B. BACKGROUND AND SIGNIFICANCE

B.1. Dengue: A worldwide public health threat. Dengue fever (DF) is caused by infection with dengue viruses (DENV), enveloped RNA viruses that occur as four serotypes: DENV-1, -2, -3, and -4 (31). These viruses are transmitted from human to human by mosquitoes (primarily *Aedes aegypti and Ae. albopictus*) (32). DENV infections can range from asymptomatic to overt illness ranging from mild symptoms to DF to severe dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (83). DF is an acute febrile illness with two or more manifestations that can include headache, retro-orbital pain, myalgia, arthralgia, rash, hemorrhagic manifestations, or leukopenia (31). DHF and DSS include thrombocytopenia, hemorrhagic manifestations, and signs of vascular leakage and can be fatal. DENV is the most rapidly spreading mosquito-borne viral disease in the world, and over 3 billion people in tropical and subtropical areas are at risk of DENV infection (26, 29). An estimated 40 million cases of DF occur each year, resulting in about 500,000 hospitalizations and over 25,000 deaths, primarily among children (26, 45). No dengue vaccine or antiviral treatment is currently available.

B.2. Cellular immune response to DENV infection. Cellular immune responses are involved in both protection and enhancement of DENV infection (40, 67). Primary (1°) infection with any of the four DENV serotypes confers life-long protection to the homologous serotype; however, secondary (2°) infection with a different DENV serotype is a major risk factor for severe disease (30, 42, 69, 70). This may be due to cross-reactive T cells (68) or to "antibody-dependent enhancement" (ADE) (33), where cross-reactive anti-DENV antibodies (Abs) facilitate entry of DENV into Fcγ receptor-bearing cells, leading to increased viral load. However, the majority of 2° DENV infections are asymptomatic or display only mild disease. The immune mechanisms underlying protection against a heterologous DENV infection are not well known. During a 2° immune response, antigen (Ag)-specific plasmablasts (PBs) are transiently present in the blood. As shown with tetanus vaccination, this population proliferates, gets activated and expresses high levels of HLA-DR and, while differentiating, acquires markers of plasma cells (PCs) such as CD138 (56). In contrast, memory B cells (MBCs) appear later in the blood (day 10 post-vaccination) and can be detected up to day 28 (8). Several reports have shown that the long-term Ab response is maintained by long-lived PCs (LLPCs), independently of re-stimulation of MBCs (76, 77). In contrast, during the 2° immune response, re-stimulation of MBCs is crucial to induce rapid secretion of high-affinity Abs (72). We have shown that pre-existing Abs secreted by LLPCs, B and T cells all contribute to protection against a second DENV infection and that re-stimulation of MBCs induce high affinity serum Abs (90). During DENV infection, increased production of both Th1- and Th2-mediated cytokines has been reported (68). Cytokine production by $CD4^+$ T cells has been shown to differ in response to different DENV serotypes, and TNF-α secretion was higher in response to heterologous serotypes after *in vitro* stimulation of $CD4^+$ T cells isolated from vaccinees who received a DENV monovalent (MV) vaccine (49).

B.3. The antibody response to DENV infection. Flaviviruses are positive-strand enveloped RNA viruses whose genome encodes 3 structural (C, prM/M, E) and 7 non-structural (NS1-5) proteins. The virion surface is composed of 180 E and M proteins in antiparallel dimers (55, 78). The E protein consists of three domains (EDI, EDII, EDIII) (54, 66), with EDIII involved in recognition of cell receptor(s) (7, 15, 37, 64) and EDII interacting with attachment factor DC-SIGN (61). The humoral immune response is dominated by anti-E Abs (66, 78), with some anti-prM and anti-NS1 Abs (12, 13, 24, 39, 60, 71, 80). PrM is cleaved from the fully mature virion during processing in the Golgi, but secreted DENV virions are often found in an immature or partially immature state (38). Anti-prM Abs may mediate protection (24) though they can also facilitate entry of immature or partially mature particles into FcγR-bearing cells *in vitro* and thus may play a role in ADE (22, 65). Abs directed against DENV NS1 can mediate protection in mice vaccinated with recombinant NS1 (1). Early mapping studies in mice determined that monoclonal Abs (mAbs) targeting the EDIII lateral ridge (LR) were type-specific and potently neutralizing (14, 48), while mAbs with EDI/II specificity were more cross-reactive and less potent *in vitro* (14, 25). Initial studies with mAbs using human DENV-immune B cells derived from symptomatic patients have identified a large number of cross-reactive mAbs targeting epitopes on EDI/II and prM and very few targeting EDIII (5, 17, 21), supported by binding studies that suggest that up to 80% of anti-DENV Abs in human serum target the fusion loop (FL) in EDII (14, 47). A separate analysis suggested that anti-EDIII Abs contribute only minimally (5-15%) to the neutralizing Ab titer in the immune serum (18). Nonetheless, human anti-EDIII-specific Abs are potently neutralizing, but other highly neutralizing mAbs have been identified that target a complex, quaternary structure epitope that spans adjacent E protein dimers only present on a virus particle (18). This is consistent with data showing that the neutralizing potency of anti-DENV human immune serum is greatly reduced when the serum is depleted using the whole virion, but only minimally reduced following depletion with recombinant E protein (rE) (81). In our dengue mouse model, we showed by depleting EDIII Abs from human seruam that anti-EDIII human Abs contribute to protection and minimize enhancement when present, but can be replaced by neutralizing Abs targeting other epitopes on the DENV virion (85). Whether serum neutralization capacity and amount of Ab produced correlates with the magnitude and quality of the B cell response has not been studied during DENV vaccination. Also, it is not clear which B cell subset provides increased serum neutralization capacity and hence cross-protection after vaccination. Measurement of serum avidity has been used to discriminate between 1° vs. 2° DENV infections (19, 20, 50); however, correlation between the DENV-specific avidity or neutralization capacity of serum and protection after

DENV infection or vaccination remains controversial. In one vaccine study in non-human primates, avidity was correlated with protection against DENV infection (75), but no correlation was found in another study (63).

B.4. Antibody-dependent enhancement. Dengue disease severity is influenced by prior DENV infection history, host genetic factors, viral genetics (56). Studies in humans (69) have demonstrated that heterotypic 2° DENV infections are a major risk factor for severe disease. The phenomenon of ADE (33) has been invoked, (34, 36, 59); most severe cases in 1° infections occur in infants (41) in endemic areas, where DENV-specific Abs are transferred transplacentally to infants from their DENV-immune mothers and wane over time until they are capable of enhancing DENV infection, potentially resulting in severe disease (35). ADE may complicate administration of a tetravalent (TV) dengue vaccine if incomplete immunity against all 4 serotypes occurs. We have recently developed a mouse model of DENV ADE and demonstrated the key role of Fc receptors in ADE *in vivo* (2). **This model will be used in Aim 2 to test serum obtained from vaccinated humans and NHPs.**

B.5. Dengue vaccines. TV dengue vaccine candidates in development include mixtures of four different recombinant live-attenuated viruses (LAV), inactivated, protein subunit or DNA vaccines (82). Three chimeric recombinant LAV dengue vaccines are under active development. Chimeric viruses based on a yellow fever virus backbone (ChimeriVax^{TM}) are in Phase 2b clinical testing in Thailand and Phase 3 testing worldwide by Sanofi Pasteur (27, 28). Recombinant DENV with a 30-nucleotide deletion in the 3'-untranslated region (UTR) of the genome of each serotype were optimized for safety and efficacy in preliminary MV Phase 1 testing (51); different TV formulations of these "delta 30" constructs are in Phase 1 clinical trials (23). Inviragen has exclusively licensed a TV dengue vaccine from the Centers of Diseases Control and Prevention (CDC), termed DENVax, which consists of the live attenuated DENV-2 PDK-53 and three chimeras expressing epitopes of DENV-1, DENV-3 and DENV-4. The DENV-2 PDK-53 was tested as a MV vaccine in Phase 1 and as a component of a TV vaccine in Phase 2 clinical trials. It was well-tolerated and generated long-lasting neutralizing Abs and cell-mediated immune responses to DENV-2 (6, 79, 87). The mutations necessary and sufficient for the attenuated phenotype of DENV-2 PDK-53 reside outside of the structural genes (11). These mutations are T-to-C at genomic nucleotide position 57 in the 5' UTR, and G-to-D and E-to-V mutations at amino acid positions 53 and 250 in NS proteins 1 and 3, respectively. The three recombinant vaccines for DENV-1, DENV-3, and DENV-4 contain the same attenuating mutations as the DENV-2 PDK-53 strain (58). Since all four DENVax components share the identical attenuating mutations, recombination between vaccine strains cannot generate more pathogenic viruses. The genetically defined attenuating mutations permit well-defined genetic quality control tests for safety. The TV DENVax vaccine generates neutralizing Abs to all four DENV serotypes in AG129 mice and in NHPs (10, 57). More recently, in Phase 1 human clinical trials in the US and Colombia, DENVax was shown to be safe and immunogenic (Osorio et al, unpublished). Currently, DENVax is being tested in Phase 2 clinical trials.

B.6. Significance. An ideal dengue vaccine should provide long-lasting protective immunity against all four DENV serotypes. The leading dengue vaccine candidate (Chimerivax) requires three doses (0, 6, and 12 months) to induce a complete balanced response (28). Current DENVax immunization schedule includes two doses (days 0, 3 months). NIH's TV vaccine (TV-003) resulted in 40% TV serconversion after a single dose in naïve subjects (Whitehead, S. personal comm.) and it is believed that a second dose is needed to increase overall seroconversion. A vaccination approach that generates neutralizing Abs to all four DENV serotypes after a single immunization would significantly facilitate prevention of DF, DHF, and DSS, as vaccine compliance in endemic countries would be challenging if repeated injection were required. In contrast, a Rapid Immunization Schedule (RIS) would likely permit widespread vaccine utilization in endemic countries. RIS can also obviate concerns regarding DENV exposure in between doses. For U.S. and EU travelers, RIS would improve uptake and compliance and it would also be of significantly greater utility to the military and for biodefense programs. A simple RIS vaccination strategy with DENVax offers the potential to prevent millions of cases of DF and thousands of DHF/DSS deaths that occur in endemic countries. Utilization of RIS for DENVax delivery would lead to significant economic savings for health care systems of tropical and subtropical countries worldwide. **Understanding how RIS works will allow us to generate effective vaccination strategies that would predictably elicit effective immune responses against all four DENV serotypes in diverse clinical settings.**

C. Preliminary data

C.1. Data on RIS. Studies in interferon (IFN)-α/β and γ-receptor-deficient AG129 mice evaluated whether immunization at two sites, administering a dose of DENVax bearing the 3:3:3:3 formulation (DENVax-1,-2,-3, & -4 at 10^3 plaque-forming units [pfu] each) intradermally (ID) on day (d) 0 (0,0) would induce higher or comparable levels of DENV serotype-specific neutralizing Ab responses to those elicited by administering two doses 42 days apart (0,42). A control group that received one dose of DENVax on d0 (0) and no boost was also included. As shown in Table 1, neutralizing Ab responses to

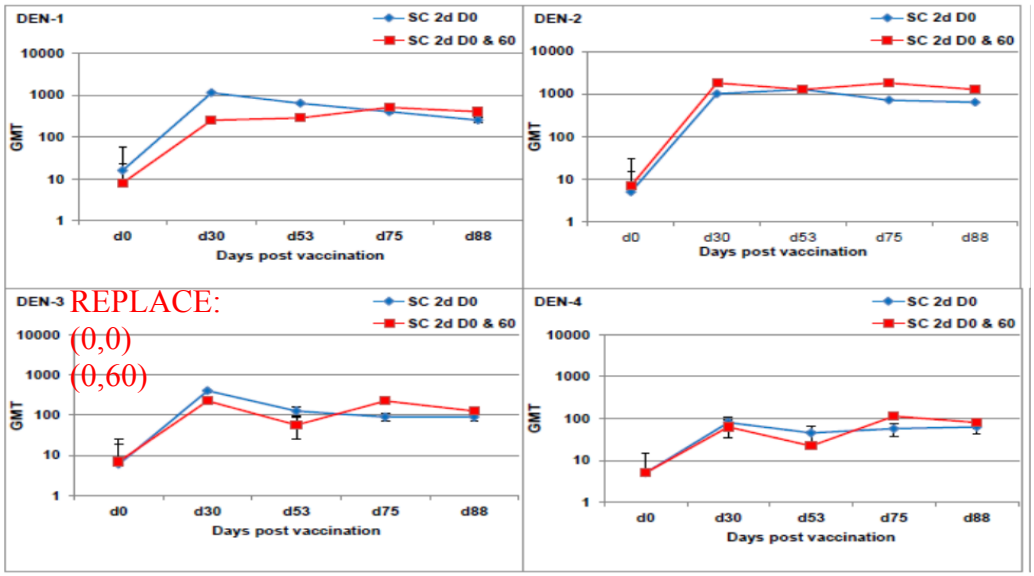
Table 1. Neutralizing antibody responses to each DENV serotype

	DENV-1		DENV-2		DENV-3		DENV-4	
Treatment	**Day 28**	**Day 56**	**Day 28**	**Day 56**	**Day 28**	**Day 56**	**Day 28**	**Day 56**
Day 0	400	800	100	200	200	400	40	40
Day 0,0	**720**	**2000**	**260**	**1000**	**480**	**1200**	**90**	**100**
Day 0,42	560	1200	320	450	180	600	40	40

each serotype in pooled serum samples collected on d28 and d56 post-prime were superior to those elicited by the 0 or 0,42 immunization schedules.

In NHPs, we investigated the potential of RIS combined with the needle-free delivery of DENVax (PharmaJet device) by subcutaneous (SC) and ID routes. Following priming with DENVax, viremia, measured by real-time RT-PCR, was only detectable for DENVax-2 virus between days 5 and 14 and ranged from 3.2 to 5.4 genome equivalents per mL (GE/mL) (data not shown). Preliminary immunogenicity data show that all animals mounted a neutralizing Ab response to all 4 DENV serotypes, and titers were comparable to those elicited after prime and boost on d0 and d60. **Fig. 1** shows results for SC administration; similar results were obtained for ID administration (data not shown). Thus, RIS could permit vaccination to occur in fewer visits, therefore increasing compliance, and induce rapid seroconversion to all 4 DENV serotypes, providing needed protection shortly after the first immunization.

Fig. 1. Kinetics of neutralizing antibody responses following SC immunization. Groups of six NHPs were injected SC with the PharmaJet needle-free device twice with the full high-dose clinical stock of DENVax on d0 at different sites (0,0) or primed and boosted on d0 and 60, respectively, with one full dose of DENVax (0,60).



C.2. B cell responses in mice and humans (Aim 1)

C.2.1. Phenotype and proliferation of B cells during acute DENV infection in mice and humans. To monitor proliferation of B cells in a 2° DENV infection in mice, spleen cells were stained with mAbs against the nuclear cell proliferation-associated antigen, Ki67, along with cell surface markers to enable phenotyping of MBCs ($B220^{+}$, $CD79b^{+}$, IgD^{-}, $CD138^{-}$) and PB/PCs ($CD20^{low}$, $CD27^{bright}$) (91). Similar analysis in human samples showed that PB/PC numbers peak at d5 post-onset of symptoms in 1° and 2° DENV infections (89).

C.2.2. Breadth and specificity of B cell response in human and mice measured by ELISPOT assays. Antigen (Ag)-specific Ab-secreting cells (ASC) that actively secrete Ab (PCs) after a 2° DENV infection were quantified *ex vivo* by ELISPOT. MBCs after a 2° DENV infection were quantified by ELISPOT after *in vitro* stimulation. Mice were infected with DENV-1 98J and 6-8 weeks later with DENV-2 D2S10. Spleen cells harvested at d3, 6 and 9 post-2° infection (p.i.) were polyclonally stimulated for 6 days *in vitro* with fixed *S. aureus* Cowan (SAC), pokeweed mitogen extract (PWM), lipopolysaccharide (LPS), and CpG. The cells were incubated for 5h in 96-well filter plates coated with DENV-1 or DENV-2 Ag, mock-infected C6/36 cellular Ag or goat anti-mouse IgG, to measure the DENV-specific and total ASC. Spots corresponding to ASC were revealed using HRP-goat anti-mouse IgG and chromagen substrate. DENV1-specific MBCs were present in greater numbers than DENV2-specific MBCs at d6 post-2° infection (81.8 DENV-1-specific vs. 23.3 DENV-2-specific ASC/10^6 splenocytes, p=0.003) (91). DENV-1-specific PCs, measured by *ex vivo* ELISPOT, peaked at d6 post-2° infection in the spleen (149.3 ASC/10^6 splenocytes) while DENV-2 specific PCs peaked at d9 post-2° infection with lower numbers (26.3 ASC/10^6 splenocytes), suggesting that cross-reactive PCs generated from cross-reactive MBCs or from cross-reactive semi-long-lived PCs are the main cells implicated in the response after a 2° heterotypic infection (90).

In humans, frozen PBMCs from d6 post-onset of symptoms, isolated from patients with a 2° DENV-3 infection, were thawed and analyzed by ELISPOT *ex vivo.* Plates were coated with DENV-2 N172 or DENV-3 N7236 prepared by ultracentrifugation and captured on anti-E mAb 4G2, to measure the number of DENV-2 and DENV-3-specific PCs circulating at the time of infection. Plates were coated with donkey anti-human IgG to measure the total number of ASCs in the blood. Duplicate samples of $1x10^5$ PBMCs per well (containing DENV antigen) and 3×10^4 cells per well (containing anti-human IgG) were plated in the first well, followed by four 2-fold dilutions. Significantly more DENV-2-specific PCs compared to DENV-3-specific PCs were found in these 2° DENV infections (DENV-2 PCs = 4,402 vs. DENV-3 PCs = 1,129 ASC/10^6 PBMCs; $p<0.0001$) (88).

C.3. A129 mouse model of protection (Aim 2). We have improved our mouse model of DENV infection and disease (3, 4, 43, 44, 73, 74, 86, 91) by performing an additional 10 passages of DENV-2 strain D2S10 between mosquito cells and AG129 mice, obtaining a more virulent strain D220 (Orozco et al, submitted). A129 mice (lacking only the IFN α/β receptor) were inoculated with different doses of D220 intravenously (IV),

and morbidity and mortality were monitored for 10 days. Morbidity was scored 4X/day using our established 5-stage scale of no/mild/severe illness. Mice were euthanized if they appeared moribund (stage 5), and time of death scored to the nearest quarter-day. Upon death, mice were inspected qualitatively for signs of vascular leak (2). IV infection of A129 mice with 10^7 pfu of D220 led to 100% lethal disease, as did ADE infection using cross-reactive serum and a sub-lethal dose of D220 (10^5 pfu) (**Fig. 2**), both with features resembling the vascular leak syndrome seen in human dengue. This improved model allows us to perform the proposed study in A129, less immunocompromised mice.

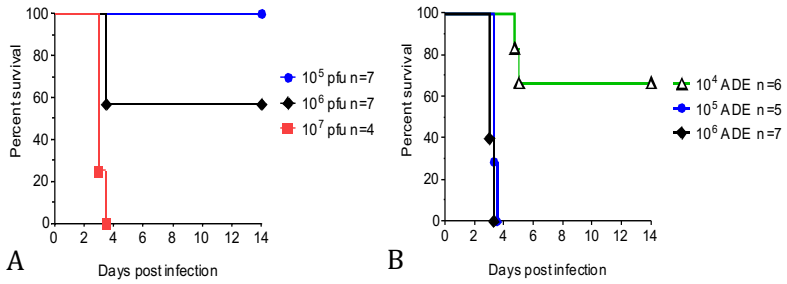


Fig. 2. DENV-2 strain D220 causes mortality in A129 mice after primary infection and at low doses under Ab-enhancing conditions. A129 mice were inoculated IV with different doses of DENV-2 D220. For 1° infections, naïve mice were inoculated (A). For ADE conditions, mice were administered a subneutralizing dose of 10 µg DENV-specific anti-envelope mAb 4G2 intraperitoneally (IP) 18 to 24 h prior to infection (B). Mice were monitored daily until d14 p.i. Kaplan-Meier curves illustrate the susceptibility of A129 mice to D220 infection. The number of mice used for each condition is depicted for each condition (n).

C.4. Immunogenicity and efficacy of DENVax formulations in mice and NHPs.

C.4.1. Immunogenicity and efficacy of DENVax formulations in AG129 and A129 mice. DENVax formulations were initially tested for safety, immunogenicity and efficacy in AG129 mice. MV formulations were safe and elicited robust neutralizing Ab responses to the homologous virus and only limited cross-reactivity to other serotypes. A single dose of MV DENVax-1, -2, or -3 vaccine provided ≥80% protection against both wild-type (WT) DENV-1 (Mochizuki strain) and DENV-2 (New Guinea C) challenge viruses. A single dose of MV DENVax-4 also provided complete protection against WT DENV-1 challenge and significantly increased survival times after challenge with WT DENV-2 (10). In TV studies, DENVax ratios were identified that: (i) caused limited viremia, (ii) induced serotype-specific neutralizing Abs to all 4 DENV serotypes with different hierarchies, and (iii) conferred full protection against clinical disease following challenge with WT DENV-1 or DENV-2 (10). We tested protection against lethal DENV infection in A129 using the new DENV-2 strain D220. A129 mice 6-8 weeks old were immunized SC with 10^5 pfu DENVax-4, DENVax (TV formulation 4345) or the FTA diluent as a control (Ctrl FTA) at d0. Mice were challenged with a lethal dose of 10^7 pfu DENV-2 D220 IV at d56 post-immunization and monitored for morbidity and mortality. Preliminary results show that Ctrl FTA mice died 3-4 days p.i., while DENVax (TV) immunized mice showed 100% survival at d10 (**Fig. 3**).

C.4.2. Efficacy of tetravalent DENVax in *Cynomolgus* macaques. DENVax formulations were tested for safety, immunogenicity, and efficacy in *Cynomolgus* macaques. SC injection of the DENVax formulations was well-tolerated. Low levels of viremia of only one of the 4 vaccine viruses were detected yet neutralizing Ab titers were induced against all 4 DENV serotypes after one or two administrations of vaccine. All animals immunized with the high-dose formulation were protected from viremia, and all immunized animals were completely protected from DENV-3 and DENV-4 challenge. A lower dose of DENVax formulation partially protected animals from DENV-1 or DENV-2 challenge. In contrast, all control animals developed high levels of viremia for multiple days after challenge with DENV-1-4 (57). This study highlights the immunogenicity and efficacy of the tetravalent DENVax formulations in nonhuman primates.

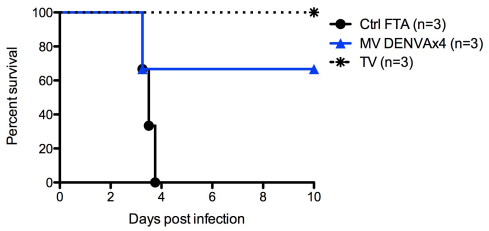


Fig. 3. DENVax tetravalent formulation protects A129 mice against against a lethal challenge with DENV2 D220. A129 mice were immunized with DENVax-4, DENVax (TV) or FTA diluent as a control (Ctrl FTA). Mice were monitored for morbidity and mortality as previously described (10).

C.5. Data for enhancement assays (Aim 2 and 3)

C.5.1. K562 assays for *in vitro* enhancement. The K562 assay has been utilized to measure the enhancing potential of both anti-DENV mAbs and polyclonal human and mouse anti-DENV sera (data not shown**)** (2, 9). K562 cells are an erythroleukemic cell line that naturally express FcγR-IIa on the cell surface and are only susceptible to DENV infection in the presence of anti-DENV Abs. The basic premise is to pre-mix the virus of interest with the anti-DENV Abs (e.g., serum) in question and add serial dilutions of the mixture to K562 cells. The cells are then fixed and stained intracellularly for the virus using a fluorescently tagged DENV-specific mAb (4G2-Alexa488). The percent of cells infected is then compared to that obtained from viral infection in the absence of Ab. An 'enhancement curve' is generated, from which the peak enhancement titer (PET) as well as the magnitude of the enhancement (power) can be derived.

C.5.2. *In vivo* enhancement assays. We examined the effects of anti-DENV-1 and anti-DENV-2 sera on DENV-2 D2S10 infection in mice over a range of doses (4). While the highest dose of anti-DENV-1 serum (400 µl; NT_{50} ~13 vs. D2S10) lethally enhanced infection (4), recipients of 400 µl (NT_{50} ~400) of anti-DENV2 serum developed no signs of illness, confirming that serum serotype-specific Abs at sufficient NT_{50} can prevent ADE.

C.6. DENV-specific serum avidity (Aim 2.1). The avidity of DENV-specific mouse serum (AG129) was measured using a modified ELISA protocol with urea washes (91). Briefly, 96-well plates were coated overnight (o/n) with either DENV-1 or DENV-2 E protein (Hawaii Biotech) at 1 µg/ml, blocked, and incubated for 1h with serum samples from 2° DENV-2 infections (91). The plates were washed for 10 min with 7M urea or PBS before adding biotin-conjugated goat anti-mouse IgG, Streptavidin-Alkaline Phosphatase (SA) and p-Nitrophenyl Phosphate (PnPP) substrate. Optical density (OD) was measured at 405 nm using KC Junior software. Background from the no-Ag coated wells was subtracted from each sample. The percentage of IgG bound was calculated by dividing the OD after the urea wash by the OD after the PBS wash. The avidity of Abs in serum against both DENV-1 and DENV-2 increased significantly 6 days after 2° DENV-2 infection when compared to pre-infection serum, and this increase in avidity was greater against DENV-1 (1° infection) than DENV-2, suggesting an increase in cross-reactive Abs during the acute phase of the 2° heterotypic infection (91). DENV-specific human serum avidity was measured using the same modified ELISA protocol, but plates were coated with DENV-2 and DENV-3 viral particles, prepared in Vero cells and concentrated by ultracentrifugation (88). In 1° DENV-3 infections (d14 post-onset of symptoms), higher avidity was found against DENV-3, with a low level of cross-reactivity against DENV-2 (% IgG bound to DENV-3 = 27.7% vs. % IgG bound to DENV-2 = 9.4%; $p<0.0001$). In contrast, in 2° DENV-3 infections (d6 post-onset of symptoms), the cross-reactive serum avidity against DENV-2 was significantly higher than the serum avidity against DENV-3 (% IgG bound to DENV-2 = 61.3% vs. % IgG bound to DENV-3 = 50.7%; $p = 0.030$) (88).

C.7. Antigen specificity of the serum (Aim 2.2)

C.7.1. Production of DENV virions and viral proteins. Binding specificities of human serum have been tested by direct ELISA using different viral proteins or whole viral particles (data not shown). ***Viral particles*** for ELISA assays were prepared in Vero cells and concentrated by ultracentrifugation (88). ***rE*** was purchased from Hawaii Biotech. If not commercially available in the future, we will produce rE using the same DENV protein expression system we previously used to express ***EDIII***. We have obtained constructs expressing EDIII from Dr. A. de Silva (University of North Carolina, Chapel Hill) (84). For production of ***EDI/II*** and ***prM***, we have received the appropriate constructs from Dr. M. Kielian (Einstein College of Medicine) to produce prM and EDI/II as previously described (46). We have obtained purified ***NS1*** from Merck and have also purified it using anti-NS1 mAb (clone 1H7.4) from the supernatants of stable replicon-transfected BHK cells (M. Diamond, Washington University in St. Louis). MTAs are in place for all of these materials.

C.7.2. Direct ELISAs to measure binding specificities of serum. We have established a number of ELISA assays to measure binding of Abs in serum to DENV virions and proteins (EDIII, NS1 and rE). Briefly, viruses and viral proteins are plated o/n at 4°C in carbonate buffer. Plates are washed and blocked with PBS-T and 5% nonfat dry milk, then serial dilutions of serum are incubated for 1h. Plates are washed and incubated with biotinylated anti-human IgG (1:1,000) for 1h, followed by SA (1:1,000) and PnPP substrate. The results are read at an OD of 450 nm using KC Junior software (data not shown).

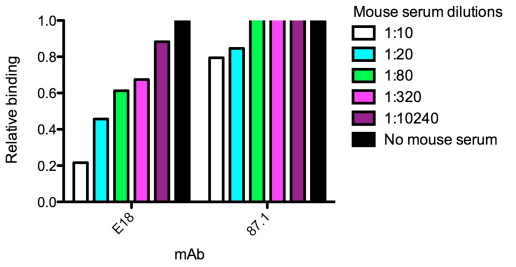


Fig. 4. Humanized/human mAb binding is out-competed by DENV immune mouse serum. DENV2 D2S10 viral particles were used as Ag. Serial dilutions of mouse serum were incubated on the plates prior to incubation with humanized mAb E18 and human mAb 87.1. Relative binding was calculated by dividing the OD measured in wells containing the mAb and mouse serum by the OD of the well containing the mAb alone.

C.7.3. Competition ELISA to evaluate the relative proportion of different epitopes in serum. To measure the relative proportion of different epitopes in mouse DENV-immune serum, we used a competition ELISA with distinct humanized or human mAbs. Briefly, we plated DENV-2 D2S10 virions, prepared using Amicon concentration tubes (Millipore), o/n at 4°C, then blocked the plates and incubated them with serial dilutions of DENV-2 immune mouse serum from 1:10 to 1:10,240. Plates were washed and incubated with 10 ug/mL of humanized mAbs targeting the EDII FL (E18) or the E A-strand (human mAb 87.1). Plates were washed and incubated with biotinylated anti-human Fc IgG (1:1,000) for 1h, followed by SA (1:1,000) and PnPP substrate. The relative binding of each mAb was calculated by dividing the OD produced by the mAb incubated with mouse serum by the OD of the mAb alone (referred to here as relative binding of 1). As shown in **Fig. 4**, the mAbs showed different patterns of decreased binding to DENV-2 D2S10 when the wells were pre-incubated with serial dilutions of anti-DENV-2 mouse serum. The relative binding of mAbs is inversely proportional to the dilution of mouse serum, as the relative binding increases when the mouse serum is diluted further. The different binding patterns are consistent with the relative proportion expected of the different Abs in mouse serum; it is known that DENV-immune serum contains a large proportion of fusion loop Abs and a lower proportion of A-strand Abs. Therefore, this method will be used to study the repertoire of serum Abs after different vaccination schedules using a defined panel of mAbs targeting characterized epitopes.

C.8. DENV-specific $CD4^+$ and $CD8^+$ T cells target NS1, NS3 and NS5 in NHP. We recently conducted a study to determine the cellular immune response to DENV in the Indian rhesus macaque (53). We infected 8 rhesus macaques with 10^5 pfu of DENV-2 NGC strain and monitored the viral load and cellular immune

response. DENV-specific $CD4^+$ and $CD8^+$ lymphocytes targeted NS1, NS3 and NS5 proteins after resolution of peak viremia (**Fig. 5**). $CD4^+$ cells expressed IFN-γ along with TNF-α, IL-2, and MIP-1β, while $CD8^+$ cells expressed IFN-γ, MIP-1β and TNF-α and were positive for the degranulation marker CD107a.

C.9. Impact of pre-existing immunity on DENVax immunogenicity. We have examined the impact of pre-existing immunity to WT DENVs on the immunogenicity of DENVax vaccine (bearing the 5:4:5:5 formulation). The vaccine was delivered SC in two groups of NHPs that had been pre-exposed to WT DENV-2 or WT DENV-4. Animals with pre-existing immunity to WT DENV-2 had neutralizing titers to the homologous virus of 1,280 (n=3), whereas those with pre-existing immunity to WT DENV-4 had a titer of 640 (n=3). When neutralizing Ab titers were compared and expressed as fold-increase to those elicited by DENVax in naïve animals (after 1° immunization) there was a 3-fold increase of neutralizing Ab titer for DENV-1 and DENV-3 on D60 and an over 10-fold increase in titer for DENV-4 antibodies (data not shown). Overall, findings from this study suggest that pre-existing immunity has no detrimental effect on DENVax immunogenicity. In contrast, neutralizing Abs were enhanced and in the case of DENVax-4, which is the least immunogenic component of DENVax, responses were significantly elevated (data not shown). This could translate into a beneficial effect for DENVax vaccination campaigns in dengue-endemic areas.

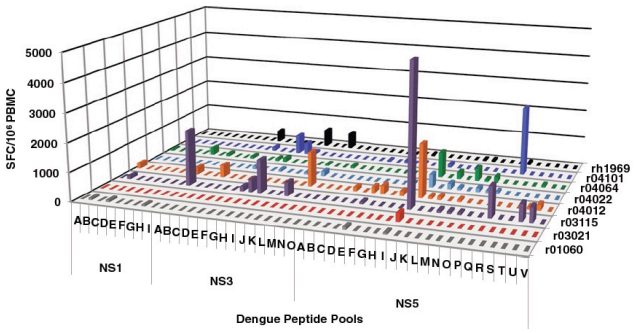


Fig. 5. T cell responses directed against the DENV NS1, NS3, and NS5 proteins. Fresh PBMCs were processed in duplicate in an IFN-γ ELISPOT assay with pools of 15-mer peptides spanning the NS1, NS3, and NS5 proteins.

C.10. Phase 1 clinical trial of DENVax in healthy adult volunteers. A Phase 1 clinical trial was performed to evaluate the safety and immunogenicity of TV DENVax formulations in healthy, flavivirus-negative adults. The study was completed in Rionegro, Colombia, a high-altitude area with no *Aedes aegypti* and no dengue exposure. Low-dose (4:3:4:5) or high-dose (4:4:5:5) formulations of DENVax were administered at 0 and 3 months by either SC or ID injection. The vaccine was well-tolerated with mostly mild and transient local or systemic reactions (data not shown). In addition, DENVax induced significant neutralizing Ab responses to all 4 dengue viruses after one or two administrations (data not shown). **Fig. 6** shows that after a single dose, only 60% seroconverted to 4 serotypes. **Based on our preliminary NHP data (Fig. 1) we anticipate that RIS (0,0) can result in 100% serconversion to 3 or more serotypes by day 30 post-vaccination.**

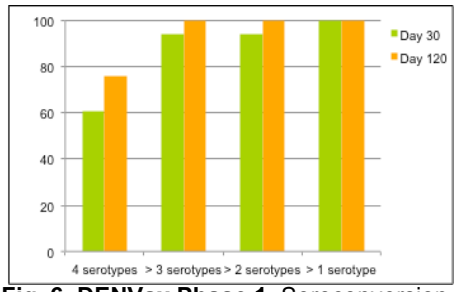


Fig. 6. DENVax Phase 1. Seroconversion % to 4 DENV serotypes.

In summary, we have established the necessary models and assays to study the impact of RIS on B and T cell responses and Ab repertoire. In addition, we have samples available from Inviragen's clinical trials that are evaluating RIS in humans. **Thus, we have preliminary data supporting all of the proposed experiments and methodologies, and are well-poised to perform the studies outlined in Section D.**

D. APPROACH

D.1. Specific Aim 1. Characterize the serotype-specific B and T cell responses elicited after immunization with DENVax using different immunization schedules in relevant mouse and NHP models and samples from human vaccine trials.

D.1.1. Compare the DENV-specific B and T cell responses induced by RIS administered at the same anatomical site or different sites in A129 mice. While several studies on dengue have focused on the role of Abs in protection or enhancement of infection or disease, little is known about the development (breadth and specificity) of the DENV-specific B and T cell responses and their role in maintenance of long-term immunity and protection. The overall hypothesis is tha**t** injection at two sites, accessing two different sets of lymph nodes, will improve the breadth and specificity of serotype-specific B and T cell responses elicited by DENVax.

D.1.1.1. Characterization of B cell responses following prime with RIS. To determine whether RIS is more efficient when performed at different anatomical sites rather than at the same site, we will use 3 groups of mice (n=8/group) that will receive a single injection or two full injections ID at one or different sites of a low-dose formulation of TV DENVax (4:3:4:5; see C.1). A control group will be injected with FTA (virus diluent). Four mice from each group will be euthanized at d6 and 28. Samples from d6 post-vaccination will be used to analyze B cell population subsets and proliferation by flow cytometry (89, 90). We will analyze the % of naïve B cells ($CD20^+$, $CD27^-$, IgD^+), MBCs ($CD20^+$, $CD27^+$, IgD^-) and circulating PB/PCs ($CD20^{low}$, $CD27^{bright}$) in the blood and splenocytes. All samples will be processed *ex vivo*. A baseline of circulating B cells will be derived from FTA immunized control mice. Total leukocyte numbers will be measured by manual cell count (using trypan blue exclusion dye). To monitor proliferation of B cells, cells from whole blood and splenocytes will be stained with anti-CD20, anti-CD27 and anti-CD38, then fixed in 70% cold ethanol for 2h at -20°C before

intracellular staining with a mAb against nuclear cell proliferation-associated Ag Ki67 (90). Results will be expressed as % of cells positive for Ki67. All data will be analyzed in FlowJo and graphed in GraphPad Prism 5.0. Absolute number and % of the different B cell populations and % Ki67-positive will be compared between the different immunization groups using the non-parametric Mann-Whitney U test. Splenocytes from the different groups will be prepared and stored in liquid nitrogen (LN_2) for use in DENV-specific B cell ELISPOT analysis. Frozen splenocytes collected on d6 and d28 will be thawed and stimulated *in vitro* for 6 days with LPS, SAC, CpG and Pokeweed mitogen (16, 89). Culture supernatant, containing Ab secreted by MBCs, will be collected and stored at -20°C for studies in Aim 2. *In vitro*-stimulated cells will be used to measure the % DENV-specific ASCs among total ASC by ELISPOT (i.e., DENV-specific MBCs). We will use separate cellular Ag for each DENV serotype to measure relative amounts of homotypic and heterotypic ASCs. Cellular Ag will be prepared using infected cell lysate from C6/36 cells infected with WT DENV strains (DENV-1, strain 16007; DENV-2, 16681 and D220; DENV-3, 16562; DENV-4, 1036). Supernatants from unstimulated cells kept in culture o/n will be collected, and Abs secreted by PCs (supernatants from unstimulated cells) will be compared to those secreted by MBCs (supernatants from stimulated cultures, see Aim 2). Splenocytes from d6 will be used *ex vivo* to measure the amount of ASCs circulating at the time of vaccination (reflecting DENV-specific PCs). Data will be graphed in Prism 5.0 as % of DENV-specific ASC. The % of DENV-specific MBCs and PCs at different time-points will be compared between the different groups using Mann-Whitney U test. These data will be correlated with protection and enhancement studies in Aim 2.

D.1.1.2. Characterization of T cell responses following prime with RIS. Three groups of mice (n=12) will receive a single ID injection of the 4:3:4:5 DENVax formulation as in Aim 1.1.1. A control group will be injected with FTA to measure basal level of T cell activation. Four mice will be euthanized at d3, 7 and 10 post-prime and splenocytes will be analyzed for $CD4^+$ and $CD8^+$ T cell populations and their activation status by flow cytometry. We will measure the % of $CD4^+$ and $CD8^+$ T cell populations by staining with CD4 and CD8α surface markers. To monitor T cell activation, cells will be stained for CD69 (early activation marker) and CD44 (cell surface receptor for hyaluronic acid), both rapidly upregulated on activated T cells. Results will be expressed as % of $CD4^+$ and $CD8^+$ T cells positive for CD69 and CD44. To determine the DENV-specific $CD4^+$ and $CD8^+$ T cell response, splenocytes collected on d7 post-prime will be stimulated *in vitro* with different concentrations of purified live or inactivated DENVs (strains as in D.1.1.1) for 24h and the Golgi blocker Brefeldin A and/or Monensin will be added for the final 6 h. Cells will also be incubated with medium (negative control) or superantigen SEB (positive control). Cells will then be stained with CD4 and CD8α surface markers, fixed and permeabilized before staining for intracellular cytokines IFN-γ, TNF-α, IL-2, IL-4, IL-6, IL-10 and cell proliferation marker Ki-67. To determine DENV-specific $CD4^+$ and $CD8^+$ T cell cytotoxicity, cells will be incubated with fluorochrome labeled anti-107a mAb, a degranulation marker, for the final 4h before straining for surface CD4 and CD8a. Samples will be analyzed by flow cytometry, and results will be expressed as % cytokine-positive, Ki-67^+ or $CD107a^+$ Ag-specific $CD4^+$ or $CD8^+$ T cells. Data will be graphed in GraphPad Prism 5.0 and p-values calculated using the non-parametric Mann-Whitney U test.

D.1.2. Evaluate the impact of a boost and the time-window between prime and boost on the DENV-specific B and T cell responses in mice elicited by RIS. Our hypothesis is that RIS is effectively priming immune responses of long duration after a single visit. In this sub-aim, we will investigate the quality and quantity of different B and T cell populations, as described in D.1.1 using samples from mice boosted at two different time-points post-primary immunization.

D.1.2.1. B cell responses after boost in mice. To determine whether a boost is needed after RIS and whether the time-window between prime and boost has an impact on the DENV-specific B cell responses, we will immunize groups of A129 mice (12 mice/group) as follows: 1) Prime on d0 (single immunization); 2) Prime on d0 and boost on d42; 3) Prime on d0 and boost on d120; 4) Prime with 0,0 (RIS; whichever site is more effective as determined in D.1.1); 5) Prime with 0,0 and boost on d42; 6) Prime with 0,0 and boost on d120; 7 & 8) Prime and boost (on d42 or 120) with FTA diluent alone. Four mice from each group will be euthanized at d6 and 28 after the boost and whole blood and splenocytes will be processed as in D.1.1.These data will be correlated with protection and enhancement studies described in Aim 2. These results will determine whether RIS without boost is equivalent to the prime and boost immunization schedule or whether a boost is necessary after RIS. Finally, these experiments will help define the best time-window between prime and boost. The remaining 4 mice from each group will be kept for serum collection at d21, 42, 90, 104, 118 and 134 post-priming to monitor the kinetics of neutralizing Ab responses and determine Ab avidity in studies in Aim 2.

D.1.2.2. T cell responses after boost in mice. Eight groups of A129 mice (n=8/group) will be immunized as above (D.1.2.1) to examine T cell responses after boost. Four mice will be euthanized on d7 and 14 post-boost and splenocytes analyzed as in D.1.1.2. To determine memory T cells, splenocytes from d14 post-boost will be stained with CD4, CD8α and memory T cells markers CD44, CD62L, CD127 and CCR7. Flow cytometry will be used to determine the frequency (%) of $CD44^{hi}$ $CD62L^{hi}$ $CD127^{hi}$ $CCR7^+$ central memory (T_{CM}) and $CD44^{hi}$ $CD62L^{low}$ $CD127^{hi}$ $CCR7^{low/-}$ effector memory (T_{EM}) $CD4^+$ and $CD8^+$ T cells. Data will be graphed in Prism 5.0 and p-values calculated using the Mann-Whitney U test.

D.1.3. Evaluate the impact of RIS on DENV-specific B and T cell responses elicited by DENVax in NHPs.

D.1.3.1. B cell responses in NHPs. Groups of 6 NHPs will be injected ID with DENVax 4:3:4:5 using the optimum RIS protocol or the standard prime-boost protocol. A control group of 6 NHP will be injected with the FTA diluent using the optimized RIS protocol. PBMCs will be collected at d0, 6, 14, 90, and 120 post prime, and 6 and 14 days following the boost and will be stored in LN_2. Frozen PBMCs from d14, and corresponding day post-boost, will be thawed and stimulated *in vitro* as in D.1.1.1. *In vitro*-stimulated cells will be used to measure the % of DENV-specific ASC among total ASC by ELISPOT, reflecting the MBC population. Frozen PBMCs from d6, and corresponding day post-boost, will be thawed and used *ex vivo* (D.1.1.1) to measure the amount of DENV-specific PCs present after prime and boost immunization. As in D.1.1.1, we will use separate Ag from the 4 DENV serotypes (DENV viral particles concentrated by ultracentrifugation captured by mouse mAb 4G2) to measure relative amounts of homotypic and cross-reactive ASC (89). The amount of viral particles will be standardized between the four serotypes by direct ELISA using pan-DENV mAb 2H12 with similar affinity against the 4 serotypes that we obtained from Dr. Gavin Screaton (52). Samples from FTA-immunized NHP will be used as negative control. Data will be graphed in Prism 5.0 as % of DENV-specific ASC (MBCs and PCs) and p-values calculated using the Mann-Whitney U test, to compare the different immunization schedules. These data will be correlated with protection studies in Aim 2.

D.1.3.2. T cell responses in NHPs. Frozen PBMC from samples collected on d0, 6, 14 after prime and 6 and 14 days after boost will be analyzed for T cell activation and expansion with mAbs against CD4 and CD8 surface molecules and cell activation markers CD69 and CD44 by flow cytometry as in D.1.1.2. To determine memory T cell numbers, samples collected on d14 post-boost will be stained for CD4, CD8, CD44, CD62L, CD127 and CCR7 and will be analyzed by flow cytometry. Absolute number and % of $CD4^+$ and $CD8^+$ T cells, $CD69^+$ and $CD44^+$ T cell subsets, as well as $CD44^{hi}$ $CD62L^{hi}$ $CD127^{hi}$ $CCR7^+$ T_{CM} and $CD44^{hi}$ $CD62L^{low}$ $CD127^{hi}$ $CCR7^{low/-}$ T_{EM} $CD4^+$ and $CD8^+$ T cells will be compared between the different immunized groups. For DENV-specific $CD4^+$ and $CD8^+$ T cell responses, PBMCs collected on d14 post-prime and post-boost will be stimulated *in vitro* with various concentrations of purified inactivated or live DENV as in D.1.1.2. Cells will then be stained for CD4 and CD8 surface markers, fixed and permeabilized before staining for intracellular cytokines IFN-γ, TNF-α, IL-2, IL-4, IL-6 and IL-10. To determine DENV-specific $CD8^+$ and $CD4^+$ T cell cytotoxicity, cells will be incubated with fluorochrome-labeled anti-107a as in D.1.1.2. Samples will be analyzed by flow cytometry and results be presented as % of cytokine-positive or $CD107a^+$ antigen-specific $CD4^+$ or $CD8^+$ T cells. All data will be graphed in Prism 5.0.

D.1.4. Evaluate the impact of RIS on the DENV-specific B and T cell responses elicited by DENVax in humans. Currently, there is an ongoing Phase 1 clinical trial funded by NIH evaluating the immunogenicity of the low-dose DENVax 4:3:4:5 formulation administered ID with the PharmaJet needle-free delivery device using RIS. In particular, 24 individuals received two administrations of the vaccine in both arms on d0 (0,0) and were boosted on d90 post-priming. A second group (n=24) received two administrations of the vaccine in both arms on d0 (0,0) without a boost, and a third group (n=24) received a single dose of the vaccine in one arm (0) on d0 and boost on d90 post-prime. Serum samples will be collected on d0, 30, 90 and 120 and PBMCs will be collected on d0, 14, 90 and 104. Here, we will leverage these samples to analyze the B and T cell responses using RIS and compare them to those elicited by the standard prime-boost schedule.

D.1.4.1. Evaluate the impact of RIS on the DENV-specific B cell responses elicited by DENVax in human samples. PBMCs from d14 and 104 will be thawed and stimulated *in vitro* for 6 days as in D.1.1.1 and analyzed as in D.1.3.1. Viral particles from DENV1-4 that have been circulating in Colombia will be used as Ag. Similarly, unstimulated PBMCs will be analyzed *ex vivo* to measure PCs. Thus, the % of DENV-specific ASC (MBCs and PCs) among total ASC will be determined by ELISPOT, and Abs secreted by MBCs and PCs in supernatants from stimulated and unstimulated cells, respectively, will be collected for Aim 2. Samples from FTA-immunized volunteers will be used as negative control. Data will be graphed in Prism 5.0 as % of DENV-specific ASC and p-values calculated using the Mann-Whitney U test.

D.1.4.2. Evaluate the impact of RIS on the DENV-specific T cell responses elicited by DENVax in human samples. Frozen PBMC from d0 and 14 will be analyzed for T cell subsets, T cell activation and expansion by flow cytometry as in D.1.3.2 with corresponding anti-human mAbs. For DENV-specific $CD4^+$ and $CD8^+$ T cell responses, PBMCs collected on d14, 90 and 104 will be stimulated *in vitro* with dengue peptide pools encompassing the amino acid sequences of the E protein from each DENV serotype (strains as in D.1.1.1) and the sequences of NS1, NS3 and NS5 from WT DENV-2 (strain 16681) that constitutes the backbone of each DENVax vaccine. We will incubate cells with peptide pools at a final concentration of 10μM. Cells will be stained as in D.1.3.2 to determine DENV peptide-specific cytokine production with Abs against IFN-γ, TNF-α, IL-2, IL-4, IL-6, IL-10 and cell proliferation marker Ki-67. To determine DENV-specific $CD8^+$ and $CD4^+$ T cell cytotoxicity, cells will be incubated with anti-107a as above. Samples will be analyzed by flow cytometry and results will be presented as % of cytokine-positive or $CD107a^+$ antigen-specific $CD4^+$ or $CD8^+$ T cells.

Anticipated results. First, we anticipate that priming with RIS will induce greater activation and expansion and of B and T cells as measured by flow cytometry and a higher number of DENV-specific PCs and MBCs and IFNγ-producing and cytotoxic T cells than priming with a single administration of one full dose of DENVax. Priming with RIS at two different sites is expected to work more efficiently than RIS administered at one site

due to Ag distribution at two different sets of lymph nodes that could permit more efficient presentation and perhaps favor less interference by immunodominant viruses such as the DENVax-2 in the DENVax TV formulation. If so, following a booster administration, particularly at a later time point post-prime, we expect a more robust expansion of Ag-specific B and T cells and a broader serotype specificity resulting in more balanced responses. This is supported by our preliminary NHP data (Fig. 1). We expect similar conclusions from the mouse, NHP and human studies.

Potential Problems and Alternative Strategies. The amount of DENV-specific ASC in mice will be measured using cellular Ag, which contain both DENV structural and non-structural Ag, as this is the optimal protocol to study ASC in mice (90). In contrast, in NHPs and humans, the optimal protocol for ASC uses viral particles (89); thus, the response to NS Ag will not be studied with these assays. If we find a difference, especially in DENV-2 reactivity, as DENVax-2 is the backbone for all the vaccine strains containing the NS proteins, we will optimize ELISPOT assays using NS1 recombinant protein as described in Aim 2 for human and NHP studies.

D.2. Specific Aim 2: Determine the repertoire and functional profile of antibody responses elicited by DENVax using RIS in mice, NHPs, and humans.

The parameters that will enable the prediction of protection vs. ADE after vaccination with a TV dengue vaccine have not been clearly established. We will use *in vitro* neutralization and enhancement assays as well as passive Ab transfer model of DENV infection to first investigate protection vs. enhancement in the A129 mouse model after lethal DENV-2 D220 challenge or by measuring viral load after sub-lethal challenge with the 4 DENV serotypes following different immunization protocols. Secondly, we will define the Ab repertoire at the domain-level after different immunization schedules by characterizing the binding activity of the serum to different DENV Ag. Finally, we will measure the overall avidity of the serum by a modified ELISA using urea washes and use several well-characterized mAbs to study which Abs are the most abundant in the serum and contribute the most to binding as measured by a competition ELISA assay. These data will be correlated with infection outcomes in mice (protection vs. ADE) and NHPs (protection), which will reveal associations between protection vs. ADE and the serum Ab repertoire and functional profile. We will then be able to apply these data to studies conducted on human samples.

D.2.1. Measure the DENV-specific neutralization and enhancement capacities and DENV-specific avidity of the serum after different immunization protocols in mice, NHPs and humans.

D.2.1.1. Neutralization and enhancement capacities of the serum. Serum samples from the different studies outlined in Aim 1 in mice, NHPs and humans will be serially diluted to measure the anti-DENV specific NT_{50} titer using microneutralization assays. Briefly, 96-well tissue culture plates will be seeded with Vero cells at a density of $2x10^5$cells/ml in 100µL/well. Two-fold serial dilutions of heat-inactivated serum will be mixed with an equal volume of virus suspension containing 100 pfu followed by incubation at 4^0C for 13-15 hours (h) or o/n (depending on the virus serotype). Culture medium will be discarded from the Vero cell plates and 30 µL of the serum-virus mixture will be added to each well in triplicate followed by incubation at 37^0C for 2h. Control positive and negative sera samples will be also included. An overlay with 100µl of 1.2% carboxy-methyl cellulose will be added per well and cells will be incubated as above for 2.5 days for DENV-1, -3 and -4, and 3 days for DENV-2. After this period of incubation, the overlay will be removed and cells will be fixed with 85% cold acetone for 10 min at room temperature (RT) or 30 min at -20^0C. After removing the acetone and prior to staining, plates will be equilibrated at RT, washed three times with PBS to rehydrate the cells and to remove remnants of the semi-solid overlay. Rabbit anti-DENV polyclonal antibody diluted (1:1000) in PBS-T containing 5% milk powder will then be added to each well, and plates will be incubated for 2h at 37^0C. Plates will be washed three times with PBS-T and incubated with anti-rabbit Ab conjugated with HRP for 2h at 37^0C. Finally, plates will be washed three times with PBS-T and incubated with the substrate (3-amino-9-ethylcarbozole) for 10-30 min or until plaques were visible. The plates will then be washed with water and air-dried. Plaques will be quantified on an ELISPOT reader. Fifty % of the average number of plaques in the negative control serum will define the cutoff point. The serum dilution closest to the cutoff will be recorded as the reciprocal neutralizing titer. The peak enhancement titer (PET) will be measured by K562 assay (9) as described in C.5.1. Enhancement data from the K562 assay will be graphed with % cell infection vs. serum dilution. The dilution that yields the highest % cell infection will be reported as the PET. For the neutralization *in vitro* assays we will use either DENV-1-4 that have been circulating recently in Colombia, for the human studies, or DENV-1-4 viruses isolated in the laboratory (D.1.1.1). For the enhancement assays we will use the DENV-2 D220 strain. The NT_{50} and PET values will be compared between each set of sera obtained from different vaccine schedules using the Mann Whitney U test.

D.2.1.2. DENV-specific serum avidity. To measure DENV-specific serum avidity, we will use our modified ELISA protocol using urea washes (C.6) (89, 90). Urea washes detach low-affinity Abs, thus decreasing the OD in an ELISA; the lower the avidity of the sera, the greater the decrease in OD. Avidity will be measured using a 1:50 or 1:100 serum dilution, as per our current protocol, and the result will be expressed as % of IgG bound after 6-9M urea washes. Serum avidity measured after prime, before boost (d42 and d90) reflects the Abs secreted by LLPCs. Serum avidity d6 post-boost may be attributed to either re-activated PCs or MBCs.

Circulating MBCs do not secrete Ab and need to differentiate into PCs *in vivo* or be stimulated *in vitro* to become ASCs. We will measure and compare the avidity of unstimulated supernatants (PBMCs in RPMI+10% FBS for 12h, reflecting Abs secreted by circulating PCs at time of infection) to the avidity measured in stimulated supernatants (PBMCs stimulated *in vitro* for 6 days with SAC/CpG/PWM, reflecting Abs secreted by circulating MBCs) and to the serum avidity before challenge (reflecting Abs secreted by LLPCs). For these *in vitro* assays, we will use concentrated viral particles prepared by ultracentrifugation or Amicon concentration. The % IgG bound values derived against DENV-1-4 will be compared between each set of sera obtained from different vaccine schedules using the Mann Whitney U test. DENV-specific serum avidity will be correlated with neutralization Ab titers obtained in D.2.1.1 and with infection and disease outcomes as described in D.2.3.

D.2.2. Characterize the serum Ab repertoire at the serotype- and domain-level using ELISA binding assays in serum obtained after different vaccination protocols in NHPs and humans. Here, we will measure the DENV-specific binding of sera after different vaccination protocols, obtained in Aim 1, using ELISA assays as well as a competition assay to estimate the contribution of different epitopes to binding capacities.

D.2.2.1. Analyze binding of serum to rE, EDI/II and EDIII, prM and NS1 for the four DENV serotypes. Serum samples, obtained in Aim 1, will be screened by direct ELISA using whole virus as Ag to determine their serotype-specificity and their binding capacity to whole virions, prepared by ultracentrifugation. These sera will then be tested for their binding specificity by ELISA using different DENV recombinant proteins as Ag. Briefly, serial 2-10X dilutions, starting at 1:3, will be incubated on ELISA plates coated with rE, EDI/II, EDIII, prM and NS1, from each of the four DENV serotypes as described in C.7.2. Binding levels for each serum sample will be expressed as the highest serum dilution that yields an OD $\geq$2X the background OD. We will produce the DENV proteins as described in C.7.1 or will obtain them from BEI, commercial sources (e.g., Microbix, Merck), or scientific collaborations. These assays will allow us to categorize the characteristics of the sera according to: i) type-specific or cross-reactive binding (depending on whether they bind to the viral protein from one or more DENV serotypes) and ii) Ag-specificity. Characteristics of serum will be visualized using descriptive statistical analysis, and the Mann-Whitney U test will be performed to compare the different vaccine protocols.

D.2.2.2. Analyze the contribution of different epitopes to the binding capacities of the serum using a competition ELISA. We will use a competition ELISA assay, as described in C.7.3, to measure the contribution of different epitopes to the binding capacity of the serum generated after different vaccination schedules. Briefly, we will coat plates with DENV-1-4 viral particles prepared by ultracentrifugation (strains as in D.1.1.1 for NHP studies, or recent DENV strains that have been circulating in Colombia for human studies), then incubate the plates with five 4-fold serial dilutions of human or NHP serum obtained from vaccinated NHPs and human volunteers, starting at 1:10. After washing, the plates will be incubated with previously characterized mouse mAbs directed to epitopes known to be the target of Abs that make up a large proportion of the human/NHP polyclonal response (e.g., FL, prM) or that are highly neutralizing (e.g., EDIII LR, EDIII A-strand, epitopes spanning adjacent E protein dimers) (B.4). Plates will be washed and incubated with biotinylated anti-human or anti-NHP IgG (1:1,000) for 1h, followed by SA (1:1,000) and PnPP substrate. Each experiment will be performed in triplicate. Relative binding of different mAbs in human and NHP serum will be calculated by dividing the OD of wells incubated with serum and mAb by the OD incubated with serum alone. The difference between the relative amount of Abs in each serum sample recognizing the different epitopes will be analyzed by the non-parametric Friedman's test for paired groups. The distribution of relative binding values to each mAb epitope between each vaccine formulation will be compared using the non-parametric Kruskal-Wallis test. This will allow defining whether Abs targeting particular epitopes are more abundant in serum after RIS, with or without boost, as compared to the single immunization protocol, with or without boost.

D.2.3. Characterize the protection vs. enhancement capacities of the serum obtained after different immunization schedules from NHPs and humans *in vivo* using the A129 mouse model. This sub-aim examines whether the sera obtained after different vaccination schedules in NHPs and humans induce protection or enhancement *in vivo* in the A129 mouse model of DENV infection and disease, following a protocol we have established for analyzing human sera in mice (85). The vaccination schedules that will be tested for NHPs are: 1) 0 and boost; 2) (0,0) and boost; 3) FTA as control. The vaccination schedules that will be tested for humans are: 1) 0 and boost; 2) 0 alone; 3) (0,0) and boost; 4) (0,0) alone; 5) FTA as control.

D.2.3.1. Protection studies using lethal challenge with DENV2 D220. Two serial 4-fold dilutions of pooled sera from the different vaccination schedules (D.1.3 and D.1.4) obtained from NHPs and humans will be transferred IP to naïve mice, with 4 mice per vaccination schedule and dose. Control mice will be administered the same volume of serum obtained from control FTA NHPs or volunteers as the experimental mice. The amount of serum that will be injected will be defined according to the neutralization potency (NT_{50}) of the serum (C.5.2). A small sample of blood will be collected using a retro-orbital or submandibular method ("pre-infection bleed") 24h after the serum transfer, approximately 4h prior to DENV-2 D220 infections. The NT_{50} of Abs against DENV-2 D220 in the mice at the time of infection will be measured in the pre-infection serum samples. The mice will then be challenged with 10^7 pfu (lethal dose) of DENV-2 D220. Morbidity and mortality will be monitored until death or for 10 days. Morbidity and mortality will be scored on a 5-point scale of no/mild/severe illness as in C.3. Upon death, the mice will be inspected qualitatively for signs of vascular leak. Survival curves

will be generated by contrasting % survival after DENV-2 D220 infection for the 2 dilutions of antiserum. Viral load in selected tissues will be measured by plaque assay and viremia will be measured by qRT-PCR at the time of death (85). Because the titer of transferred sera in the mice can fall below the limit of detection for *in vitro* NT assays (3), we will use the NT_{50} of the pre-transfer Ab against DENV-2 multiplied by a dilution factor as a proxy. For instance, we have determined that the dilution factor between the NT_{50} against DENV-2 D2S10 of pre- and post-transfer serum (when the latter is above the detection limit) is 5-10 for anti-DENV-2 sera. Mann-Whitney rank sum tests will then be used to compare resulting viremia levels between titers of antisera transferred between different immunization protocols. Data will be plotted using GraphPad Prism 5.0 and p-values will be generated by Mann-Whitney U test to compare the efficacy of the different vaccine protocols.

D.2.3.2. Protection studies against the 4 DENV serotypes using sublethal challenge. Two 4-fold dilutions of pooled sera from the different vaccination schedules (D.1.3 and D.1.4) obtained from NHPs and humans will be transferred IP to naïve mice, with 4 mice per vaccination schedule and dose. Control mice will be administered the same volume of sera obtained from control FTA NHPs or volunteers as the experimental animals. A small sample of blood will be collected 24h after serum transfer (D.2.3.1), ~4h prior to DENV-1-4 infections. The following viral strains injected at 10^5 pfu/mouse will be used: DENV-1 West Pac, DENV-2 D220, DENV-3 3009 and DENV-4 TVP-360. Viral load in selected tissues will be measured by plaque assay, and viremia will be measured by qRT-PCR at the time of death (85). Data will be plotted using Prism 5.0 and p-values will be generated by Mann-Whitney U test to compare the efficacy of different vaccine protocols.

D.2.3.3. Enhancement studies against the DENV2 D220 serotypes using sub-lethal challenge. We will test enhancement *in vivo* by using serum from vaccinated volunteers or NHPs as described in D.1.3 and D1.4. Based on previous work described in C.5.2 (2), we know that the *in vitro* NT_{50} can be used to determine the amount of serum in passive transfer that will induce enhancement *in vivo*. As a negative control, we will use serum from NHPs infected with DENV-2 or DENV-2 WHO human serum and transfer at an NT_{50} titer known not to cause enhancement (C.5.2) (2). As a positive control, we will use serum from NHPs infected with DENV-1 (strain) or DENV-1 WHO human serum and transfer the same volume of sera (at an NT_{50} known to cause enhancement) (C.5.2). We will compare the pooled sera from NHPs and humans vaccinated with the TV formulation to the anti-DENV-1 and anti-DENV-2 results with the expectation that the sera from the RIS vaccinated NHPs and humans will not enhance infection. Additional control mice will be administered the same volume of serum from NHPs or humans given FTA alone. All sera will be transferred IP into naïve mice. A small pre-infection bleed will be collected 24h after serum transfer, ~4h prior to DENV-2 D220 infection. Survival curves will be generated contrasting % survival after DENV-2 D220 infection comparing anti-DENV-1, anti-DENV-2, DENVax-immune sera and FTA non-immune sera. Viral load in selected tissues will be measured by plaque assay, and viremia will be measured by qRT-PCR at d3 p.i. Data will be plotted using Prism 5.0, and p-values will be generated by Mann-Whitney U test.

Anticipated results. We expect to find a correlation between *in vitro* neutralization and *in vivo* protection and between the strength and serotype-specificity of the B cell response and *in vivo* protection assays. We expect higher serum neutralization capacity after RIS as compared to single immunization and after boost compared to prime immunization. We expect higher avidity after boost when compared to that observed after prime immunization. We expect FL and prM Abs to make up a larger proportion of binding capacity of the serum after boost compared to prime immunization. We expect serum from TV DENVax immunizations to protect against DENV-2 D220 lethal challenge and to reduce viral load against the 4 serotypes in mice. We do not expect passive transfer of serum from TV DENVax vaccinated humans and NHPs to enhance DENV in mice.

Potential Problems. The ELISA data will be useful for determining the overall quality of the sera and the range of binding signal. Low-avidity Abs, however, may be undetected by ELISA, and could be detected by Surface Plasmon Resonance (SPR). We are currently collaborating with Dr. Alam Munir at Duke University to measure serum avidity in DENV-immune individuals by SPR, and this technique could be applied in the future to analyze serum samples from vaccinated animals and humans.

D.3. Specific Aim 3: Examine the impact of pre-existing immunity to WT DENVs on the immunogenicity of DENVax administered with RIS. An effective vaccine against DENV must be safe, immunogenic, and capable of eliciting a long-term protective immunity against all 4 serotypes. Pre-existing immunity to flaviviruses can significantly impact the safety and immunogenicity of TV dengue vaccines (62). Here the immunogenicity of DENVax administered using the optimized RIS protocol will be assessed in previously WT DENV-exposed A129 mice, NHPs, and humans. Serotype-specific B and T cell responses and the functional profile of serum Abs will be analyzed.

D.3.1. Impact of pre-existing immunity to WT DENVs on the serotype-specific B and T cell responses to DENVax administered by RIS in mice. Groups (n=60) of 6-8 week old A129 mice will be injected ID on d0 with 10^5 pfu of WT DENV-2 (16681) or WT DENV-4 (1036). A control group of mice will be injected with PBS. Mice will be bled on d59 post-DENV infection to monitor seroconversion against all 4 DENV serotypes. The following day (d60) all groups of mice will be immunized ID with TV DENVax (4:3:4:5) formulation using the optimum RIS priming (D.1.2). Mice will be bled on d3 post-DENVax prime to measure viremia against each

DENVax by qRT-PCR and on d74 to measure serotype-specific neutralizing Ab titers. On d6 and d28 post-1^0 immunization, 4 mice from each group will be euthanized and splenocytes analyzed for serotype-specific B and T cell responses as in D.1.1 and 1.2. The remaining mice will be boosted on d120 post-DENVax prime. Six and 28 days post-2° DENVax immunization, 4 mice from each group will be euthanized and splenocytes analyzed for serotype-specific B and T cell responses as in D.1.1 and 1.2. The remaining mice will be bled every two weeks for two months to measure the duration of DENV serotype-specific neutralizing Ab responses (D.2.2.1).

D.3.2. Impact of pre-existing immunity to WT DENVs on the serotype-specific B and T cell responses to DENVax administered by RIS in NHPs. The 6 control animals from the NHP study in D.1.3 together with those that have been vaccinated will be challenged SC with 10^5 pfu WT DENV-2 NGC (n=3) or WT DENV-4 Dominica/81 (n=3) 2 weeks post-boost (see D.1.3.1). Protection will be assessed by measuring viremia in serum samples collected on d3, 5, 7, 9, 11, 13 and 15 post-challenge by qRT-PCR. In addition, serum samples collected just prior to challenge and 2 weeks post-challenge will be used for Ab analysis as in Aim 2. On d60 post-challenge, control NHPs that received only FTA will be injected ID with the 4:3:4:5 DENVax formulation using the optimized RIS. Viremia to DENVax vaccine viruses will be measured in serum samples collected on d0, 3, 5, 7, 9,12 and 15 post-DENVax prime and boost (depending on the optimum time window per D.1.2) using qRT-PCR. Neutralizing Ab responses will be measured in serum samples collected on d0, 15, 30 and 60 post-primary and secondary vaccination and Abs will be analyzed as in Aim 2. PBMCs will be collected at d0, 6, 14, and corresponding days following the boost and will be stored in LN_2 until use for analysis of B and T cell responses as described in D.1.3.1 and D.1.3.2.

D.3.2. Impact of pre-existing immunity to WT DENVs on the serotype-specific B and T cell responses to DENVax administered by RIS in humans.
Invivagen is currently conducting clinical trials evaluating RIS in human subjects in dengue-endemic areas (Puerto Rico, Colombia, Singapore). PBMCs and sera will be available from these studies compared to standard prime-boost schedules to test B and T cell responses and Ab repertoire to DENV as described above.

Anticipated results. We expect that in the face of pre-existing immunity, DENVax priming with RIS will induce significantly higher activation and expansion of B and T cells to all 4 serotypes and a greater number of DENV-specific ASC B cells and IFNγ-producing and cytotoxic T cells compared to the naïve group. We anticipate that responses to all 4 serotypes will be balanced following the DENVax boost. Also we expect DENVax to be safe with no viremia detected to any of the vaccine viruses after prime or boost due to circulating Abs.

Potential Problems and Alternative Strategies. As in Aim 1, the optimal protocol to study ASC in NHPs uses viral particles (89) rather than cellular Ag as used in mice (90); thus, the response to NS Ag will not be studied with these assays. If we find a difference between mice and NHP, especially in the reactivity against DENV-2 (backbone) we will optimize ELISpot assays using NS1 recombinant protein as described in Aim 2.

Conclusions. The studies performed in this proposal will reveal the mechanistic basis for the improved immunological outcomes associated with RIS dengue vaccination and will determine the optimal immunization schedule. We take advantage of expertise within our group in the areas of B and T cell immunology and analysis of DENV antibody repertoire, utilize our existing dengue mouse and NHP models, and leverage ongoing human clinical vaccine trials. The results are significant, as **this new RIS could permit vaccination to occur in fewer visits, thus increasing compliance, and induce rapid seroconversion to all four DENV serotypes**, providing needed protection shortly after the first immunization. Thus, this would enable a safer, more effective vaccine for endemic countries where dengue is a major public health problem as well as for travelers to tropical and subtropical countries.

Timeline:

Aim	Milestone	Year1		Year2		Year3		Year4		Year5	
1.1.1	Characterize serotype-specific B cell responses in mice (injection site) - UCB										
1.1.2	Characterize serotype-specific T cell responses in mice (injection site) - Invivagen										
1.2.1	Characterize the serotype-specific B cell responses after boost in mice - UCB										
1.2.2	Characterize the serotype-specific T cell responses after boost in mice - Invivagen										
1.3.1	Characterize the serotype-specific B cell responses in NHPs - UCB										
1.3.2	Characterize the serotype-specific T cell responses in NHPs - Invivagen										
1.4.1	Evaluate the serotype-specific B cell responses in humans - UCB										
1.4.2	Evaluate the serotype-specific T cell responses in humans - Invivagen										
2.1	Characterize serum functionality in mice, NHPs and humans - UCB										
2.2	Characterize serum Ab repertoire in NHPs and humans										
2.3	Characterize in vivo protection vs. enhancement capacities of the serum - UCB										
3.1	Examine impact of pre-existing DENV immunity on B cell responses in mice - UCB										
3.1	Examine impact of pre-existing DENV immunity on T cell responses in mice - Invivagen										
3.2	Examine impact of pre-existing DENV immunity on B cell responses in NHPs - UCB										
3.2	Examine impact of pre-existing DENV immunity on T cell responses in NHPs - Invivagen										