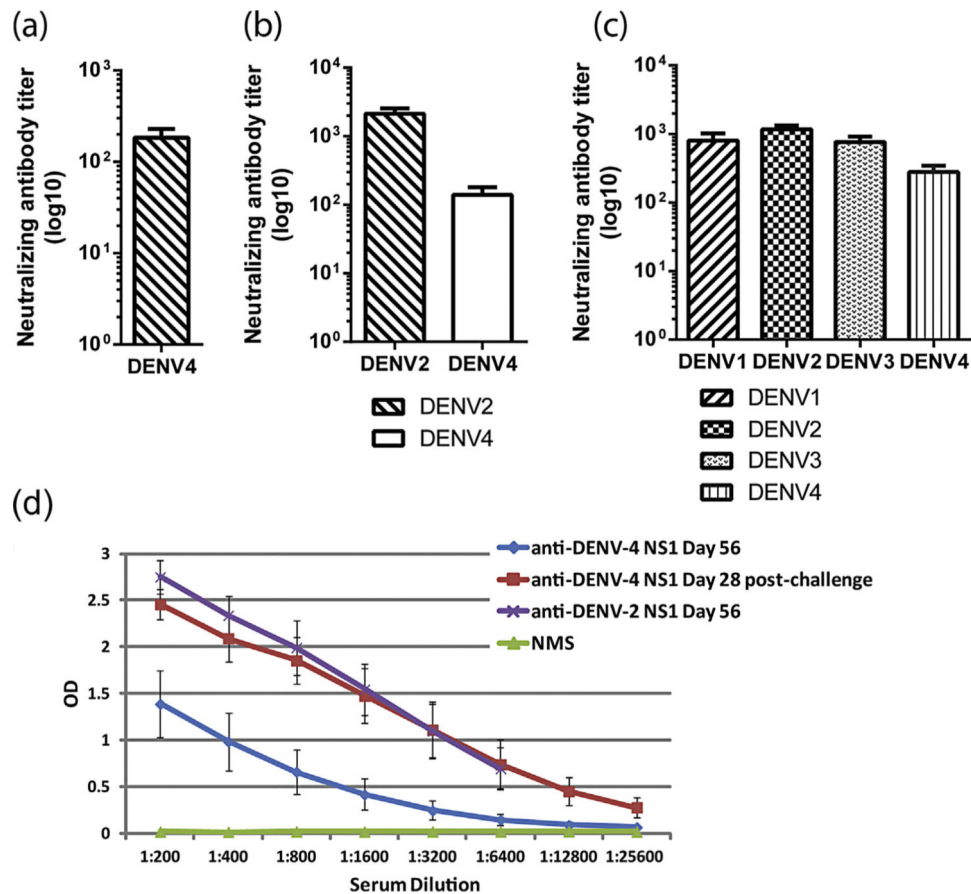
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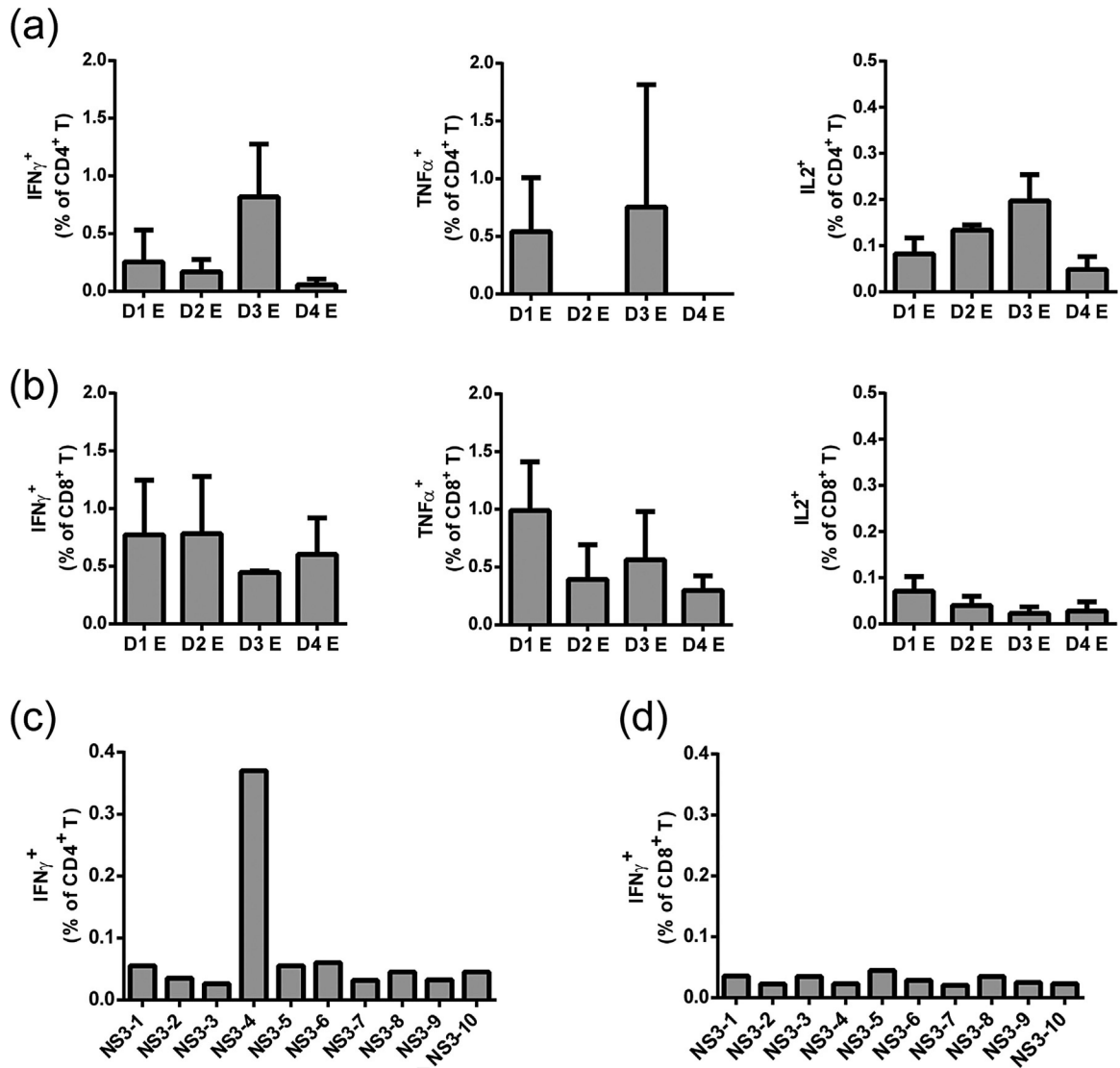
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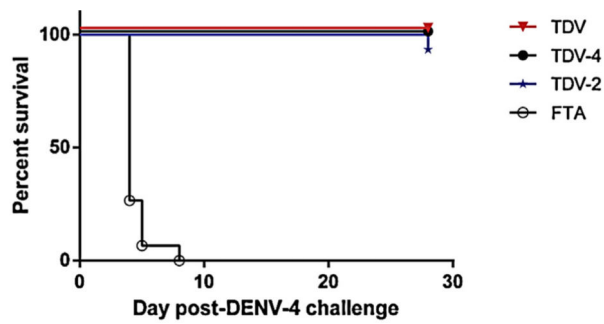


**Fig. 1.** Neutralizing and anti-NS1 antibodies. (a–c) Neutralizing antibody responses elicited after a single immunization with TDV-4 (a) or TDV-2 (b) vaccines or tetraivalent TDV (c). Individual serum samples collected from each group (day 56 post-primary immunization) were tested for neutralizing activity. Sera from group A were tested for neutralizing activity against DENV-4, sera from group B were tested for neutralizing activity against DENV-2 and DENV-4, and those from group C for tetraivalent neutralizing antibody responses. Data present mean of neutralizing antibody titers against each virus  $\pm$  SE. (d) Titration of immune serum collected from animals vaccinated with TDV on day 56 post-immunization or day 28 post-DENV-4 challenges against DENV-2 and DENV-4 NS1 antigens coated on an ELISA plate. Data represent the mean  $\pm$  SD of titrations of individual serum for each time point tested against the DENV-2 and DENV-4 NS1 antigens  $\pm$  SD. Normal mouse serum was tested as negative control (green line).



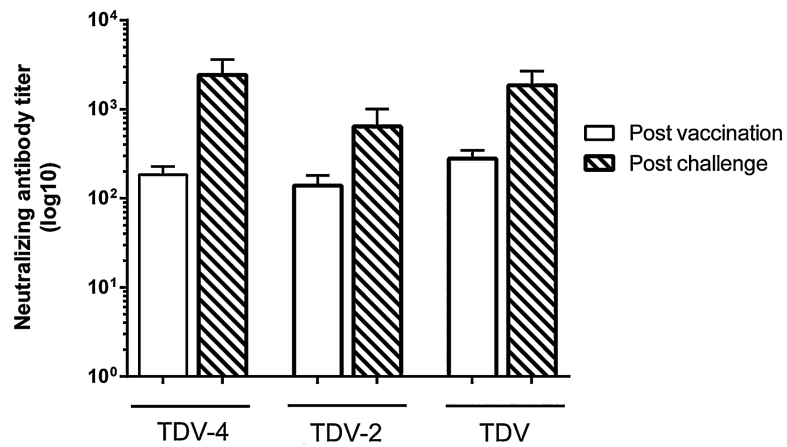
**Fig. 2.** T cell responses to DENV E and NS proteins. (a and b) CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses to E proteins of each DENV serotype elicited after a single subcutaneous immunization with TDV. Immune splenocytes collected 6 weeks post-primary immunization were restimulated in vitro with pools of peptides encompassing the entire sequence of DENV-1–4 E (each pool consisted of the entire peptide array for a given E protein). Cells were stained for surface markers CD4, CD8 and intracellular cytokines (IFN $\gamma$ , IL-2 or TNF- $\alpha$ ). Samples were analyzed by flow cytometry (FACS Calibur). The percentage of cytokine producing cells in medium treated groups was subtracted from each sample. Data are presented as mean  $\pm$  SD ( $n = 3$ ). T cells derived from control FTA group did not produce any significant cytokine response above the medium background (data not shown). Peptide arrays representing the entire sequence of E protein for each serotype and NS3 protein of DENV-4 were obtained through the NIH Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH (<http://www.beiresources.org>). For E peptide arrays: Dengue virus type 1, Singapore/S275/1990, NR-4551; Dengue virus type 2, New Guinea C (NGC), NR5-7;

Dengue virus type 3, Sleman/1978, NR-511; Dengue type 4, Dominica/814669/1981, NR-512. (c and d) CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses to the NS3 protein of DENV-4. Immune splenocytes collected seven weeks post-primary immunization with TDV-4 were restimulated in vitro with pools of peptides (10 peptides/pool) encompassing the entire sequence of DENV-4 NS3 protein. Cells were stained for surface markers CD4, CD8 and intracellular cytokines (IFN $\gamma$ ). Samples were analyzed by flow cytometry (FACS Calibur). The percentage of cytokine producing cells in medium treated groups was subtracted from each sample. Data are presented as mean of two individual mice.

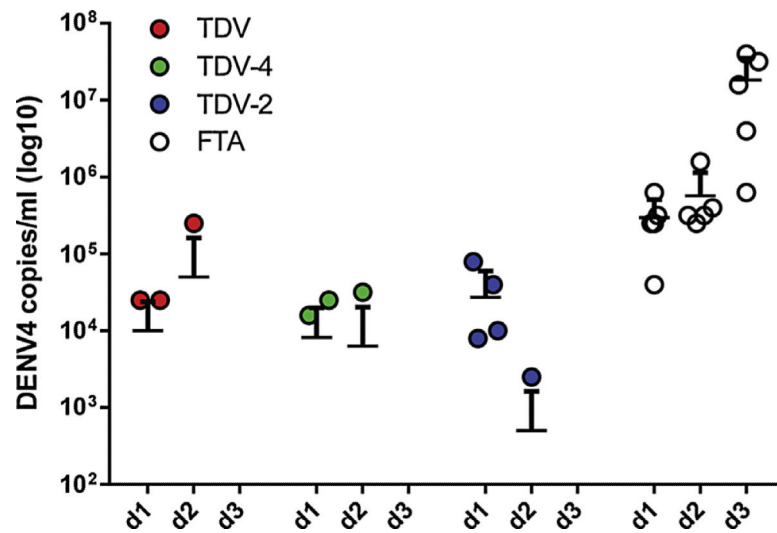


Group	Challenge Virus	Number Dead (%)	Survivors with > 10% Weight Loss (%)	Total with Signs (%)
TDV	DENV-4	0/15 (0%)	1/15 (6.7%)	1/15 (6.7%)
TDV-4	DENV-4	0/15 (0%)	0/15 (0%)	0/15 (0%)
TDV-2	DENV-4	1/15 (6.7%)	0/14 (0%)	1/15 (6.7%)
FTA	DENV-4	15/15 (100%)	-	15/15 (100%)

**Fig. 3.** Impact of immunization on survival following DENV-4 virus challenge. Groups of 15 AG129 mice were immunized with a single dose of TDV-4, TDV-2 or TDV vaccines. Control animals were immunized with FTA (virus diluents). On day 60 post-primary immunization animals were challenged with mouse adapted DENV-4 and morbidity and mortality was monitored over a period of four weeks.



**Fig. 4.** Anti-DENV-4 neutralizing antibody responses at day 56 post-primary vaccination or day 28 post-DENV-4 virus challenge. Groups of 15 AG129 mice were immunized with a single dose of TDV-4, TDV-2 or TDV vaccines. Control animals were immunized with FTA (virus diluents). On day 60 post-primary immunization animals were challenged with mouse adapted DENV-4. Individual sera collected post-vaccination or post-challenge (from all surviving animals) were tested for neutralizing activity against DENV-4. Data represent the mean neutralizing titer from each animal in each group  $\pm$  SE.



**Fig. 5.** Viremia data following DENV-4 challenge of animals immunized with TDV-2, TDV-4 or TDV vaccines. Levels of DENV-4 RNA in the serum post-challenge were quantified by qRT-PCR using a Fam labeled 5 prime nuclease assay designed to target the 3 prime non-coding region of dengue virus. Limit of detection is 2.5 log<sub>10</sub> genomes/ml.

**Table 1**

Cross-neutralization of diverse DENV-4 isolates by antibodies induced against TDV and TDV-4 vaccines. Pooled serum samples collected on day 56 post primary immunization from groups of AG129 mice ( $n = 15$ ) with TDV or TDV-4 were tested for neutralizing activity against each DENV-4 isolate. Control animals ( $n = 15$ ) were immunized with FTA (virus diluents).

DENV-4 isolates	Neutralizing antibody titers						
	1036	1*	2*	3*	4*	5*	6*
TDV immune serum	320	320	80	80	160	160	320
TDV-4 immune serum	160	320	160	80	160	160	160
FTA control serum	<20	40	<20	<20	20	<20	<20

1\*: CL/DB039/2006; 2\*: EC/DB041/1999; 3\*: MC/DB043/1995; 4\*: MX/DB042/2006; 5\*: SC/DB045/1994; 6\*: TH/DB060/2006.