

Evolution of a Functionally Intact but Antigenically Distinct DENV Fusion Loop

Reviewed Preprint

Revised by authors after peer review.

[About eLife's process](#)

Reviewed preprint version 2

September 8, 2023 (this version)

Reviewed preprint version 1

July 12, 2023

Posted to bioRxiv

March 27, 2023

Sent for peer review

March 22, 2023

Rita M. Meganck, Deanna Zhu, Stephanie Dong, Lisa J. Snoderly-Foster, Yago R. Dalben, Devina Thiono, Laura J. White, Aravinda M. DeSilva, Ralph S. Baric, Longping V. Tse 

Department of Molecular Microbiology and Immunology, Saint Louis University • Department of Epidemiology, University of North Carolina at Chapel Hill • Department of Microbiology, University of North Carolina at Chapel Hill

 https://en.wikipedia.org/wiki/Open_access

 <https://creativecommons.org/licenses/by/4.0/>

Abstract

A hallmark of Dengue virus (DENV) pathogenesis is the potential for antibody-dependent enhancement, which is associated with deadly DENV secondary infection, complicates the identification of correlates of protection, and negatively impacts the safety and efficacy of DENV vaccines. ADE is linked to antibodies targeting the fusion loop (FL) motif of the envelope protein, which is completely conserved in mosquito-borne flaviviruses and required for viral entry and fusion. In the current study, we utilized saturation mutagenesis and directed evolution to engineer a functional variant with a mutated FL (D2-FL) which is not neutralized by FL-targeting monoclonal antibodies. The FL mutations were combined with our previously evolved prM cleavage site to create a mature version of D2-FL (D2-FLM), which evades both prM- and FL-Abs but retains sensitivity to other type-specific and quaternary cross-reactive (CR) Abs. CR serum from heterotypic (DENV4) infected non-human primates (NHP) showed lower neutralization titers against D2-FL and D2-FLM than isogenic wildtype DENV2 while similar neutralization titers were observed in serum from homotypic (DENV2) infected NHP. We propose D2-FL and D2-FLM as valuable tools to delineate CR Ab subtypes in serum as well as an exciting platform for safer live attenuated DENV vaccines suitable for naïve individuals and children.

eLife assessment

This **valuable** study describes engineered dengue virus variants that can be used to dissect epitope specificities in polyclonal sera, and to design candidate vaccine antigens that dampen antibody responses against undesirable epitopes. While the major claims are supported by **solid** evidence, experiments to distinguish the impact on antibody binding from neutralizing activities would have strengthened the study. This work will be of interest to virologists and structural biologists working on antibody responses to flaviviruses.

Dengue virus (DENV) is a member of the *Flavivirus* genus and is a major global public health threat, with four major serotypes of DENV found worldwide. Dengue causes ~400 million infections each year, of which ~20% of cases present clinically, a subset of which may progress to severe Dengue Hemorrhagic Fever/Dengue Shock Syndrome (DHF/DSS).^{1,2} DENV is transmitted through *Aedes* mosquito vectors, and globalization and global warming are increasing the endemic range of Dengue worldwide.^{3,4} The pathogenesis of Dengue is complex, as first-time infections are rarely severe and lead to serotype-specific immunity. However, re-infection with a different serotype increases the risk of developing DHF/DSS.⁵ This is thought to be due to the phenomenon of antibody-dependent enhancement (ADE), in which poorly neutralizing cross-reactive (CR) antibodies (Abs) lead to enhanced viral uptake and infection of unique cell populations in an Fcγ-receptor-mediated manner.⁶

ADE remains a major challenge for DENV vaccine development.⁷ The leading DENV vaccine platforms in clinical testing are tetravalent live attenuated virus mixtures of all four serotypes. However, creating formulations that elicit a balanced response has proven challenging.⁸ Additionally, lab-grown strains differ from patient-derived DENVs in both maturation status and antigenicity.⁹ In particular, Abs targeting the fusion loop (FL) have been reported to neutralize lab and patient strains with differing strengths and have been observed to facilitate Fcγ-receptor uptake *in vitro* and therefore ADE.^{10–12} Currently, there is a single FDA-approved DENV vaccine, Dengvaxia. However, it is only approved for use in individuals aged 9–16 with previous DENV infection living in endemic areas and is contraindicated for use in naïve individuals and younger children. In naïve children, vaccination stimulated non-neutralizing CR Abs that increased the risk of severe disease after DENV infection.^{13,14} Other DENV vaccines have been tested or are currently undergoing clinical trial, but thus far none have been approved for use in the United States.¹⁵ The vaccine Qdenga has been approved in the European Union, Indonesia, and Brazil, although vaccine efficacy in adults, naïve individuals, and with all serotypes has not yet been shown.

The DENV FL is located in Envelope (E) protein domain II (EDII) and is involved in monomer-monomer contacts with EDIII.¹⁶ During the DENV infection cycle, low pH triggers a conformational change in the E protein.¹⁷ The structure of the virion rearranges, and individual monomers form a trimer with all three FLs in the same orientation, ready to initiate membrane fusion.^{16,17} The core FL motif (DRGWGNGCGLFGK, AA 98–110) is highly conserved, with 100% amino acid conservation in all DENV serotypes and other mosquito-borne flaviviruses, including Yellow fever virus (YFV), Zika virus (ZIKV), West Nile virus (WNV), Kunjin virus (KUNV), Murray Valley encephalitis virus (MVEV), Japanese encephalitis virus (JEV), Usutu virus (USUV), and Saint Louis encephalitis virus (SLEV; **Figure 1A**). Although the extreme conservation and critical role in entry have led to it being considered extremely difficult to change the FL, we successfully tested the hypothesis that massively parallel directed-evolution could produce viable DENV FL mutants that were still capable of fusion and entry, while altering the antigenic footprint. The FL mutations, in combination with optimized prM cleavage site mutations, ablate neutralization by the prM- and FL-Abs, retain sensitivity to other protective Abs, and provide a novel vaccine strategy for DENV.

Results

To engineer a virus with a novel antigenic footprint at the FL, we targeted the core conserved FL motif. We generated two different saturation mutagenesis libraries, each with 5 randomized amino acids: DRGXGXGXXFGK (Library 1; AA 101, 103, 105–107) and DRGXXXXXGLFGK (Library 2 AA 101–105). Library 1 was designed to mutate known residues targeted by FL mAbs while

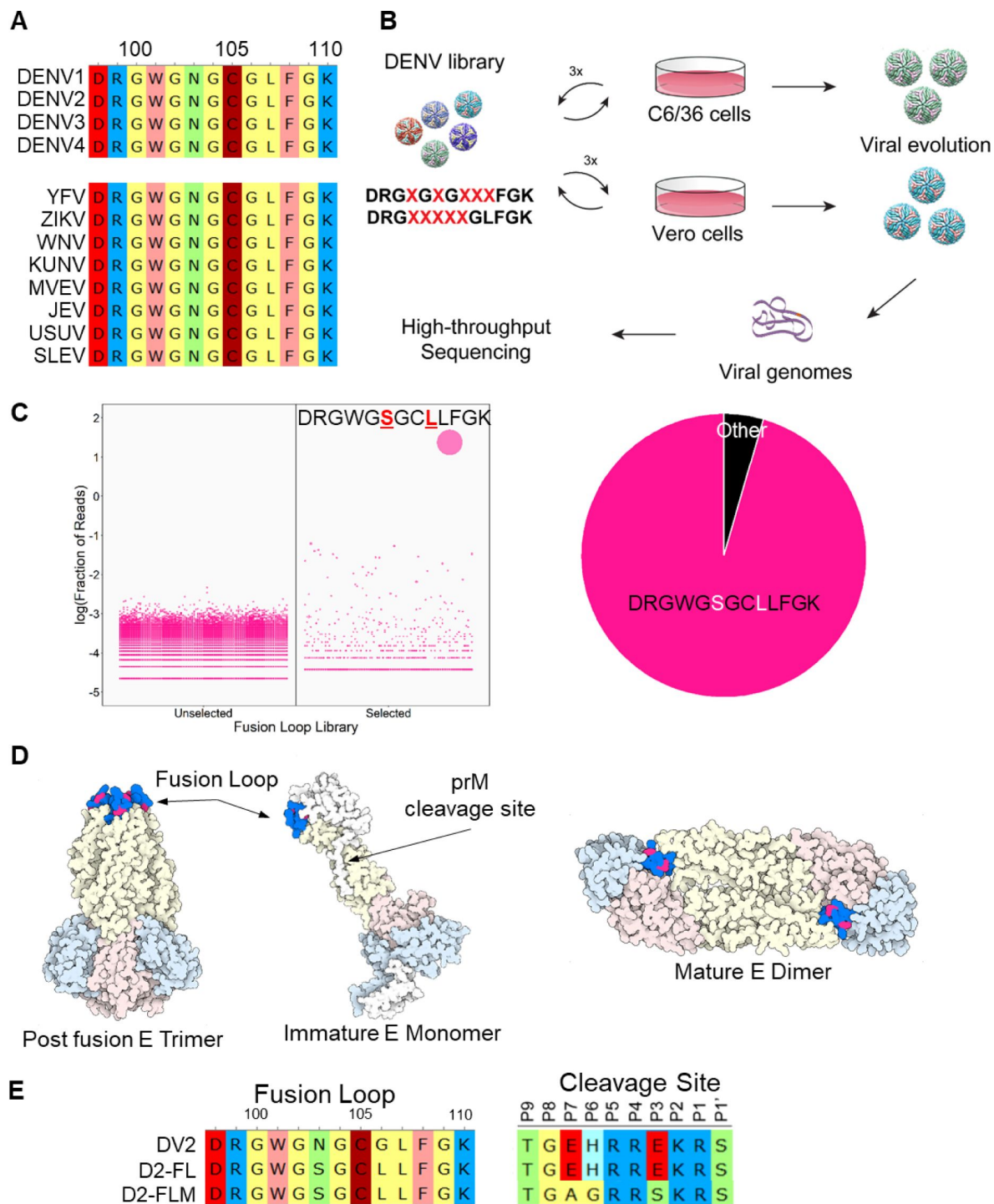


Figure 1

Generation of DENV2 fusion loop mutants via directed evolution. A) Alignment of Top: Dengue virus fusion loops; Bottom: Mosquito-borne flavivirus fusion loops, including Yellow Fever virus (YFV), Zika virus (ZIKV), West Nile virus (WNV), Kunjin virus (KUNV), Murray Valley Encephalitis virus (MVEV), Japanese Encephalitis virus (JEV), Usutu virus (USUV), and Saint Louis Encephalitis virus (SLEV). Amino acids are colored by functional groups: negatively charged (red), positively charged (blue), nonpolar (yellow), polar (green), aromatic (pink), and sulfide (dark red). B) Schematic of directed evolution procedure. Saturation mutagenesis libraries were used to produce viral libraries, which were passaged three times in either C6/36 or Vero 81 cells. At the end of the selection, viral genomes were isolated and mutations were identified by high-throughput sequencing. C) Left: Bubble plot of the sequences identified from either the unselected or selected (passage 3) C6/36 DENV libraries. Right: Pie chart of the sequences from passage 3 C6/36 DENV libraries. D) Structure of the DENV envelope with the fusion loop mutations highlighted in red. E) Sequences of the fusion loop and furin cleavage site of DENV2, D2-FL, and D2-FLM.

Library 2 focused on a continuous linear peptide that is the epitope for FL-Abs to maximally alter antigenicity.¹⁸ Saturation mutagenesis plasmid libraries were used to produce viral libraries in either C6/36 (*Aedes albopictus* mosquito) or Vero 81 (African green monkey) cells and passaged three times in their respective cell types. Following directed evolution, viral genomes were extracted and subjected to deep sequencing to identify surviving and enriched variants (**Figure 1B**). Due to the high level of conservation, it was not surprising that most mutational combinations failed to yield viable progeny. In fact, evolutions carried out on Library 2 only yielded wild-type sequences. For Library 1, wild-type sequences dominated in Vero 81 evolved libraries. However, a novel variant emerged in C6/36 cells with two amino acid changes: DRGWGSGCLLF GK. The major variant comprised ~95% of the population, while the next most populous variant (DRGWGSGCWLF GK) comprised only 0.25% (**Figure 1C**). Bulk Sanger sequencing revealed an additional Env-T171A mutation outside of the FL region. Residues W101, C105, and L107 were preserved in our final sequence, supporting the importance of these residues.¹⁶ When modeled on the pre-fusion DENV2 structure, the N103S and G106L mutations are located at the interface with the neighboring monomer EDIII domain, protected from the aqueous environment. In the post-fusion form, the two residues are located between W101 and F108 and form the bowl concavity above the chlorine ion in the post-fusion trimer (**Figure 1D**). We used reverse genetics to re-derive the FL N103S/G106L/T171A mutant, which we term D2-FL. As enhancing Abs also target prM,¹⁹ we also created a mature version of D2-FL termed D2-FLM, containing both the evolved FL motif and our previously published evolved prM furin cleavage site, which results in a more mature virion like those found in infected patients (**Figure 1E**).^{9,20}

We performed growth curves comparing DENV2, D2-FL, and D2-FLM in both C6/36 and Vero 81 cells. In C6/36 cells, the growth of all three viruses was comparable, reaching high titers of 10^6 – 10^7 FFU/mL. However, in Vero 81 cells, both FL mutant viruses were highly attenuated, with a 2–2.5 log reduction in titer (**Figure 2A**). The species-specific phenotype in culture involved a change from insect to mammalian cells, as well as a change in growth temperature. To investigate if the mutant viruses were more unstable at higher temperatures, we performed a thermostability assay, comparing viruses incubated at temperatures ranging from 4–55°C before infection. The three viruses had comparable thermostabilities, indicating that this does not explain the attenuation of the FL mutants (**Figure 2B**). Because the D2-FLM virus contains mutations that increase prM cleavage frequency, we also assayed the maturation status of the three viruses by western blot. D2-FL had a comparable prM:E ratio to the isogenic wildtype DENV2 (DV2-WT), while, as expected, D2-FLM had a reduced prM:E ratio, indicating a higher degree of maturation (**Figure 2C**).

Next, we characterized the ability of Abs targeting the FL to recognize DV2-WT, D2-FL, and D2-FLM with a panel of monoclonal antibodies (mAbs). Importantly, D2-FL and D2-FLM were resistant to mAbs targeting the FL. Neutralization by 1M7 is reduced by ~2-logs in both variants, 1N5 neutralization is reduced by ~1-log for D2-FL and reduced to background levels for D2-FL, and no neutralization was observed for 1L6 or 4G2 for either variant (**Figure 3A**).¹⁸ Focusing on the D2-FLM virus containing both evolved motifs, we then characterized the antigenicity of the whole virion with a panel of mAbs. As expected, D2-FLM was unable to be neutralized by the prM Abs 1E16 and 5M22; the Ab 2H2 does not neutralize either DV2-WT or D2-FLM (**Figure 3B**). For Abs targeting epitopes in non-mutated regions, including the ED1 and EDE epitopes that target EDII and EDIII, FRNT₅₀ values were generally comparable, although EDE1-C10 shows a moderate but statistically significant reduction between DV2-WT and D2-FLM, indicating that the overall virion structural integrity was intact (**Figure 3B**).

Next, we analyzed neutralization of the D2-FLM virus using serum derived from convalescent humans and experimental infected non-human primates (NHPs). Overall, we tested serum from 6 humans and 9 NHPs at different time points with a total of 27 samples. Serum from a homotypic infected NHP (n=3) did not display a difference in neutralization between DV2-WT, D2-FL, and D2-

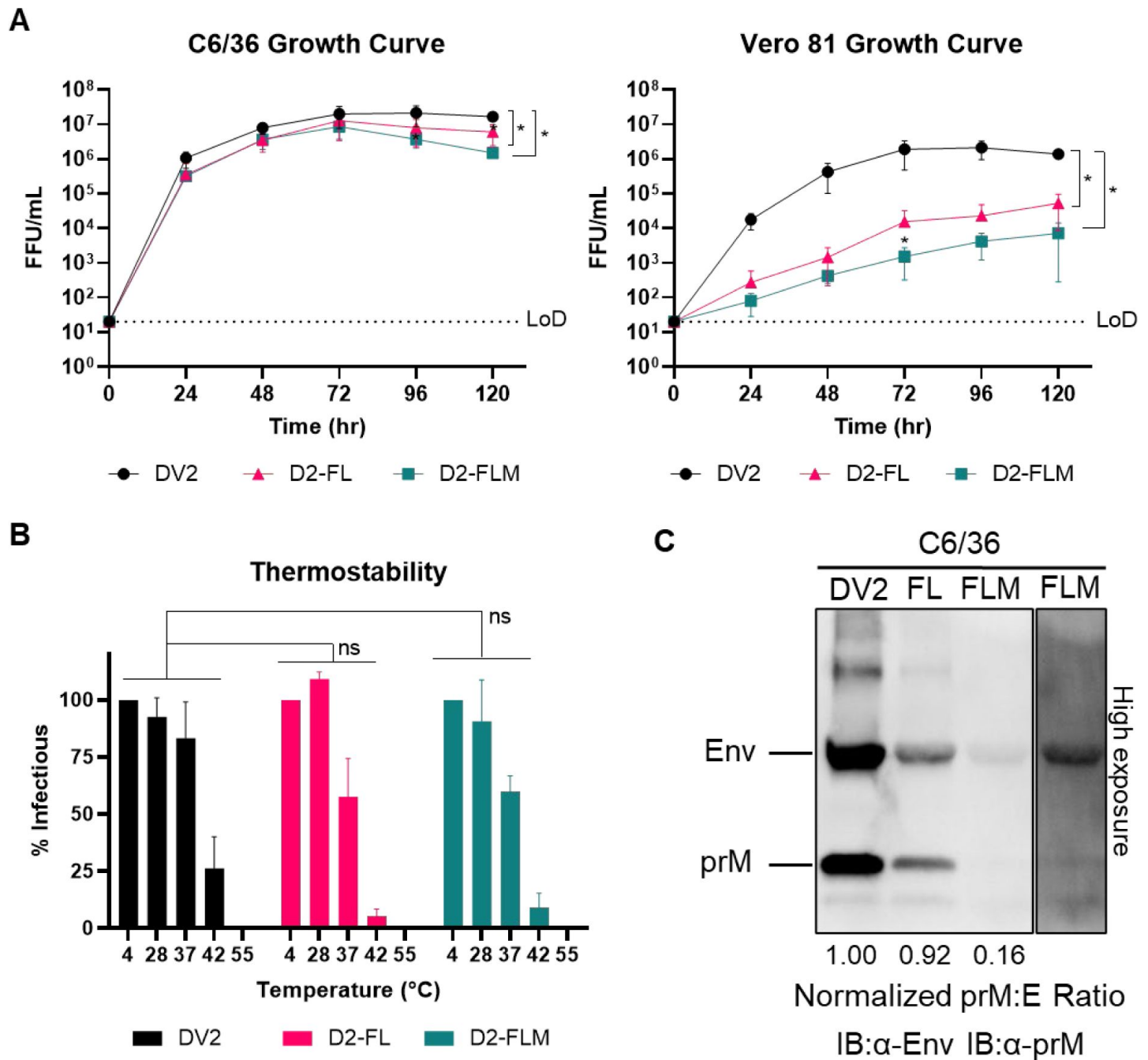


Figure 2

Biological and physical properties of mature DENV2 fusion loop mutants. A) Multistep growth curves (MOI = 0.05-0.1) of DV2-WT, D2-FL, and D2-FLM on C6/36 cells (left) or Vero 81 cells (right). B) Thermostability assay on DV2-WT, D2-FL, and D2-FLM. C) Western blot of virions, blotted against Envelope and prM proteins. The prM:E ratio was determined and normalized to the DV2-WT ratio. Averages of 3 biological replicates are shown. Two-way ANOVA was used for statistical comparison of growth curves and thermostability: ns = not significant; * < 0.05; ** < 0.005; *** < 0.0005.

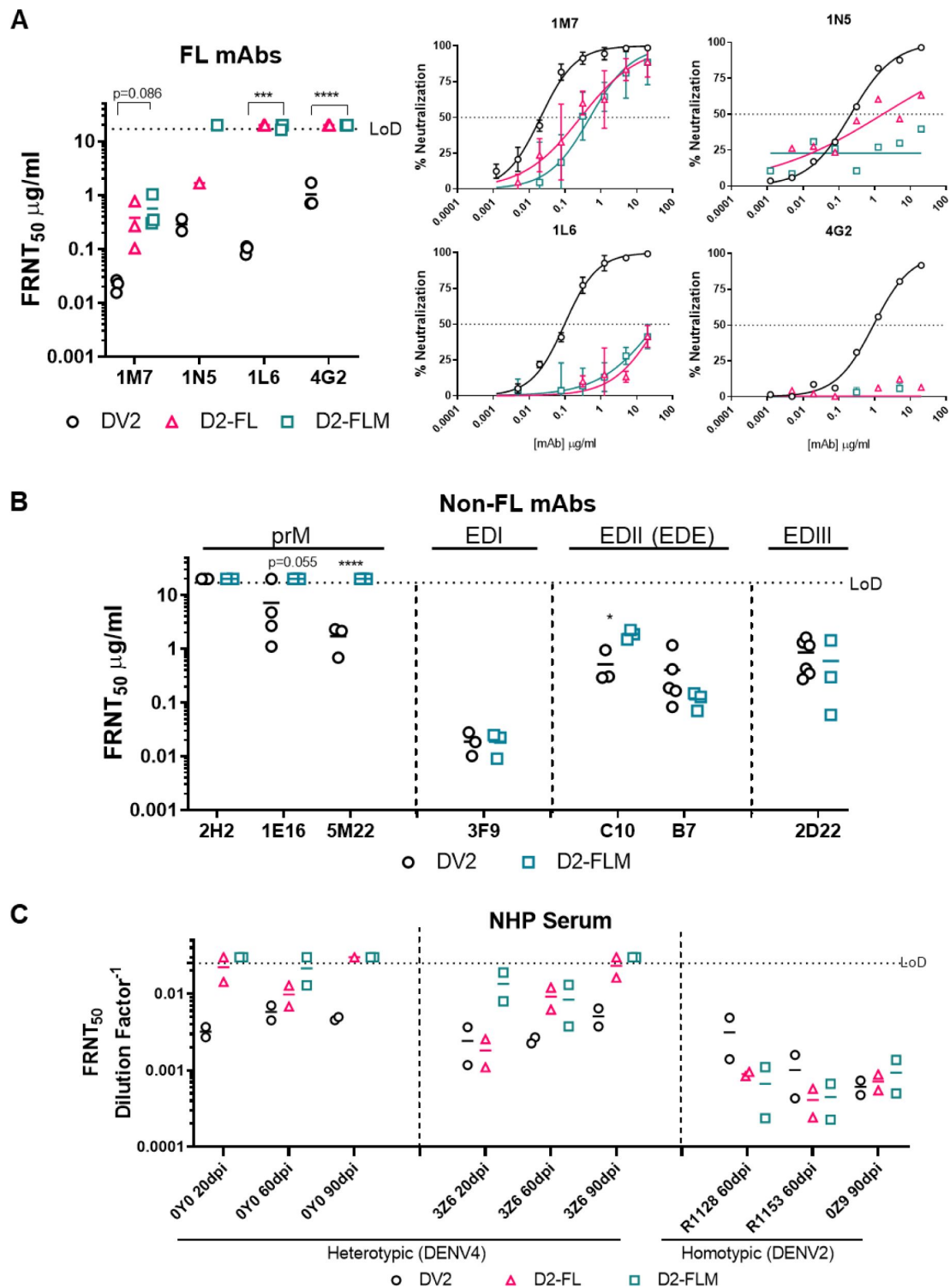


Figure 3

Fusion loop mutant is insensitive to fusion loop mAbs, the major target for cross-reactive Abs in NHPs. A) Left: FRNT₅₀ values for neutralization of DV2-WT, D2-FL, and D2-FLM with mAbs against the FL (1M7, 1N5, 1L6, 4G2). All Abs were tested in at least n=3 independent experiments, except 1N5 due to limited Ab. Right: Average neutralization curves for neutralization of DV2-WT, D2-FL, and D2-FLM with mAbs against the DENV2 fusion loop. B) FRNT₅₀ values for neutralization of DV2-WT and D2-FLM with mAbs against DENV2 prM (2H2, 1E16, 5M22), EDI (3F9), EDE (C10, B7), and EDIII (2D22). All Abs were tested in at least n=3 independent experiments. C) Neutralization of DV2-WT, D2-FL, and D2-FLM with sera from NHPs infected with either DENV4 or DENV2. FRNT₅₀s were compared using Student's t-test. Significant symbols are as follows: *, P < 0.05; **, P < 0.005; ***, P < 0.0005; ****, P < 0.00005. The data are graphed as means ± standard deviations.

FLM, confirming that prM and FL epitopes are not significant contributors to the homotypic type-specific (TS) neutralizing Ab response in primates (**Figure 3C**). In heterotypic vaccination and infection, most of the serum (18/24) did not cross-neutralize (FRNT₅₀ < 1:40) DV2-WT, confirming the serotypic difference of DENVs (**Table 1**). However, in two NHPs infected with DENV4, strong neutralization potency (FRNT₅₀ between 1: 100 – 1:1,000) was demonstrated against DV2-WT (**Figure 3C**). Heterologous cross-neutralization was significantly reduced to background levels (FRNT₅₀ < 1:40) against the D2-FLM virus at 90 days post-infection (dpi). Of note, one DENV4 animal (3Z6) showed low levels of neutralization against D2-FLM at early time points (20- and 60-days post-infection), which was eventually lost at later time points. Neutralization observed against D2-FL, in general, fell between DV2-WT and D2-FLM. Interestingly, in animal 3Z6, at 20 dpi, neutralization against DENV-FL was comparable to DV2-WT, while D2-FLM was greatly reduced, indicating that antibodies in the sera targeting the immature virion formed a large portion of the CR response. In contrast, animal 0Y0 displayed less difference in neutralization between D2-FL and D2-FLM, suggesting that FL antibodies were more prominent in this animal. These data suggest that after a single infection, much of the CR Ab responses target prM and the FL and tend to wane over time (**Figure 3C**). The collection of FL, mature, and FL-mature variants provides new opportunity to delineate antibody composition in complex polyclonal serum from DENV natural infection and vaccination.

Discussion

Mechanistic understanding of vaccine protection and identification of correlates of protection are immensely important for DENV vaccine development. The dual protective and enhancing properties of DENV Abs create major challenges for dissecting the role of various Ab populations in disease protection. Cross-reactive weakly neutralizing prM- and FL-Abs are often immunodominant after primary DENV infection,^{10, 21–24} and can lead to overestimation of the levels of heterotypic protection in traditional neutralization assays. Since these same antibodies are also associated with ADE,¹⁹ inaccurate conclusions could have dire consequences if protection *in vitro* translates to the enhancement of disease in human vaccinees. Unfortunately, Ab profiling in polyclonal serum is mainly performed by ELISA, and a neutralization assay that can discriminate Abs does not exist. The D2-FLM variant is not neutralized by FL- and prM-mAbs and appears insensitive to neutralization by these Abs in polyclonal serum. Of note, EDE1-C10 neutralization potency was also reduced in our FL-M variant, further indicating our mutations are affecting the tip of the EDII region which partially overlaps with the EDE1 epitope.²⁵ In combination with other chimeric DENVs,^{26–28} D2-FLM provides a reagent to distinguish between TS, protective CR (e.g. E dimer epitope, EDE),^{29, 30} and ADE-prone CR (e.g. FL and prM).²⁴ Ab subclasses in neutralization assays after infection and vaccination.

Due to the ADE properties of DENV Abs, studies to understand and eliminate ADE phenotype are under active investigation. For example, mAbs can be engineered to eliminate binding to the Fcγ receptor, abolishing ADE.³¹ While this methodology holds potential for Ab therapeutic development and passive immunization strategies, it is not relevant for vaccination. As FL and prM targeting Abs are the major species demonstrated to cause ADE *in vitro* and are thought to be responsible for ADE-driven negative outcomes after primary infection and vaccination,^{10–12, 32} we propose that genetic ablation of the FL and prM epitopes in vaccine strains will minimize the production of these subclasses of Abs responsible for undesirable vaccine responses. Indeed, efforts have been made to reduce the availability of the FL or reduce the ability of FL Abs to drive ADE. Covalently locked E-dimers and E-dimers with FL mutations have been engineered as subunit vaccines that reduce the availability of the FL, thereby reducing the production of FL Abs.^{33–36} DENV subunit vaccines are an area of active study;³⁷ however, monomer/dimer subunits can also expose additional, interior-facing epitopes not normally exposed to the cell. Furthermore, dimer subunits are not a complete representation of the DENV virion which presents other structurally important interfaces such as the 3-fold and 5-fold symmetries.

Human #	Infected Serotype	Collection Time	DV2	D2-FL	D2-FLM
DS1500	DENV1	Convalescent serum	1 : 75	ND	1 : 53
DS2499			1 : 80	ND	1 : 52
DS1136	1 : 133		ND	1 : 45	
DS1160	1: 86		ND	1: 208	
DS0275	1: 94		ND	1 : 94	
DS2239	1: 156		ND	1 : 42	
NHP #	Infected Serotype	Collection Time	DV2	D2-FL	D2-FLM
R628	DENV4	30dpi	< 1:40	< 1:40	< 1:40
		60dpi	< 1:40	< 1:40	< 1:40
		90dpi	1 : 42*	< 1:40	< 1:40
R737		30dpi	< 1:40	< 1:40	< 1:40
		60dpi	< 1:40	< 1:40	< 1:40
		90dpi	< 1:40	< 1:40	< 1:40
R1132		30dpi	< 1:40	< 1:40	< 1:40
		60dpi	< 1:40	< 1:40	< 1:40
		90dpi	< 1:40	< 1:40	< 1:40
R1160		30dpi	1 : 58*	< 1:40	< 1:40
		60dpi	1 : 57*	1 : 50*	< 1:40
		90dpi	< 1:40	1 : 57*	1 : 91*

Table 1

Summary of FRNT₅₀s of human convalescent serum and NHP infection serum against DV2, D2-FL and D2-FLM.

Concerns about balanced immunity to all four serotypes also apply to subunit vaccine platforms. Given the complexity of the immune response to DENV, live virus vaccine platforms have thus far been more successful. However, the fusion loop is strongly mutationally intolerant. Previous reported mutations in the fusion loop of mosquito-borne flaviviruses include a L107F mutation in WNV³⁸ and JEV associated with attenuation, and mutations at position 106 in ZIKV(G106A) and DENV(G106V) which were tolerated. Interestingly, we also recovered a mutation in position 106 (G106L) Using directed-evolution, we successfully generated our D2-FLM variant that combines viability with the desired Ab responses. Therefore, the D2-FLM variant is a novel candidate for a vaccine strain which presents all the native structures and complex symmetries of DENV necessary for T-cell mediated responses and which can elicit more optimal protective Ab responses.^{39–41}

Other considerations of high importance when designing a live DENV vaccine include strain selection and serotype balance.⁴² In the current study we used DENV2 S16803, a prototype for DENV2.⁴³ However, S16803 was isolated several decades ago, and it may be beneficial to utilize more contemporaneous strains.^{8, 44} Work is currently ongoing to demonstrate the portability of the evolved FL motif on additional DENV2 strains and other serotypes, which is essential for tetravalent vaccine production. D2-FLM was highly attenuated in Vero cells, creating a challenge for vaccine production. Therefore, further adaptation of this strain to grow efficiently in mammalian cells while retaining its antigenic properties is needed. Taken together, the FLM variant holds exciting new possibilities for a new generation of DENV vaccines, as well as a platform to readily measure TS and CR ADE-type responses and thereby assess the true protective potential of any DENV vaccine trials and safeguard approval of DENV vaccines for human use.

Acknowledgements

We thank members of the Tse, Baric, and DeSilva laboratories for helpful discussions. This work was supported by NIAID R01AI107731 to A.D. and R.S.B., P01AI106695 to R.S.B., NIAID F30AI160898 to D.R.Z. L.J.W. is supported by NIAID P01 5112869.

Author contribution

R.M.M. and L.V.T. designed the study. R.M.M. performed high-throughput sequencing preparation and analysis. R.M.M., D.Z., S.D., L.J.S., Y.D., and L.V.T. performed experiments. D.T., L.J.W., A.M.D.S., and R.S.B. provided reagents. L.V.T. and R.S.B. provided oversight of the project and funding. R.M.M. wrote the manuscript. L.V.T. reviewed and revised the final version. All authors approved the final version of the manuscript.

Conflict disclosure

R.M.M., R.S.B., and L.V.T. are inventors on a patent application filed on the subject matter of the manuscript.

Cells and viruses

C6/36 (ATCC CRL-1660) were grown in MEM (Gibco) with 5% FBS (HyClone), 1% penicillin/streptomycin (Gibco), 0.1mM nonessential amino acids (Gibco), 1% HEPES (Gibco), and 2mM GlutaMAX (Gibco), cultured at 32°C with 5% CO₂. Vero 81 cells (ATCC CCL-81) were grown in DMEM/F12 (Gibco) with 10% FBS, 1% penicillin/streptomycin (Gibco), 0.1mM nonessential amino acids (Gibco), and 1% HEPES (Gibco), cultured at 37°C with 5% CO₂. DENV viruses were grown in C6/36 or Vero 81 cells maintained in infection media. C6/36 infection media consists of Opti-MEM (Gibco) with 2% FBS (HyClone), 1% penicillin/streptomycin (Gibco), 0.1mM nonessential amino acids (Gibco), 1% HEPES (Gibco), and 2mM GlutaMAX (Gibco). Vero 81 infection media consists of DMEM/F12 (Gibco) with 2% FBS, 1% penicillin/streptomycin (Gibco), 0.1mM nonessential amino acids (Gibco), and 1% HEPES (Gibco). DENV2 strain S16803 was used in this study.⁴³ Sequences used for the alignments include DENV1 WestPac-74 (U88535.1), DENV2 S-16803 (GU289914.1), DENV3 3001 (JQ411814.1), DENV4 Sri Lanka-92 (KJ160504.1), YFV 17D (NC_002031.1), SLEV Kern217 (NC_007580.2), JEV (NC_001437.1), USUV Vienna-2001 (NC_006551.1), MVEV (NC_000943.1), WNV-1 NY99 (NC_009942.1), and ZIKV MR-766 (NC_012532.1).

DENV Reverse Genetics

DENV2 S16803 was used in this study. Recombinant viruses were created using a four-plasmid system as previously described,⁴⁵ consisting of the DENV genome split into four segments, each cloned into a separate plasmid. The DENV plasmids were digested and ligated to form a single template for *in vitro* transcription. The resulting RNA was electroporated into either C6/36 or Vero cells. Virus-containing supernatant was harvested at 4-5 days post electroporation and passaged. DENV variants were created through site-directed mutagenesis of the DENV plasmids.

Library Generation and Directed Evolution

DENV fusion loop libraries were generated through saturation mutagenesis of the indicated residues, based on a previously published protocol.^{20, 46} Degenerate NNK oligonucleotides were used to amplify the region, generating a library of mutated DNA fragments. Q5 DNA Polymerase was used with less than 18 cycles to maintain accuracy. The resulting library was cloned into the DENV reverse genetics system. The ligated plasmids were electroporated into DH10B ElectroMax cells (Invitrogen) and directly plated on 5,245mm² dishes (Corning) to avoid bias from suspension culture. Colonies were pooled and purified using a Maxiprep kit (Qiagen), and the plasmid library used for DENV reverse genetics (above). Viral libraries were passaged three times in the corresponding cell type.

High-throughput Sequencing and Analysis

Viral RNA was isolated with a QIAamp viral RNA kit (Qiagen), and cDNA produced using the Superscript IV Reverse Transcriptase (Invitrogen). Amplicons were prepared for sequencing using the Illumina TruSeq system with two rounds of PCR using Q5 Hot Start DNA polymerase (NEB). For the first round of PCR, primers were specific to the DENV2 E sequence surrounding the fusion loop motif with overhangs for the Illumina adapters. After purification, this product was used as the template for the second round of PCR using Illumina P5 and P7 primers containing 8-nucleotide indexes. Purified PCR products were analyzed on a Bioanalyzer (Agilent Technologies) and quantified on a Qubit 4 fluorometer (Invitrogen). Amplicon libraries were run on a MiSeq system with 2×150bp reads. Plasmid and P0 libraries were sequenced at a depth of ~4.5 million reads; later passages were sequenced at a depth of ~750,000 reads. Custom perl and R scripts were used to analyze and plot the data as previously published.²⁰

DENV Growth Kinetics

One day before infection, 5×10^5 cells were seeded in every well of a 6-well plate. Cells with infected with an MOI of 0.05-0.1, estimating 1×10^6 cells on the day of infection. Infection was carried out for one hour in the incubator, followed by 3x washes with PBS and replenishment with fresh infection medium. 300 μ L of viral supernatant was collected at 0, 24, 48, 72, 96, and 120 hours and stored at -80°C . All experiments were performed independently at least three times.

DENV Focus-Forming Assay

Titers of viral supernatant were determined using a standard DENV focus-forming assay. In brief, cells were seeded at 2×10^4 cells per well of a 96-well plate one day before infection. The next day, 50 μ L of 10-fold serial dilution of viral supernatant were added to each well for 1 hour in the incubator. After, 125 μ L of overlay (Opti-MEM, 2% FBS, NEAA, P/S, and methylcellulose) was added to each well. Infection was allowed to continue for 48 hours in the incubator. Overlay was removed, and each well rinsed 3x with PBS followed by a 30 minute fixation with 10% formalin in PBS. Cells were blocked in permeabilization buffer (eBioscience) with 5% nonfat dried milk. Primary Abs anti-prM 2H2 and anti-E 4G2 from nonpurified hybridoma supernatant were used at a 1:500 dilution in blocking buffer. Goat anti-mouse HRP secondary (SeraCare KPL) were used at a 1:1000 dilution in blocking buffer. Followed washing, foci were developed using TrueBlue HRP substrate (SeraCare) and counted using an automated Immunospot analyzer (Cellular Technology).

Thermal Stability Assay

The indicated viruses were thawed and incubated at temperatures ranging from 4°C to 55°C for one hour. Following, viral titers were determined by focus-forming assay as described above.

Western Blotting

Viral supernatants were combined with 4X Laemmli Sample Buffer (Bio-Rad) and boiled at 95°C for 5 minutes. After SDS-PAGE electrophoresis, samples were transferred to PVDF membrane and blocked in 3% nonfat milk in PBS-T. A polyclonal rabbit anti-prM (1:1000; Invitrogen PA5-34966) and polyclonal rabbit anti-Env (Invitrogen PA5-32246) in 2% BSA in PBS-T were incubated on the blot for 1 hour at 37°C . Goat anti-rabbit HRP (1:10,000 Jackson ImmunoLab) in 3% milk in PBS-T was incubated on the blot for 1 hour at room temperature. Blots were developed by SuperSignal West Pico Plus chemiluminescent substrate (ThermoFisher). Blots were imaged on an iBright FL1500 imaging system (Invitrogen). The pixel intensity of individual bands was measured using ImageJ, and the relative maturation was calculated by using the following equation: $(\text{prMExp}/\text{EnvExp})/(\text{prMWT}/\text{EnvWT})$. All experiments were performed independently a minimum of three times.

FRNT Assay

Focus reduction neutralization titer (FRNT) assays were performed as described previously with C6/36 cells. 1×10^5 cells were seeded in a 96-well plate the day prior to infection. Abs or sera were serially diluted and mixed with virus (~ 100 FFU/well) at a 1:1 volume and incubated for 1 hour in the incubator. The mixture was added onto the plate with cells and incubated for 1 hour in the incubator, then overlay was added (see Focus-Forming Assay) and plates were incubated for 48 hours. Viral foci were stained and counted as described above (Focus-Forming Assay). A variable slope sigmoidal dose-response curve was fitted to the data, and values were calculated with top or bottom restraints of 100 and 0 using GraphPad Prism version 9.0. All experiments were performed independently at least two times, due to limited amounts of human serum.

Statistical Analysis

GraphPad Prism version 9.0 was used for statistical analysis. Titer and % infection of D2-FL and D2-FLM were compared to the DV2 using two-way ANOVA. FRNT₅₀s were compared using Student's t-test. Significant symbols are as follows: *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$; ****, $P < 0.00005$. The data are graphed as means $\pm$ standard deviations.

References

1. Bhatt S., et al. (2013) **The global distribution and burden of dengue** *Nature* **496**
2. Brady O. J., et al. (1970) **Refining the Global Spatial Limits of Dengue Virus Transmission by Evidence-Based Consensus** <https://doi.org/10.1371/journal.pntd.0001760>
3. Ebi K. L., Nealon J. (2016) **Dengue in a changing climate** *Environ Res* **151**:115–123
4. Messina J. P., et al. (1970) **The current and future global distribution and population at risk of dengue** *Nat Microbiol* <https://doi.org/10.1038/s41564-019-0476-8>
5. Halstead S. B. (1988) **Pathogenesis of Dengue: Challenges to Molecular Biology** *Science* (1979) **239**:476–481
6. Katzelnick L. C., et al. (2017) **Antibody-dependent enhancement of severe dengue disease in humans** *Science* (1979) **358**:929–932
7. Rey F. A., Stiasny K., Vaney M., Dellarole M., Heinz F. X. (2018) **The bright and the dark side of human antibody responses to flaviviruses: lessons for vaccine design** *EMBO Rep* **19**:206–224
8. Rabaa M. A., et al. (2017) **Genetic epidemiology of dengue viruses in phase III trials of the CYD tetravalent dengue vaccine and implications for efficacy** *Elife* **6**:1–22
9. Raut R., et al. (2019) **Dengue type 1 viruses circulating in humans are highly infectious and poorly neutralized by human antibodies** *Proc Natl Acad Sci U S A* **116**:227–232
10. Lai C. Y., et al. (2013) **Analysis of Cross-Reactive Antibodies Recognizing the Fusion Loop of Envelope Protein and Correlation with Neutralizing Antibody Titers in Nicaraguan Dengue Cases** *PLoS Negl Trop Dis* **7**:1–11
11. Beltramello M., et al. (2010) **The human immune response to dengue virus is dominated by highly cross-reactive antibodies endowed with neutralizing and enhancing activity** *Cell Host Microbe* **8**:271–283
12. Costin J. M., et al. (2013) **Mechanistic Study of Broadly Neutralizing Human Monoclonal Antibodies against Dengue Virus That Target the Fusion Loop** *J Virol* **87**:52–66
13. Wilder-Smith A., et al. (2019) **Personal View Deliberations of the Strategic Advisory Group of Experts on Immunization on the use of CYD-TDV dengue vaccine The Strategic Advisory Group of Experts (SAGE) on Immunization advises WHO on global policies for vaccines.** In *Lancet Infect Dis* **19**:e31–e38
14. Dengue vaccine: WHO position paper (2019) **2018 – Recommendations** *Vaccine* **37**:4848–4849
15. Izmirly A. M., Alturki S. O., Alturki S. O., Connors J., Haddad E. K. (2020) **Challenges in Dengue Vaccines Development: Pre-existing Infections and Cross-Reactivity** *Front Immunol* **11**
16. Modis Y., Ogata S., Clements D., Harrison S. C. (2004) **Structure of the dengue virus envelope protein after membrane fusion** *Nature* **427**:313–319

17. Klein D. E., Choi J. L., Harrison S. C. (2013) **Structure of a Dengue Virus Envelope Protein Late-Stage Fusion Intermediate** *J Virol* **87**:2287–2293
18. Smith S. A., et al. (2013) **The potent and broadly neutralizing human dengue virus-specific monoclonal antibody 1C19 reveals a unique cross-reactive epitope on the bc loop of domain II of the envelope protein** *mBio* **4**
19. Rodenhuis-Zybert I. A., et al. (2010) **Immature Dengue Virus: A Veiled Pathogen?** *PLoS Pathog* **6**
20. Tse L. v., et al. (2022) **Generation of Mature DENVs via Genetic Modification and Directed Evolution** *mBio* **13**:1–18
21. Smith S. A., et al. (2012) **Persistence of Circulating Memory B Cell Clones with Potential for Dengue Virus Disease Enhancement for Decades following Infection** *J Virol* **86**:2665–2675
22. Oliphant T., et al. (2006) **Antibody Recognition and Neutralization Determinants on Domains I and II of West Nile Virus Envelope Protein** *J Virol* **80**:12149–12159
23. Lai C.-Y., et al. (2008) **Antibodies to Envelope Glycoprotein of Dengue Virus during the Natural Course of Infection Are Predominantly Cross-Reactive and Recognize Epitopes Containing Highly Conserved Residues at the Fusion Loop of Domain II** *J Virol* **82**:6631–6643
24. Dejnirattisai W., et al. (2010) **Cross-Reacting Antibodies Enhance Dengue Virus Infection in Humans** *Science (1979)* **328**:745–748
25. Sharma A., et al. (2021) **The epitope arrangement on flavivirus particles contributes to Mab C10's extraordinary neutralization breadth across Zika and dengue viruses** *Cell* **184**:6052–6066
26. Andrade D. v., et al. (2019) **Tracking the polyclonal neutralizing antibody response to a dengue virus serotype 1 type-specific epitope across two populations in Asia and the Americas** *Scientific Reports* **2019** *9*:1–12
27. Young E., et al. (2020) **Identification of Dengue Virus Serotype 3 Specific Antigenic Sites Targeted by Neutralizing Human Antibodies** <https://doi.org/10.1016/j.chom.2020.04.007>
28. Gallichotte E. N., et al. (2015) **A new quaternary structure epitope on dengue virus serotype 2 is the target of durable type-specific neutralizing antibodies** *mBio* **6**
29. Barba-Spaeth G., et al. (2016) **Structural basis of potent Zika-dengue virus antibody cross-neutralization** *Nature* **2016** *536*:7614 **536**:48–53
30. Dejnirattisai W., et al. (2014) **A new class of highly potent, broadly neutralizing antibodies isolated from viremic patients infected with dengue virus** *Nature Immunology* **2014** *16*:2 **16**:170–177
31. Kotaki T., et al. (2021) **An affinity-matured human monoclonal antibody targeting fusion loop epitope of dengue virus with in vivo therapeutic potency** *Sci Rep* **11**:1–14
32. de Alwis R., et al. (2014) **Dengue Viruses Are Enhanced by Distinct Populations of Serotype Cross-Reactive Antibodies in Human Immune Sera** *PLoS Pathog* **10**

33. Rouvinski A., et al. (2017) **Covalently linked dengue virus envelope glycoprotein dimers reduce exposure of the immunodominant fusion loop epitope** *Nat Commun* **8**
34. Rockstroh A., et al. (2015) **Recombinant Envelope-Proteins with Mutations in the Conserved Fusion Loop Allow Specific Serological Diagnosis of Dengue-Infections** *PLoS Negl Trop Dis* **9**:1–12
35. Slon-Campos J. L., et al. (2019) **A protective Zika virus E-dimer-based subunit vaccine engineered to abrogate antibody-dependent enhancement of dengue infection** *Nature Immunology* **20**:10 20:1291–1298
36. Metz S. W., et al. (2017) **In Vitro Assembly and Stabilization of Dengue and Zika Virus Envelope Protein Homo-Dimers** *Scientific Reports* **7**:1 7:1–8
37. Kudlacek S. T., et al. (2021) **Designed, highly expressing, thermostable dengue virus 2 envelope protein dimers elicit quaternary epitope antibodies** *Sci Adv* **7**
38. Zhang S., et al. (2006) **A mutation in the envelope protein fusion loop attenuates mouse neuroinvasiveness of the NY99 strain of West Nile virus** *Virology* **353**:35–40
39. Tian Y., Grifoni A., Sette A., Weiskopf D. (2019) **Human T Cell Response to Dengue Virus Infection** *Front Immunol* **10**
40. Ngono A. E., Shresta S. (2019) **Cross-reactive T Cell immunity to dengue and zika viruses: New insights into vaccine development** *Front Immunol* **10**
41. Waickman A. T., et al. (2019) **Dissecting the heterogeneity of DENV vaccine-elicited cellular immunity using single-cell RNA sequencing and metabolic profiling** *Nature Communications* **10**:1 10:1–16
42. Gallichotte E. N., et al. (2018) **Genetic Variation between Dengue Virus Type 4 Strains Impacts Human Antibody Binding and Neutralization** *CellReports* **25**:1214–1224
43. Halstead S. B., Marchette N. J. (2003) **BIOLOGIC PROPERTIES OF DENGUE VIRUSES FOLLOWING SERIAL PASSAGE IN PRIMARY DOG KIDNEY CELLS: STUDIES AT THE UNIVERSITY OF HAWAII** *Am J Trop Med Hyg* **69**:5–11
44. Juraska M., et al. (2018) **Viral genetic diversity and protective efficacy of a tetravalent dengue vaccine in two phase 3 trials** *Proc Natl Acad Sci U S A* **115**:E8378–E8387
45. Messer W. B., et al. (2012) **Development and characterization of a reverse genetic system for studying dengue virus serotype 3 strain variation and neutralization** *PLoS Negl Trop Dis* **6**
46. Tse L. V., et al. (2017) **Structure-guided evolution of antigenically distinct adeno-associated virus variants for immune evasion** *Proc Natl Acad Sci U S A* **114**:E4812–E4821

Author information

Rita M. Meganck

Department of Molecular Microbiology and Immunology, Saint Louis University

Deanna Zhu

Department of Epidemiology, University of North Carolina at Chapel Hill

Stephanie Dong

Department of Epidemiology, University of North Carolina at Chapel Hill

Lisa J. Snoderly-Foster

Department of Molecular Microbiology and Immunology, Saint Louis University

Yago R. Dalben

Department of Molecular Microbiology and Immunology, Saint Louis University

Devina Thiono

Department of Microbiology, University of North Carolina at Chapel Hill

Laura J. White

Department of Microbiology, University of North Carolina at Chapel Hill

Aravinda M. DeSilva

Department of Microbiology, University of North Carolina at Chapel Hill

Ralph S. Baric

Department of Epidemiology, University of North Carolina at Chapel Hill

Longping V. Tse

Department of Molecular Microbiology and Immunology, Saint Louis University

For correspondence: victor.tse@health.slu.edu

ORCID iD: [0000-0001-7582-8396](https://orcid.org/0000-0001-7582-8396)

Editors

Reviewing Editor

Leslie Goo

Fred Hutchinson Cancer Research Center, United States of America

Senior Editor

Detlef Weigel

Max Planck Institute for Biology Tübingen, Germany

Reviewer #1 (Public Review):

Summary of the major findings -

1. The authors used saturation mutagenesis and directed evolution to mutate the highly conserved fusion loop (98 DRGWGNGCGLFGK 110) of the Envelope (E) glycoprotein of Dengue virus (DENV). They created 2 libraries with parallel mutations at amino acids 101, 103, 105-107, and 101-105 respectively. The in vitro transcribed RNA from the two plasmid libraries was electroporated separately into Vero and C6/36 cells and passaged thrice in each of these cells. They successfully recovered a variant N103S/G106L from Library 1 in C6/36 cells, which represented 95% of the sequence population and contained another mutation in E outside the fusion loop (T171A). Library 2 was unsuccessful in either cell type.

2. The fusion loop mutant virus called D2-FL (N103S/G106L) was created through reverse genetics. Another variant called D2-FLM was also created, which in addition to the fusion loop mutations, also contains a previously published, evolved, and optimized prM-furin cleavage sequence that results in a mature version of the virus (with lower prM content). Both D2-FL and D2-FLM viruses grew comparably to wild type virus in mosquito (C6/36) cells but their infectious titers were 2-2.5 log lower than wildtype virus when grown in mammalian (Vero) cells. These viruses were not compromised in thermostability, and the mechanism for attenuation in Vero cells remains unknown.

4. Next, the authors probed the neutralization of these viruses using a panel of monoclonal antibodies (mAbs) against fusion loop and domain I, II and III of E protein, and against prM protein. As intended, neutralization by fusion loop mAbs was reduced or impaired for both D2-FL and D2-FLM, compared to wild type DENV2. D2-FLM virus was equivalent to wild type with respect to neutralization by domain I, II, and III antibodies tested (except domain II-C10 mAb) suggesting an intact global antigenic landscape of the mutant virion. As expected, D2-FLM was also resistant to neutralization by prM mAbs (D2-FL was not tested in this batch of experiments).

5. Finally, the authors evaluated neutralization in the context of polyclonal serum from convalescent humans (n=6) and experimentally infected non-human primates (n=9) at different time points (27 total samples). Homotypic sera (DENV2) neutralized D2-FL, D2-FLM, and wild type DENV similarly, suggesting that the contribution of fusion loop and prM epitopes is insignificant in a serotype-specific neutralization response. However, heterotypic sera (DENV4) neutralized D2-FL and D2-FLM less potently than wild type DENV2, especially at later time points, demonstrating the contribution of fusion loop- and prM-specific antibodies to heterotypic neutralization.

Impact of the study-

1. The engineered D2-FL and D2-FLM viruses are valuable reagents to probe antibodies targeting the fusion loop and prM in the overall polyclonal response to DENV.
2. Though more work is needed, these viruses can facilitate the design of a new generation of DENV vaccine that does not elicit fusion loop- and prM-specific antibodies, which are often poorly neutralizing and lead to antibody-dependent enhancement effect (ADE).
3. This work can be extended to other members of the flavivirus family.
4. A broader impact of their work is a reminder that conserved amino acids may not always be critical for function and therefore should not be immediately dismissed in substitution/mutagenesis/protein design efforts.

Appraisal of the results -

The data largely support the conclusions, but some improvements and extensions can benefit the work.

1. In Figure 3A, the authors concluded that the engineered dengue virus fusion loop mutant viruses are insensitive to monoclonal antibodies (mAbs) targeting the fusion loop. However, the reduction in neutralization sensitivity varied depending on the mAb tested. The contribution of the optimized prM cleavage site (D2-FLM) to sensitivity to fusion loop mAbs also varied.

- a) Are the epitopes known for these mAbs? It would be useful to discuss how the epitope of 1M7 differs from the other mAbs. What are the critical residues?
- d) Maybe the D2-FL mutant can be further evolved with selection pressure with fusion loop mAbs 1M7 +/-1N5 and/or other fusion loop mAbs.

2. It would have been useful to include D2-M for comparison (with evolved furin cleavage sequence but no fusion loop mutations).

3. Data for polyclonal serum can be better discussed. Table 1 is not discussed much in the text.

Suggestions for further experiments-

1. It would be interesting to see the phenotype of single mutants N103S and G106L, relative to double mutant N103S/G106L (D2-FL).

2. The fusion capability of these viruses can be gauged using liposome fusion assay under different pH conditions and different lipids.

3. Correlative antibody binding vs neutralization data would be useful.

Reviewer #2 (Public Review):

Antibody-dependent enhancement (ADE) of Dengue is largely driven by cross-reactive antibodies that target the DENV fusion loop or pre-membrane protein. Screening polyclonal sera for antibodies that bind to these cross-reactive epitopes could increase the successful implementation of a safe DENV vaccine that does not lead to ADE. However, there are few reliable tools to rapidly assess the polyclonal sera for epitope targets and ADE potential. Here the authors develop a live viral tool to rapidly screen polyclonal sera for binding to fusion loop and pre-membrane epitopes. The authors performed a deep mutational scan for viable viruses with mutations in the fusion loop (FL). The authors identified two mutations functionally tolerable in insect C6/36 cells, but lead to defective replication in mammalian Vero cells. These mutant viruses, D2-FL and D2-FLM, were tested for epitope presentation with a panel of monoclonal antibodies and polyclonal sera. The D2-FL and D2-FLM viruses were not neutralized by FL-specific monoclonal antibodies demonstrating that the FL epitope has been ablated.

Overall the central conclusion that the engineered viruses can predict epitopes targeted by antibodies is supported by the data and the D2-FL and D2-FLM viruses represent a valuable tool to the DENV research community.

Author Response

The following is the authors' response to the original reviews.

Reviewer #1 (Public Review):

Summary of the major findings -

1. The authors used saturation mutagenesis and directed evolution to mutate the highly conserved fusion loop (98 DRGWNGCGLFGK 110) of the Envelope (E) glycoprotein of Dengue virus (DENV). They created 2 libraries with parallel mutations at amino acids 101, 103, 105-107, and 101-105 respectively. The in vitro transcribed RNA from the two plasmid libraries was electroporated separately into Vero and C6/36 cells and passaged thrice in each of these cells. They successfully recovered a variant N103S/G106L from Library 1 in C6/36 cells, which represented 95% of the sequence population and contained another mutation in E outside the fusion loop (T171A). Library 2 was unsuccessful in either cell type.

1. The fusion loop mutant virus called D2-FL (N103S/G106L) was created through reverse genetics. Another variant called D2-FLM was also created, which in addition to the fusion loop mutations, also contains a previously published, evolved, and optimized prM-furin cleavage sequence that results in a mature version of the virus (with lower prM content). Both D2-FL and D2-FLM viruses grew comparably to wild type virus in mosquito (C6/36) cells but their infectious titers were 2-2.5 log lower than wild type virus when grown in mammalian (Vero) cells. These viruses were not compromised in thermostability, and the mechanism for attenuation in Vero cells remains unknown.

1. Next, the authors probed the neutralization of these viruses using a panel of monoclonal antibodies (mAbs) against fusion loop and domain I, II and III of E protein, and against prM protein. As intended, neutralization by fusion loop mAbs was reduced or impaired for both D2-FL and D2-FLM, compared to wild type DENV2. D2-FLM virus was equivalent to wild type with respect to neutralization by domain I, II, and III antibodies tested (except domain II-C10 mAb) suggesting an intact global antigenic landscape of the mutant virion. As expected, D2-FLM was also resistant to neutralization by prM mAbs (D2-FL was not tested in this batch of experiments).

1. Finally, the authors evaluated neutralization in the context of polyclonal serum from convalescent humans (n=6) and experimentally infected non-human primates (n=9) at different time points (27 total samples). Homotypic sera (DENV2) neutralized D2-FL, D2-FLM, and wild type DENV similarly, suggesting that the contribution of fusion loop and prM epitopes is insignificant in a serotype-specific neutralization response. However, heterotypic sera (DENV4) neutralized D2-FL and D2-FLM less potently than wild type DENV2, especially at later time points, demonstrating the contribution of fusion loop- and prM-specific antibodies to heterotypic neutralization.

Impact of the study-

1. The engineered D2-FL and D2-FLM viruses are valuable reagents to probe antibodies targeting the fusion loop and prM in the overall polyclonal response to DENV.

1. Though more work is needed, these viruses can facilitate the design of a new generation of DENV vaccine that does not elicit fusion loop- and prM-specific antibodies, which are often poorly neutralizing and lead to antibody-dependent enhancement effect (ADE).

1. This work can be extended to other members of the flavivirus family.

1. A broader impact of their work is a reminder that conserved amino acids may not always be critical for function and therefore should not be immediately dismissed in substitution/mutagenesis/protein design efforts.

Evaluating this study in the context of prior literature -

The authors write "Although the extreme conservation and critical role in entry have led to it being traditionally considered impossible to change the fusion loop, we successfully tested the hypothesis that massively parallel directed evolution could produce viable DENV fusion-loop mutants that were still capable of fusion and entry, while altering the antigenic footprint."

".....Previously, a single study on WNV successfully generated a viable virus with a single mutation at the fusion loop, although it severely attenuated neurovirulence. Otherwise, it has not been generated in DENV or other mosquito-borne flaviviruses"

The above claims are a bit overstated. In the context of other flaviviruses:

- A previous study applied a similar saturation mutagenesis approach to the full length E protein of Zika virus and found that while the conserved fusion loop was mutationally constrained, some mutations, including at amino acid residue 106 were tolerated (PMID 31511387).
- The Japanese encephalitis virus (JEV) SA14-14-2 live vaccine strain contains a L107F mutation in the fusion loop (in addition to other changes elsewhere in the genome) relative to the parental JEV SA14 strain (PMID: 25855730).
- For tickborne encephalitis virus (TBEV-DENV4 chimera), H104G/L107F double mutant has been described (PMID: 8331735)

There have also been previous examples of functionally tolerated mutations within the DENV fusion loop:

- Goncalvez et al., isolated an escape variant of DENV 2 using chimpanzee Fab 1A5, with a mutation in the fusion loop G106V (PMID: 15542644). G106 is also mutated in D2-FL clone (N103S/G106L) described in the current study.
- In the context of single-round infectious DENV, mutation at site 102 within the fusion loop has been shown to retain infectivity (PMID 31820734).

We thank the reviewer for these comments. We have adjusted the text above to better reflect and credit the prior literature. Text is modified as follows in the discussion session.

“Previous reported mutations in the fusion loop are mainly derived from experimental evolution using FL-Ab to select for escape mutant or by deep mutational scanning (DMS) of the Env protein for Ab epitope mapping. Mutations in the FL epitope were observed in a DENV2-NGC-V2 (G106V)39, attenuated JEV vaccine strain SA14-14-2 (L107F)40, attenuated WNV-NY99 (L107F)41. While most of the mutations, including the double mutations reported here lead to attenuation of the virus. A recent DMS study showed that Zika-G106A has no observable impact on viral fitness42. Interestingly, we also recovered a mutation G106L, suggesting position 106 and 107 might be the most tolerable position for mutation in mosquito borne flavivirus FL. On the other hand, tick borne flavivirus as well as vector only flavivirus show a more diverse FL composition. The inflexibility of mosquito borne flavivirus might be due to the evolution constraint of the virus to switch between mosquito and vertebrate hosts.”

Appraisal of the results -

The data largely support the conclusions, but some improvements and extensions can benefit the work.

1. Line 92-93: "This major variant comprised ~95% of the population, while the next most populous variant comprised only 0.25% (Figure 1C)".

What is the sequence of the next most abundant variant?

The sequence of the next most abundant variant has been added to the text.

1. Lines 94-95: "Residues W101, C105, and L107 were preserved in our final sequence, supporting the structural importance of these residues." L107F is viable in other flaviviruses.

We acknowledge that the L107F mutation has been described in other flaviviruses, including the tick-borne flaviviruses DTV and POWV. This mutation in JEV is associated with viral attenuation. This sentence is referring to the fact that, in our libraries, we did not recover variants with mutations at these positions, in contrast to D2-FL with variants at N103 and G106, indicating less mutational tolerance. However, we want to re-direct the focus of this manuscript to engineer a viable DENV that is antigenically different in the FL epitope, but not which residue is more tolerance for mutation.

1. *Figure 2c: The FLM sample in the western blot shows hardly any E protein, making E/prM quantitation unreliable.*

The samples used in Figure 2C derive from the growth curve endpoint (Figure 2A), in which there is a 1-log difference in viral titer between D2 and D2-FLM. Equivalent volumes of viral supernatant were loaded in the gel, explaining the reduced intensity of the E band in D2-FLM. The higher exposure on the right shows the E band more clearly for D2-FLM. The Western blot assay comparing prM/E ratio as a measure of maturation state was described and validated in our previous study (Tse et al. 2022, mbio). The methods and figure legend have been updated to include greater detail. The polyclonal E antibody was specifically chosen for this study as our previously used monoclonal antibody targeted the fusion loop. The polyclonal antibody was raised against a fragment of E (AA 1-495) and should have minimal effect by the fusion loop mutations.

1. *Lines 149 -151: "Importantly, D2-FL and D2-FLM were resistant to antibodies targeting the fusion loop. While neutralization by 1M7 is reduced by ~2-logs, no neutralization was observed for 1N5, 1L6, and 4G2 for either variant (Figure 3 A)".*

a) Partial neutralization was observed for 1N5, for D2-FL.

The text has been updated to more accurately describe the 1N5 neutralization data.

b) Do these mAbs cover the full spectrum of fusion loop antibodies identified thus far in the field?

We did not test every known fusion loop antibody that has been described, instead focusing on 1M7, 1N5, 1L6, and 4G2, which were previously described by Smith et al and Crill et al. We also modified the text in discussion to reflect the possibility of other FL-Ab that are not affected by our mutations.

"We have tested a panel of FL-Ab; however, we cannot exclude the possibility that other FL-Abs may not be affected by N103S and G106L. However, we have shown that saturation mutagenesis could generate mutants with multiple amino acid changes, and we are currently using D2-FLM as backbone to iteratively evolve additional mutations in FL to further deviate the FL antigenic epitope."

c) Are the epitopes known for these mAbs? It would be useful to discuss how the epitope of 1M7 differs from the other mAbs? What are the critical residues?

Critical residues for these antibodies have been described. They are as follows: 1M7: W101R, W101C, G111R; 1N5: W101R, L107P, L107R, G111R; 1L6: G100A, W101A, F108A; 4G2: G104H, G106Q, L107K. The critical residues for 1M7 are slightly different than the others, perhaps explaining the residual binding to D2-FL. Note that the critical residue identified previously for 1M7 and 1N5 do not overlap with D2-FLM mutations, suggesting the FL mutations has extending effect on the antigenic FL epitope.

d) Maybe the D2-FL mutant can be further evolved with selection pressure with fusion loop mAbs 1M7 +/-1N5 and/or other fusion loop mAbs.

We agree that it may be possible to further evolve D2-FL using antibody selection, although we have not yet performed these experiments, we are currently performing iterative saturation mutagenesis and directed evolution to further evolve away from the natural FL.

1. It would have been useful to include D2-M for comparison (with evolved furin cleavage sequence but no fusion loop mutations).

Neutralization data for some of the mAbs against D2-M can be found in our previous study (Tse et al. 2022 mBio), in which no difference in neutralization was observed compared to DV2 wildtype. Given the limited resources of the anti-DENV NHP and human serum, we did not add D2-M for comparison. Although some insight can be deduced from the D2-FL vs D2-FLM comparison, we agree future studies that are designed to delineate CR-Ab population between prM, FL and other CR-epitopes should include D2-M for comparison.

1. Data for polyclonal serum can be better discussed. Table 1 is not discussed much in the text. For the R1160-90dpi-DENV4 sample, D2-FL and D2-FLM are neutralized better than wild type DENV2? The authors' interpretation in lines 181-182 is inconsistent with the data presented in Figure 3C, which suggests that over time, there is INCREASED (not waning) dependence on FL- and prM-specific antibodies for heterotypic neutralization.

We remade Table 1 to show dilution factors instead of dilution factor-1 of FRNT50.

In general, our human convalescent sera from heterotypic infection (DENV1, 3 and 4) showed none to low neutralization against our DENV2. FRNT50s were between 1: 40 – 1:200. Given the weak potency of the antiserum, it is difficult to compare the FRNT50s between DV2-WT and D2-FLM.

Similarly, in a different NHP cohort (2nd NHP cohort shown in Table 1), only one DENV4 infected NHP (R1160) showed a low heterotypic titer against DENV2. The detectable FRNT50s were between 1: 50 – 1:90. The value was extrapolated based on a single data point (1:40) which has above 50% neutralization. Given the Hill slope of all the neutralization curves were below 0.5, the FRNT50 values is should not be

In conclusion, we do not think serum from Table 1 is potent enough to shows difference between the viruses. The intension to show the negative data in Table 1 is to highlight the difference in serum heterogeneity in DENV infected patients and experimental infected NHPs.

As the reviewer pointed out, the dependence of FL-Ab in later time points increased (the difference between DV2 and D2-FL at 20dpi vs 60dpi vs 90dpi), suggesting non-FL CR-Ab is waning but not prM- and FL-Abs. We rewrote the sentence as follow:

“These data suggest that after a single infection, many of the CR Ab responses target prM and the FL and the reliance on these Abs for heterotypic neutralization increase overtime (Figure 3C).”

Suggestions for further experiments-

1. It would be interesting to see the phenotype of single mutants N103S and G106L, relative to double mutant N103S/G106L (D2-FL).

1. *The fusion capability of these viruses can be gauged using liposome fusion assay under different pH conditions and different lipids.*

1. *Correlative antibody binding vs neutralization data would be useful.*

We thank the reviewer for the suggestions; we agree these would be of interest and, indeed, these studies are currently underway. In regard to single mutants, these were present in the initial plasmid library but did not enrich after viral production and passage. Two possible explanations can be drawn, 1) The stochastic of directed evolution prevents a single mutant with similar fitness to enriched. 2) The two mutations are compensatory to each other to make a functional mutant. The 2nd hypothesis highlights the difference between saturation mutagenesis (this study) and DMS (in previous studies).

Fusion capability is indeed very interesting, however, the mechanistic difference or not between wildtype FL and the mutated FL in supporting fusion is not the focus of this study. Instead, we are currently working on adapting the D2-FLM in mammalian cells. If successful, the difference in fusion mechanism between the Vero adapted and D2-FLM in different lipid, insect vs mammalian would be of interest.

We are currently developing whole virus ELISA; we avoid using rE monomer for the study as it might neglect the conformation Ab.

Reviewer #2 (Public Review):

Antibody-dependent enhancement (ADE) of Dengue is largely driven by cross-reactive antibodies that target the DENV fusion loop or pre-membrane protein. Screening polyclonal sera for antibodies that bind to these cross-reactive epitopes could increase the successful implementation of a safe DENV vaccine that does not lead to ADE. However, there are few reliable tools to rapidly assess the polyclonal sera for epitope targets and ADE potential. Here the authors develop a live viral tool to rapidly screen polyclonal sera for binding to fusion loop and pre-membrane epitopes. The authors performed a deep mutational scan for viable viruses with mutations in the fusion loop (FL). The authors identified two mutations functionally tolerable in insect C6/36 cells, but lead to defective replication in mammalian Vero cells. These mutant viruses, D2-FL and D2-FLM, were tested for epitope presentation with a panel of monoclonal antibodies and polyclonal sera. The D2-FL and D2-FLM viruses were not neutralized by FL-specific monoclonal antibodies demonstrating that the FL epitope has been ablated. However, neutralization data with polyclonal sera is contradictory to the claim that cross-reactive antibody responses targeting the pre-membrane and the FL epitopes wane over time.

Overall, the central conclusion that the engineered viruses can predict epitopes targeted by antibodies is supported by the data and the D2-FL and D2-FLM viruses represent a valuable tool to the DENV research community.

Reviewer #1 (Recommendations For The Authors):

1. Line 51-52: "Currently, there is a single approved DENV vaccine, Dengvaxia." Line 56-57: "Other DENV vaccines have been tested or are currently undergoing clinical trial, but thus far none have been approved for use."

It should be specified for the global audience that this applies to the United States. Takeda's DENV vaccine, QDENG is approved in Indonesia, European Union, and Brazil.

The text has been modified to include this information.

1. Line 62-63: - *"The core fusion loop-motif DRGWGNGCGLFGK is highly conserved..."* Lines 78-80: - *We generated two different saturation mutagenesis libraries, each with 5 randomized amino acids: DRGXGXGXXXFGK (Library 1) and 79 DRGXXXXXGLFGK (Library 2).*

It may be useful for the readers if the amino acid numbers are stated. The core fusion loop motif DRGWGNGCGLFGK (Eaa98-110) is highly conserved. We generated two different saturation mutagenesis libraries, each with 5 randomized amino acids: DRGXGXGXXXFGK (Library 1; Xaa 101, 103, 105-7) and DRGXXXXXGLFGK (Library 2; Xaa 101-105).

This information has been added to the text.

1. Line 91-92: *"Bulk Sanger sequencing revealed an additional Env-91 T171A mutation outside of the fusion-loop region."*

It looks like the mutation T171A is in domain I of the E protein and does not seem to interface with the fusion loop. Is that why it wasn't pursued further?

The E171A mutation was included in the infectious clone for D2-FL and D2-FLM. The text has been modified to clarify this inclusion.

1. Lines 82-85: *"Saturation mutagenesis plasmid libraries were used to produce viral libraries in either C6/36 (Aedes albopictus mosquito) or Vero 81 (African green monkey) cells and passaged three times in their respective cell types."*

a) What was the size of the libraries? How does one make sure that the experimental library actually has all the amino acid combinations that were intended?

Each library has 5 randomized amino acids, so there are $205 = 3.2$ million combinations. In these experiments, sequencing of the plasmid libraries revealed about 2 million unique amino acid sequences, or approximately 62.5% library coverage. The actual plasmid diversity is expected to be higher than 2 million as our deep sequencing has limited coverage.

b) The wild type sequence was excluded from the libraries, correct?

The wild-type sequence was not specifically excluded from the libraries, as there is no easy method to do so. Wild-type sequence was detected in the plasmid libraries but was not selected in the C6/36 library. However, in the Vero library, we recovered WT virus.

1. Table 1: - *Please include in the table description, what the colors indicate.*

We remade Table 1 to show dilution factors instead of dilution factor-1 of FRNT50 and removed the unnecessary color code. We also added all relevant information in the table legend.

1. Lines 246-248: *"Previously, a single study on WNV successfully generated a viable virus with a single mutation at the fusion loop, although it severely attenuated neurovirulence."*

It may be worthwhile to mention the WNV mutation (L107F) as some readers may be curious about where this mutation is relative to the ones described in this study.

This information has been added to the text. We also included the previously described FL mutations in flaviviruses in the text.

Reviewer #2 (Recommendations For The Authors):

Major Critique:

- *There is a disconnect between Fig 2A and 2C. FL and FLM viruses have much lower levels of prM-E expression in the viral supernatants based on the western blot in 2C. Why isn't E being detected in the Western? Is the particle-to-pfu ratio skewed in the mutant viruses? Is it possible that the polyclonal is targeting the cross-reactive prM and FL epitopes, and if so would using a monoclonal antibody targeting a known DIII-epitope (2D22) yield a different western result? Also, the legend and methods for Fig 2C are not clear. What is actually being tested in the Western blot? Were equivalent volumes of the different viral preps used?*

The samples used in Figure 2C derive from the growth curve endpoint (Figure 2A), in which there is a 1-log difference in viral titer between D2 and D2-FLM. Equivalent volumes of viral supernatant were loaded in the gel, explaining the reduced intensity of the E band in D2-FLM. The higher exposure on the right shows the E band more clearly for D2-FLM. The Western blot assay comparing prM/E ratio as a measure of maturation state was described and validated in our previous study (Tse et al. 2022, mBio) and the methods have been updated to include greater detail. The polyclonal E antibody was specifically chosen for this study as our previously used monoclonal antibody targeted the fusion loop. The polyclonal antibody was raised against a fragment of E (AA 1-495) and should not be affected by the fusion loop mutations. 2D22 is a conformational antibody and does not work in western blot.

- *Table 1: The data within Table 1 is ignored in the text, and some of this data contradicts the central conclusions of the manuscript.*

o A.) Some of the convalescent data contradicts the hypothesis. DS0275 had an equivalent neut between DV2 and D2-FLM, DS1660, and R1160 (90) had better neut against the D2-FLM than DV2. Discussion of these samples is warranted.

o C.) The description in the legend does not adequately describe the table. What do the colors represent? What are the numerical values being displayed? What is in parentheses, (I assume the challenge strain)? The limit of detection is reported as 1:40; 0.25. 1:40 is 0.025 which matches most of the data? There is inadequate description of these experiments in the materials and methods.

We remade Table 1 to show dilution factors instead of dilution factor-1 of FRNT50 and removed the unnecessary color code. We also added discussion for Table 1 and clarify the difference between the three cohorts of serum in the text with the corresponding references.

In general, our human convalescent sera from heterotypic infection (DENV1, 3 and 4) showed none to low neutralization against our DENV2. FRNT50s were between 1: 40 – 1:200. Given the weak potency of the antiserum, it is difficult to compare the FRNT50s between DV2-WT and D2-FLM.

Similarly, in a different NHP cohort (2nd NHP cohort shown in Table 1), only one DENV4 infected NHP (R1160) showed a low heterotypic titer against DENV2. The detectable FRNT50s were between 1: 50 – 1:90. The value was extrapolated based on a single data point (1:40) which was above 50% neutralization. Given the Hill slope of all the neutralization curves were below 0.5, the FRNT50 values are not reliable.

In conclusion, we do not think sera from Table 1 is potent enough to show difference between the viruses. The intention to show the negative data in Table 1 is to highlight the difference in serum heterogeneity in DENV infected patients and experimental infected NHPs.

Minor critique:

Figure 1C: Legend is not clear for this panel. What is on the x-axis of the bubble plots? Are these mutations across the entire viral genome or is this just the prM-E sequence?

The X-axis is a scatter of all of the sequences contained in the library, similar to graphs used for plotting CRISPR screen results. These represent individual sequences from the saturation mutagenesis libraries in the fusion loop of E as described in Figure 1B.

The wording in Lines 92-94 is not clear. It looks like the T171A mutation was present in 95% of the sequences (Line 92). Yet this sequence was not incorporated into the variant virus. What is the rationale for omitting this mutation in downstream variant virus generation?

The 95% in Line 92 refers to the variant containing N103S/G106L mutations as seen in Figure 1C. The high-throughput sequencing approach did not include residue 171, so the presence of the T171A mutation in combination with fusion loop mutations cannot be determined. However, the E171A mutation was included in the infectious clone for D2-FL and D2-FLM. The text has been modified to clarify this inclusion.

The authors discuss the potential of the D2-FL or D2-FLM virus as a potential vaccine platform in the abstract, introduction, and conclusion. This is a good idea, but the authors provide no evidence of feasibility in this manuscript.

The ultimate goal to engineer a viable DENV with distinct FL antigenic epitope is for it use as live attenuated vaccine. As this is the rationale for the study, we introduce the concept throughout the manuscript. The current study demonstrated the possibility to mutate a novel fusion loop motif in DENV and provided evidence to show the favorable antigenic properties of D2-FLM. We agree with the reviewer that definitive work in animal to show vaccine efficacy need to be done and are currently undergoing. To avoid misleading our audience, we tone down the emphasis of vaccine use in the text.

Line 150-153: Figure 3A demonstrates that the FL-specific antibodies broadly do not neutralize the mutant viruses. However, the conclusions are overstated in the text. 1N5 neutralizes the D2-FL variant.

The text has been updated to more accurately describe the 1N5 neutralization data.

Lines 175-182: The authors make a lot of assumptions about the target of the polyclonal target without any evidence.

These lines reference studies that showed greater enhancement by antibodies targeting the fusion loop and prM as compared to other cross-reacting antibodies. The assumption that both our manuscript and others have drawn was that Abs that are cross-reactive and weakly neutralizing are more prone for ADE. As discussed, other groups have attempted to mutate the FL from recombinant E protein to achieve similar goal to remove the fusion loop epitope to reduce ADE. We have re-written the sentence in the followings:

“As FL and prM targeting Abs are the major species demonstrated to cause ADE in vitro, we and others hypothesized these Abs are responsible for ADE-driven negative outcomes after

primary infection and vaccination,^{10–12,32} we propose that genetic ablation of the FL and prM epitopes in vaccine strains will minimize the production of these subclasses of Abs responsible for undesirable vaccine responses. Indeed, covalently locked E-dimers and E-dimers with FL mutations have been engineered as potential subunit vaccines that reduce the availability of the FL, thereby reducing the production of FL Abs.^{33–36}