Evolution of a Functionally Intact but Antigenically Distinct DENV Fusion Loop

Rita M. Meganck¹, Deanna Zhu², Stephanie Dong², Lisa J. Snoderly-Foster¹, Yago R. Dalben¹, Devina Thiono³, Laura J. White³, Aravinda M. DeSilva³, Ralph S. Baric², Longping V. Tse¹*

¹ Department of Molecular Microbiology and Immunology, Saint Louis University
² Department of Epidemiology, University of North Carolina at Chapel Hill
³ Department of Microbiology, University of North Carolina at Chapel Hill

*Corresponding author: victor.tse@health.slu.edu
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. Antibody-dependent enhancement 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 fusion-loop (D2-FL) which is not neutralized by fusion-loop-targeting monoclonal antibodies. The fusion-loop mutations were combined with our previously evolved pre-membrane cleavage site to create a mature version of D2-FL (D2-FLM), which evades both pre-membrane and fusion-loop antibodies but retains sensitivity to other type-specific and quaternary cross-reactive antibodies. Cross-reactive serum from heterotypic (DENV4) infected non-human primates 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 non-human primates. We propose D2-FL and D2-FLM as valuable tools to delineate cross-reactive antibody subtypes in serum as well as an exciting platform for safer live attenuated DENV vaccines suitable for naïve individuals and children.
INTRODUCTION

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).\textsuperscript{1,2} DENV is transmitted through Aedes mosquito vectors, and globalization and global warming are increasing the endemic range of Dengue worldwide.\textsuperscript{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.\textsuperscript{5} This is thought to be due to the phenomenon of antibody-dependent enhancement, in which poorly neutralizing cross-reactive antibodies lead to enhanced viral uptake and infection of unique cell populations in an Fc\textgamma-receptor-mediated manner.\textsuperscript{6}

Antibody-dependent enhancement remains a major challenge for DENV vaccine development.\textsuperscript{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.\textsuperscript{8} Additionally, lab-grown strains differ from patient-derived DENVs in both maturation status and antigenicity.\textsuperscript{9} In particular, antibodies targeting the fusion loop have been reported to neutralize lab and patient strains with differing strengths and have been observed to facilitate Fc\textgamma-receptor uptake \textit{in vitro} and therefore antibody-dependent enhancement.\textsuperscript{10–12} Currently, there is a single approved DENV vaccine, Dengvaxia. However, it is only FDA-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 cross-reactive antibodies that increased the risk of severe disease after DENV infection.\textsuperscript{13,14} Other DENV vaccines have been tested or are currently undergoing clinical trial, but thus far none have been approved for use.\textsuperscript{15}

The DENV fusion loop is located in Envelope protein domain II (EDII) and is involved in monomer-monomer contacts with EDIII.\textsuperscript{16} During the DENV infection cycle, low pH triggers a conformational change in the E protein.\textsuperscript{17} The structure of the virion rearranges, and individual monomers form a trimer with all three fusion loops in the same orientation, ready to initiate membrane fusion.\textsuperscript{16,17} The core fusion-loop motif (DRGWNGCGLFGK) 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 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. The fusion-loop mutations, in combination with optimized pre-membrane cleavage site mutations, ablate neutralization by the pre-membrane and fusion-loop antibodies, retain sensitivity to other protective antibodies, and provide a novel vaccine strategy for DENV.
RESULTS

To engineer a virus with a novel antigenic footprint at the fusion loop, we targeted the core conserved fusion-loop motif. We generated two different saturation mutagenesis libraries, each with 5 randomized amino acids: DRGXGXGXXXFGK (Library 1) and DRGXXXXXGLFGK (Library 2). Library 1 was designed to mutate known residues targeted by fusion-loop antibodies while Library 2 focused on a continuous linear peptide that is the epitope for fusion-loop antibodies to maximally alter antigenicity.\textsuperscript{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: DRGWGSGLFGK. Bulk Sanger sequencing revealed an additional Env-T171A mutation outside of the fusion-loop region. This major variant comprised \(~95\%\) of the population, while the next most populous variant comprised only \(0.25\%\) (Figure 1C).

Residues W101, C105, and L107 were preserved in our final sequence, supporting the structural importance of these residues.\textsuperscript{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 fusion-loop N103S/G106L mutant, which we term D2-FL. As enhancing antibodies also target pre-membrane,\textsuperscript{19} we also created a mature version of D2-FL termed D2-FLM, containing both the evolved fusion-loop motif and our previously published evolved pre-membrane furin cleavage site, which results in a more mature virion like those found in infected patients (Figure 1E).\textsuperscript{9,20}
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.

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 fusion-loop 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 fusion-loop mutants (Figure 2B). Because the D2-FLM virus contains mutations that increase pre-membrane cleavage frequency, we also assayed the maturation status of the three viruses by western blot. D2-FL had a comparable pre-membrane-to-envelope ratio to the isogenic wildtype DENV2 (DV2-WT), while, as expected, D2-FLM had a reduced pre-membrane-to-envelope ratio, indicating a higher degree of maturation (Figure 2C).
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 envelopes and pre-membrane proteins. The pre-membrane-to-envelope 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.

Next, we characterized the ability of antibodies targeting the fusion loop to recognize DV2-WT, D2-FL, and D2-FLM with a panel of monoclonal antibodies.
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).\textsuperscript{18} Focusing on the D2-FLM virus containing both evolved motifs, we then characterized the antigenicity of the whole virion with a panel of antibodies. As expected, D2-FLM was unable to be neutralized by the pre-membrane antibodies 1E16 and 5M22; the antibody 2H2 does not neutralize either DV2-WT or D2-FLM (Figure 3B). For antibodies targeting epitopes in non-mutated regions, including the ED1 and envelop-dimer-epitope that target EDII and EDIII, FRNT\textsubscript{50} 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. Overall, we tested serum from 6 humans and 9 non-human primates at different time points with a total of 27 samples. Serum from a homotypic infected non-human primate (n=3) did not display a difference in neutralization between DV2-WT, D2-FL, and D2-FLM, confirming that pre-membrane and fusion-loop epitopes are not significant contributors to the homotypic type-specific neutralizing antibody response in primates (Figure 3C). In heterotypic vaccination and infection, most of the serum (18/24) did not cross-neutralize (FRNT\textsubscript{50} < 1:40) DV2-WT, confirming the serotypic difference of DENVs (Table 1). However, in two non-human primates infected with DENV4, strong neutralization potency (FRNT\textsubscript{50} between 1: 100 – 1:1,000) was demonstrated against DV2-WT (Figure 3C). Heterologous cross-neutralization was significantly reduced to background levels (FRNT\textsubscript{50} < 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-fusion-loop 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 cross-reactive response. In contrast, animal 0Y0 displayed less difference in neutralization between D2-FL and D2-FLM, suggesting that fusion-loop antibodies were more prominent in this animal. These data suggest that after a single infection, much of the cross-reactive antibody responses target the pre-membrane and the fusion loop and tend to wane over time (Figure 3C). The collection of fusion-loop, mature, and fusion-loop-mature variants provides new opportunities to delineate antibody composition in complex polyclonal serum from DENV natural infection and vaccination.
**Figure 3:** Fusion loop mutant is insensitive to fusion loop antibodies, the major target for cross-reactive antibodies in non-human primates. A) Left: FRNT\textsubscript{50} values for neutralization of DV2-WT, D2-FL, and D2-FLM with antibodies against the fusion-loop (1M7, 1N5, 1L6, 4G2). All antibodies were tested in at least n=3 independent experiments, except 1N5 due to limited antibodies. Right: Average neutralization curves for neutralization of DV2-WT, D2-FL, and D2-FLM with antibodies against the DENV2 fusion loop. B) FRNT\textsubscript{50} values for neutralization of DV2-WT and D2-FLM with antibodies against DENV2 pre-membrane (2H2, 1E16, 5M22), EDI (3F9), envelop-dimer-epitope (C10, B7), and EDIII (2D22). All antibodies were tested in at least n=3 independent experiments. C) Neutralization of DV2-WT, D2-FL, and D2-FLM with sera from non-human primates infected with either DENV4 or DENV2. FRNT\textsubscript{50}s 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.

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* Limit of detection (1:40; 0.25)

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**Table 1:** Summary of FRNT\textsubscript{50}s of human convalescent serum and non-human primate infection serum against DV2, D2-FL, and D2-FLM.
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 antibodies create major challenges for dissecting the role of various antibody populations in disease protection. Cross-reactive weakly neutralizing pre-membrane and fusion loop antibodies 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 antibody-dependent enhancement,\(^ {19}\) inaccurate conclusions could have dire consequences if protection \textit{in vitro} translates to the enhancement of disease in human vaccinees. Unfortunately, antibody profiling in polyclonal serum is mainly performed by ELISA, and a neutralization assay that can discriminate antibodies does not exist. The D2-FLM variant is not neutralized by fusion-loop and pre-membrane antibodies and appears insensitive to neutralization by these antibodies in polyclonal serum. Of note, EDE1-C10 neutralization potency was also reduced in our FLM 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 type-specific, protective cross-reactive (e.g. envelop-dimer-epitope),\(^ {29,30}\) and antibody-dependent enhancement-prone cross-reactive (e.g. fusion-loop and pre-membrane)\(^ {24}\) antibody subclasses in neutralization assays after infection and vaccination.

Due to the antibody-dependent enhancement properties of DENV antibodies, studies to understand and eliminate antibody-dependent enhancement phenotypes are
under active investigation. For example, antibodies can be engineered to eliminate
binding to the Fcγ receptor, abolishing antibody-dependent enhancement. While this
methodology holds potential for antibody therapeutic development and passive
immunization strategies, it is not relevant for vaccination. As fusion-loop and pre-
membrane targeting antibodies are the major species demonstrated to cause antibody-
dependent enhancement \textit{in vitro} and are thought to be responsible for antibody-
dependent enhancement-driven negative outcomes after primary infection and
vaccination, we propose that genetic ablation of the fusion-loop and pre-membrane
epitopes in vaccine strains will minimize the production of these subclasses of antibodies
responsible for undesirable vaccine responses. Indeed, efforts have been made to
reduce the availability of the fusion-loop or reduce the ability of fusion-loop antibodies to
drive antibody-dependent enhancement. Covalently locked envelope-dimers and
everse-dimers with fusion-loop mutations have been engineered as subunit vaccines
that reduce the availability of the fusion-loop, thereby reducing the production of fusion-
loop antibodies. DENV subunit vaccines are an area of active study; 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. 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. 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. Using directed-evolution, we successfully generated our D2-FLM variant that combines viability with the desired antibody 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 antibody responses.\textsuperscript{39–41}

Other considerations of high importance when designing a live DENV vaccine include strain selection and serotype balance.\textsuperscript{42} In the current study we used DENV2 S16803, a prototype for DENV2.\textsuperscript{43} However, S16803 was isolated several decades ago, and it may be beneficial to utilize more contemporaneous strains.\textsuperscript{8,44} Work is currently ongoing to demonstrate the portability of the evolved fusion-loop 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 type-specific and cross-reactive antibody-dependent enhancement-type responses and thereby assess the true protective potential of any DENV vaccine trials and safeguard approval of DENV vaccines for human use.
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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.
MATERIALS AND METHODS

Cells and viruses

C6/36 (ATCC cross-reactiveL-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. 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, 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.\textsuperscript{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 5,245mm\textsuperscript{2} dishes (Corning) to avoid bias from the suspension culture. Colonies were pooled and purified using a Maxiprep kit (Qiagen), and the plasmid library was 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 was 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 2x150bp 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, 5x10⁵ cells were seeded in every well of a 6-well plate. Cells with infected with an MOI of 0.05-0.1, estimating 1x10⁶ 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 uL 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 2x10⁴ cells per well of a 96-well plate one day before infection. The next day, 50 uL of 10-fold serial dilution of viral supernatant were added to each well for 1 hour in the incubator. After, 125uL 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 antibodies anti-pre-membrane 2H2 and anti-E 4G2 from non-purified hybridoma supernatant were used at a 1:500 dilution in blocking buffer. Goat anti-mouse HRP secondary (SeraCare KPL) was used at a 1:1000 dilution in a blocking buffer. Following 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-pre-membrane (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 fusion-loop1500 imaging system (Invitrogen).

**FRNT Assay**

Focus reduction neuralization titer (FRNT) assays were performed as described previously with C6/36 cells. 1x10⁵ cells were seeded in a 96-well plate the day prior to infection. antibodies 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 ± standard
deviations.
REFERENCES


