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The mechanism of differential neutralization of dengue serotype 3 strains by monoclonal antibody 8A1

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Abstract

While previous studies have demonstrated that envelope (E) glycoprotein variation between dengue viruses (DENV) genotypes can influence antibody neutralization potency, the mechanisms of variable neutralization remain incompletely understood. Here we characterize epitope antibody interactions of a DENV-3 EDIII binding mouse mAb 8A1 which displays highly variable neutralizing activity against DENV-3 genotypes. Using a DENV-3 reverse genetics platform, we characterize ability of 8A1 to bind and neutralize naturally occurring DENV-3 E genotypic variant viruses. Introduction of single and multiple amino acid mutations into the parental clone background demonstrates that mutations at positions 301 and 383 on EDIII are responsible for 8A1 differential neutralization phenotypes. ELISA and surface plasmon resonance (SPR) studies indicate differences in binding are responsible for the variable neutralization. Variability at position 301 primarily determined binding difference through influencing antibody-EDIII dissociation rate. Our findings are relevant to the many groups focusing on DENV EDIII as a vaccine target.

Introduction

Dengue virus (DENV) is a mosquito borne virus that is endemic in most of the tropical areas in the world, putting 2.5 billion people in risk (WHO 2009). Four serotypes of DENV co-circulate and infection with one serotype does not provide life-long immunity against other serotypes (Halstead 1988). Many DENV infections are asymptomatic, while symptomatic disease can manifest as classical Dengue Fever (DF), or can develop into more severe form of disease called Dengue Hemorrhagic Fever (DHF) or Dengue Shock Syndrome (DSS) (Shepard, Suaya et al. 2004). It is estimated that DHF/DSS leads to 10,000 – 15,000 deaths annually (WHO 2009). Epidemiologic data suggests that pre-existing antibodies, either from

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previous heterotypic DENV infection or, in the case of newborns and infants, maternally acquired anti-DENV antibodies, are associated with development of the more severe disease (Halstead and O'Rourke 1977). This phenomenon, known as Antibody-Dependent Enhancement (ADE), has been demonstrated *in vitro* using sub-neutralizing concentration of antibodies to facilitate infection of otherwise non-permissive cells such as monocytes via Fc-γ receptor mediated endocytosis (Halstead and O'Rourke 1977). This particular feature of DENV potentially confounds vaccine implementation and design strategies.

DENV is a single-stranded, positive-sense RNA virus in the family *Flaviviridae*, genus flavivirus. It is closely related to several other important human pathogens such as Japanese encephalitis (JEV), West Nile (WNV), Yellow fever (YFV) and Tick-borne encephalitis (TBEV). The DENV genome is translated into a single polyprotein and then cleaved into structural proteins (C-prM-E) and non-structural proteins (NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5). The Envelope protein (E) is the major surface exposed antigen and the principal target of host antibody response. The envelope proteins of different DENV serotypes have been crystallized and their structures characterized (Rey, Heinz et al. 1995; Modis, Ogata et al. 2003; Zhang, Zhang et al. 2004; Modis, Ogata et al. 2005; Cockburn, Navarro Sanchez et al. 2012). On the mature DENV virion, E forms a homodimer and is arranged on the virion surface in a herringbone pattern with dimers arranged in sets of three rafts. The ectodomain of E protein has three domains: domain I, II and III (EDI-EDIII). Importantly, EDIII is believed to be involved in cell receptor binding (Crill and Roehrig 2001). EDIII belongs to the Ig superfamily and is composed of several beta-strands linked by loops (Figure 1A, 1B). A subset of these loops form a surface exposed structure called the lateral ridge, which is the target of many monoclonal antibodies that strongly neutralize DENV (Modis, Ogata et al. 2005; Pierson and Diamond 2008).

EDIII is a potent immunogen that elicits type-specific antibodies in various animal models (Schmitz, Roehrig et al. 2011). Consequently, recombinant EDIII based vaccines have been under development with the hypothesis that EDIII type-specific antibodies will be protective but less likely to enhance dengue virus infection. Many type-specific monoclonal antibodies against EDIII target the most diversified/non-conserved region – the lateral ridge and hence mutations within this region may have important immunological consequences.

There are four distinct DENV-3 genotypes: I, II, III and IV (Lanciotti, Lewis et al. 1994). Currently genotype I and II are circulating in Asia, genotype III is circulating in the Indian subcontinent, Africa and Latin America, while genotype IV appears to have been displaced but occurred throughout the Caribbean in the 1960s and 70s (Messer, Gubler et al. 2003; Araújo, Nogueira et al. 2009). Wahala *et al.* revealed difference of sensitivity between DENV-3 genotypes to certain type-specific neutralizing mAbs (Wahala, Donaldson et al. 2010). Other researchers have also shown that genotypes play a role in antibody neutralization and protection (Brien, Austin et al. 2010; Shrestha, Brien et al. 2010; Sukupolvi-Petty, Austin et al. 2010; Pitcher, Gromowski et al. 2012), including the finding that intra-genotypic variations can elicit different immune response that fail to effectively neutralize virus of the same serotype (Wong, Abd-Jamil et al. 2007). Since multiple genotypes co-circulate worldwide (Nogueira, Stella et al. 2008; Jiang, Yu et al. 2012), it becomes imperative to understand how viral genotypic variation affects neutralization and define its mechanism. The constant evolution of dengue viruses further justifies studying how mutations influence interactions with antibodies (de Mora, Andrea et al. 2009; Kukreti, Mirtal et al. 2010; Ramirez, Fajardo et al. 2010).

To better understand the role of genotypic variation in DENV-3 neutralization, we tested the mouse monoclonal antibody 8A1 against a panel of recombinant DENV-3 viruses that expressed complete envelope genes from each of the four genotypes. We then constructed

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additional mutant recombinant viruses containing single or multiple amino acid mutations to identify the residues critical to 8A1 neutralization of DENV-3. We found that the sensitivity of genotype I and II, compared to resistant genotype III, are attributed to only two amino acid differences in EDIII region. Further study revealed that the amino acids work independently to confer the sensitivity to 8A1. Variation at two amino acid positions led to different on and off rates of epitope/antibody binding and thus different affinity. Our studies provided insights into neutralization mechanism and how binding kinetics affect virus sensitivity to different antibodies.

Methods and materials

Cells

Mosquito *Aedes albopictus* C6/36 cells were maintained in MEM (Gibco) media at 28°C. Human monocyte lymphoma cell line U937 expressing DC-SIGN (U937 DC-SIGN) were maintained in RPMI-1640 (Gibco) at 37°C supplemented with 50mM beta mercaptoethanol. Vero-81 cells were maintained in DMEM at 37°C. All media used were also supplemented with 5% FBS, 100U/ml penicillin, 100mg/ml streptomycin, 0.1mM non-essential amino acids (Gibco) and 2mM glutamine and all cells were incubated in the presence of 5% CO_2. The 5% FBS was reduced to 2% to make infection media for each cell line.

DENV-3 Molecular Clone Strategy

The four fragment cloning strategy for the DENV-3 clone was recently described (Messer, Yount et al. 2012). In brief, plasmids containing the four DENV fragments DNAs (A–D) were propagated in *E. coli*, purified, restriction enzyme digested and directionally ligated to create a full-length cDNA of the dengue viral genome. The cDNAs were subsequently transcribed with T7 polymerase (Ambion). This RNA produced infectious dengue virus when electroporated into Vero E6 cells. E variant clones have been previously characterized (Messer, Yount et al. 2012). Briefly, E genes from representative DENV-3 isolates (D3/InJ-16-82, D3/D95-0400, D3/Cuba21/02, UNC3001, D3/1339, each from genotype I, II, III, III, IV) were synthesized and introduced into the parent clone A and B fragments using unique Type IIS restriction enzymes. The resulted constructs were each named as Indonesia ‘82 (I), Thailand ‘95 (II), Cuba ‘02 (III), Sri Lanka ‘89 (III), Puerto Rico ‘77 (IV), to indicate the isolates’ location, year of isolation and genotype.

To generate A fragments with EDIII variants, synthesized EDIII constructs were amplified with the DenBglI #1+ (5′-gaagccaagagggcgcaatgcataccgcactg-3′) and the Den2kb- (nnnnncgtctcgcctcaatattgacaggctcc) primers. These amplicons were digested with BglI and ligated to another amplicon which had been made using Den 985+ (5′-nnnnnnacccagaaggtggtcattttcatactac-3′) and DenBglI#2- (5′-gcgccctcttggcttccaaggacgactacttcttg-3′) and had been similarly digested. The ligated products were gel isolated and cloned into the pCR-XL TOPO cloning vector. Consensus clones were digested with BstEII and BsmBI and ligated into the parent A plasmid which had also been digested with BstEII and BsmBI. Variants at position 383 were generated either using parent B plasmid (N383) or Thailand ’95 B plasmid (K383). The B plasmid sequences were otherwise identical.

Recombinant Virus Recovery

The virus clones were recovered following a protocol that was recently described by Messer *et al.* (Messer, Yount et al. 2012). Briefly, each plasmid was transformed, propagated, cloned to *E. coli* and expanded in LB media. Plasmid purified (Qiagen Mini-Spin Kit) and digested as follows according to manufacturer’s instructions. Fragments were gel-isolated (Qiagen Gel Extraction Kit) on 0.8% agarose gel, mixed in equivalent copy number and

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ligated with T4 ligase (NEB) overnight at 4°C. Full-length transcripts of DENV-3 cDNA constructs were generated in vitro and mixed with Vero cells trypsinized and resuspended in RNAse free PBS in an electroporation cuvette. After electroporation, the Vero cells were then incubated at 37°C for 4 days. Supernatant from transfected Vero cells were further passaged to Vero cells and these supernatants were harvested at day 7 as working virus stocks.

Virus Titration and Focus Reduction Neutralization Test (FRNT)

The FRNT procedure is based on a method previously described (Durbin, Karron et al. 2001). Briefly, twenty-four well plates were seeded with Vero cells and grown to 80% confluence in incubator (37°C, 5% CO_2 unless otherwise specified). For virus titration, virus stocks were serially diluted, added to wells and after 1 hr incubation at 37°C overlaid with 0.8% methylcellulose in Opti-MEM (Gibco) supplemented with 2% FBS, antibiotic mix (Gibco) and non-essential amino acids (Gibco). Following incubation for 5 days, the overlay was removed, the wells washed twice with PBS and cells fixed in 80% methanol/PBS. Fixed monolayers were either stored at −80°C or developed for foci visualization immediately. Briefly, wells were blocked for 10 minutes with 5% non-fat milk in 1x PBS, followed by 1 hr incubation with anti-flavivirus mAb 4G2 diluted in blocking buffer at 37°C, then washed, incubated with horseradish peroxidase (HRP) conjugated goat anti-mouse Ab (Sigma) dilutions, washed again and foci developed by the addition of TrueBlue HRP substrate (KPL). Foci were counted on a light box and viral titers calculated by standard methods. For FRNT, mAbs were serially diluted, mixed with approximately 600 focus forming units (ffu)/ml of virus to a final volume of 400 ul, The virus-mAb mixes were incubated for 1 hr and added in triplicate (100 ul volumes) to 24-well plates and incubated and developed as described above.

Monoclonal Abs

Purified mAbs 8A1 (IgG1), 7.5 mg/ml and 14A4 (IgG1), 9.6 mg/ml were kindly provided by Robert Putnak (Walter Reed Army Institute of Research, MD) (Matsui, Gromowski et al. 2010; Wahala, Donaldson et al. 2010). Hybridoma cells producing mAb 1H9 (IgM) were kindly provided by John Aaskov (Queensland University of Technology, Australia) (Serafin and Aaskov 2001).

Software and statistics

FRNT counts were entered into GraphPad Prism (Version 5.00 for OSX, GraphPad Software, San Diego California USA, www.graphpad.com). $FRNT_{50}$ values were calculated by sigmoid dose-response curve fitting with upper and lower limits of 100 and 0 respectively. All error bars show 95% confidence intervals unless otherwise specified. Mean log values were compared by one-way ANOVA followed by Tukey HSD multiple comparison test with significance level alpha (P) set at <0.05. EDIII structure figure was made using The PyMOL Molecular Graphics System, Version 1.5.0.4 Schrödinger, LLC. (http://www.pymol.org). Alignment of the DENV-3 genotype DIII sequences was prepared using ALINE (Bond and Schuttelkopf 2009).

ELISA

High binding 96-well ELISA plates were coated with 300 ng human monoclonal antibody 3H4 (which binds E protein, but not EDIII) per well in 0.1 M carbonate buffer. Plates were incubated at 4°C overnight, washed three times (All washes were performed three times with Tris-buffered saline containing 0.2% Tween-20), then blocked with dilution buffer on a shaker at 37°C for 1hr. Antigen was captured by adding virus in Tris-buffered saline containing 0.05% Tween-20 and 3% Normal Goat Serum to wells and incubating on a

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shaker at 37°C for 1 hr. Unbound antigen was washed off, primary mouse monoclonal antibody (either 8A1 or 1H9) was serially diluted and added to wells, and plates were incubated on a shaker at 37°C for 1 hr. Unbound primary antibody was washed off and 1:1000 alkaline phosphatase conjugated diluted human sera-adsorbed, goat anti-mouse secondary antibody added; and plate was incubated on a shaker at 37°C for 1 hr. Unbound secondary antibody was washed off, pNPP substrate added, and absorbance readings were taken at 405 nm in Epoch Microplate Spectrophotometer (Biotek Instruments Inc. Winooski, VT).

Mutagenesis and production of rEDIII

Recombinant EDIII constructs were created as described by Wahala *et al.* (Wahala, Donaldson et al. 2010). E gene fragments encoding EDIII each genotype of viruses were engineered into pMAL c2X vector to generate recombinant EDIII-MBP fusion protein construct. Individual or combinations of mutations at E301 and E383 were further introduced to the rEDIII-MBP construct using QuikChange multi kit (Stratagene, LaJolla, CA) according to manufacturer's instructions and were confirmed by sequencing. The recombinant EDIII-MBP constructs, including the mutants, were expressed in *E. coli* DH5a (Invitrogen) and purified using amylose resin affinity chromatography (NEB) as instructed by manufacturer.

Surface Plasmon Resonance (SPR)

Kinetic information on the interaction between anti-dengue antibodies and DIII variants was obtained using a Biacore T100 instrument. Anti-mouse capture antibodies (GE Life Sciences) were immobilized at high density using amine coupling to a Series S CM5 chip. The anti-dengue antibodies or control (WNV E16) antibody were flowed over the surface of the chip and captured at 700 RUs (Response Units) or less. A two-fold dilution series of the DENV-DIII MBP fusion protein variants were injected over the chip at a flow rate of 65 ul/minute for 180 seconds and allowed to dissociate for 450 seconds. The flow cell was regenerated with 10 mM glycine pH 1.7 for 180 seconds, followed by a second pulse for 30 seconds, at 30ul/min. Data was processed using the Biacore Evaluation Software (Version 1.1.1) by double referencing (control antibody and buffer alone subtraction) and a global 1:1 Langmuir fit of the curves. Results were generated from at least three independent experiments.

Results

Mouse Monoclonal antibody (mAb) neutralization against four DENV-3 isogenic clones containing E protein from different genotypes

It was commonly believed that only inter-serotype variation matters in virus-antibody interactions. However, several recent studies challenged this assumption by showing that intra-serotype variation plays a role in this interaction too (Wong, Abd-Jamil et al. 2007; Brien, Austin et al. 2010; Wahala, Donaldson et al. 2010). To study this problem we used a DENV-3 reverse genetic platform to capture naturally occurring variation in DENV-3 envelope glycoprotein. Because each variant E gene is expressed from an isogenic genome backbone, the recombinant viruses allow us to probe the impact of micro-variation on the antigenic properties of variant E glycoproteins. Focus Reduction Neutralization Test (FRNT) for the parent and isogenic clones with variant E genes were performed against a panel of mouse monoclonal antibodies (14A4, 1H9 and 8A1) and $FRNT_{50}$ values were calculated for each of the mAbs against each of the clones. Mouse mAb 14A4 is a DENV-3 specific IgG1 mAb that has been mapped to the A-strand of DENV-3 E domain III (Matsui, Gromowski et al. 2010; Wahala, Donaldson et al. 2010). 14A4 is known to neutralize all four genotypes within DENV-3 and would be expected to neutralize all of the isogenic

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clones (Wahala, Donaldson et al. 2010). As expected, 14A4 neutralized the entire recombinant DENV-3 panel at equivalent concentrations (Figure 2A). Calculated $FRNT_{50}$ values were not significantly different and ranged from 12 ug/ml for genotype I to 34 ug/ml for genotype III (Cuba '02) (Figure 2A).

Mouse mAb 1H9 is an IgM antibody that is specific to DENV-3 and has been previously mapped to the lateral ridge of domain III of the E glycoprotein (Wahala, Donaldson et al. 2010). Serafin *et al.* demonstrated that mutation from lysine to asparagine in position E386 enabled DENV-3 prototype strain H87 to escape neutralization from 1H9 (Serafin and Aaskov 2001). Subsequently, Wahala *et al.* demonstrated by both binding and FRNT that clinical isolate genotype IV PR '77, via a naturally occurring K to R mutation at 386, also allows for escape from neutralization by 1H9 (Wahala, Donaldson et al. 2010). 1H9 was tested against the parent and isogenic clones using FRNT. As expected, the genotype IV was not neutralized by 1H9 (Figure 2B), consistent with the wild type PR '77 phenotype. In contrast, genotype I and genotype II were neutralized at very low antibody concentrations - 0.046 ug/ml and 0.061 ug/ml respectively (Figure 2B). Genotype III clones Cuba '02 and SL '89 were also effectively neutralized by 1H9, but at antibody concentrations significantly greater - 1 log - than genotype I and II clones ($P < 0.01$) (Figure 2B). 1H9 had essentially three neutralization phenotypes - an escape phenotype (IV), an intermediately sensitive phenotype (III), and a highly sensitive phenotype (I and II).

Mouse mAb 8A1 is a DENV-3 specific IgG1 mAb that binds EDIII, via lateral ridge residues 301, 302, 380 and 386, similar to that observed for mAb 1H9 (Wahala, Donaldson et al. 2010). The DENV-3 clones recapitulate genotypic variation at those residues. Purified 8A1 was tested for ability to neutralize parent and isogenic clones (Figure 2C and Table 1). Previous studies have demonstrated that mAb 8A1 binds EDIII from DENV-3 genotypes I, II and III but not genotype IV (Wahala, Donaldson et al. 2010). As predicted from these binding studies, the genotype IV clone was extremely resistant to neutralization, with a calculated $FRNT_{50}$ of 413 ug/ml, which was 2–3 logs greater than values recorded for the other isogenic recombinant viruses ($P < 0.01$). $FRNT_{50}$s for genotype III recombinant viruses Cuba '02 and SL '89 were 13.5 ug/ml and 9.9 ug/ml, respectively, virtually identical and forty-fold less than genotype IV (Table 1). The remaining genotype I and II clones were again significantly more sensitive to neutralization by 8A1, with calculated $FRNT_{50}$s of 0.2 ug/ml and 0.37 ug/ml respectively, 800-fold more sensitive to neutralization than genotype IV ($P < 0.01$). For mAb 8A1, as with mAb 1H9, a distinct pattern of neutralization was observed; genotype IV requires a high concentration of antibody for neutralization, genotype III requires intermediate concentration of antibody and finally genotype I and II are neutralized by low concentrations of antibody (Figure 2C).

Amino Acid Variations in EDIII lateral ridge

Wahala *et al.* previously demonstrated that 8A1 bound to DENV-3 genotypes I, II and III but not to IV. Moreover, Wahala *et al.* mapped loss of 8A1 binding to genotype IV to a K386R (lysine to arginine) mutation on the lateral ridge of DENV-3 genotype IV (Wahala, Donaldson et al. 2010). We analyzed EDIII sequence differences between DENV-3 genotypes I, II and III to explore why genotypes I and II were more sensitive to neutralization than genotype III, despite the antibody binding to all three genotypes. The EDIII sequence differences between the different DENV-3 genotypes are summarized in Figure 1B. The more sensitive genotype I and genotype II clones have two common non-conservative variations compared to the less-sensitive genotype III clones: T301L and N383K. These two sites are located in adjacent loops of the lateral ridge, with position 301 at the apex of the N-terminal linker and position 383 in the FG loop. Genotype I and genotype III (Cuba '02) viruses also each have variations at 303 and 329 in the lateral ridge (Figure 1A), but as these variations are not conserved within sensitive genotypes (I and II)

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or within genotype III they are not considered critical to the phenotype. We hypothesized that variations at one or both sites (301, 383) are responsible for the sensitivity difference of mAb 8A1 and our reverse genetics system provided us a chance to test this hypothesis and further study the mechanism of this differential neutralization at molecular level.

To test our hypothesis residues T301 and N383 were mutated from the parental DENV-3 genotype III background both individually and in combination to amino acids - T301L and N383R respectively, recapitulating the residues in the sensitive genotype I and II strains. FRNTs for the parent and mutant variants against 8A1 were done and $FRNT_{50}$ values calculated. We found that both mutations were required to increase sensitivity to neutralization (Figure 3). Single mutations led to partial increases in neutralization sensitivity but only the double mutant was as sensitive to neutralization as the DENV-3 genotype I and II sensitive strains. This suggests that the two amino acids at 301 and 383 both confer sensitivity to mAb 8A1, with double mutations at these two positions conferring the greatest effect.

The Mutations Altered Binding Affinity

To further explore the mechanism of neutralization sensitivity by the individual and combined effect of 301 and 383 site mutations, we studied the binding affinity difference of mutant viruses to mAb 8A1. ELISA plates were coated with equal quantities of purified antigen from DENV-3 genotype II and III for binding studies with mAb 8A1. The binding curve indicated that genotype II has higher binding affinity to 8A1 than genotype III. The EC50 concentration for DENV-3 genotype III virus and genotype II virus are 0.098 ug/ml, and 0.050 ug/ml respectively. This EC50 difference is consistent with the observed $FRNT_{50}$ differences. We further studied the binding affinity of 8A1 to genotype II, genotype III and mutant virus variant using capture ELISA. After optimization, equal amount of virus was captured and 8A1 concentration at 0.2 ug/ml was chosen for comparison for its sensitivity of detecting affinity variation. As shown in Figure 4, the sensitive genotype II strain had 50% increase of OD value compared to non-sensitive genotype III in the capture ELISA and introduction of single mutation T301L can increase the OD value to the same level of genotype II, suggesting the mutation at position 301 fully restores binding affinity. Introducing N383K alone increased the OD value only slightly, indicating that N383K mutation failed to increase binding affinity. This is consistent with previous observation that mutation at this site did not cause change of 8A1 binding of EDIII recombinant protein (Wahala, Donaldson et al. 2010). In other words, our ELISA data demonstrated that site 301 determines the differential binding affinity between genotype II and genotype III.

8A1 affinity is determined by off-rate

We next employed surface plasmon resonance (SPR) to better understand how specific residues affect binding affinity and kinetics of 8A1 to the EDIII protein from each genotype. Recombinant EDIII proteins from genotypes I, II and III were engineered as fusion proteins with maltose binding protein (MBP), produced and purified as described and validated by Wahala *et al.* (Wahala, Donaldson et al. 2010). The recombinant EDIII proteins differ at two critical sites: position 301 and 383 (Table 1). The two EDIIIs from potently neutralized genotypes (I and II) display distinct binding curves relative to those of the poorly neutralized genotype III EDIII (Figure 5A–C). EDIII from genotype I and genotype II had a half-life of 60.5 seconds and 59.0 seconds respectively (Table 1). These values contrast with those of the genotype III EDIII-MBP, which had a half-life of 16.6 seconds (Table 1). The association rate clearly cannot explain the half-life difference or neutralization sensitivity variation. The dissociation rate data are consistent with the neutralization data and half-life data as slower dissociation results in the longer half-life of binding, and subsequently a higher 8A1 occupancy of the virus at any given moment, which ultimately leads to

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neutralization (Pierson, Xu et al. 2007). Generally, our data suggests that the discrepancies of 8A1 binding affinity for DENVs EDIIIs are primarily due to distinct dissociation rates.

EDIII Residues at Site 301 Alone Determines Dissociation Rate and Half-Life

Expanding our kinetic data characterizing 8A1 binding to EDIII, we sought to determine the role of specific residues involved in this interaction. Our neutralization experiments suggested that sites 301 and 383 both contribute to neutralization sensitivity. To study how each site contributes to the kinetics of 8A1 binding, especially dissociation rate, EDIII-MBP from the genotype III background was mutated to the corresponding residues found in the genotype II virus (L301 or K383) and tested by SPR as above. Mutation of position 301 from a threonine to a leucine resulted in a half-life of 68.2 seconds (Figure 5D and Table 1), comparable to that of the wild-type genotype II EDIII. In contrast, mutation of position 383 from an asparagine to a lysine resulted in a decrease of only association rate and a half-life comparable to parental genotype III EDIII (11.9 seconds; Figure 5E and Table 1). These results suggest that the dissociation rate is determined by position 301 alone as T301 leads to short half-life (12–16 seconds) while L301 leads to long half-life (50–68 seconds).

Discussion

How natural strain variation within each DENV serotype influences virus-antibody interactions has important implications for rational vaccine design and virus evolution (Wong, Abd-Jamil et al. 2007; Brien, Austin et al. 2010; Wahala, Donaldson et al. 2010). We and others have recently reported genotype dependent variable neutralization within serotypes using both mAbs and human DENV-3 sera (Wahala, Donaldson et al. 2010; Messer, Yount et al. 2012). Despite the accumulated evidence, the detailed mechanism of genotype dependent variable neutralization remains unknown. To better understand this mechanism, we employed a DENV-3 reverse genetic clone system to capture the amino acid variation in E glycoprotein of all the four genotypes into the otherwise isogenic background of genotype III. The resulting isogenic viruses recapitulated the previously reported sensitivity differences of the DENV-3 genotypes to these mAbs (Wahala, Donaldson et al. 2010). Using these isogenic viruses, we mapped the mutations critical to mAb 8A1 neutralization to two sites - 301 and 383 - on the lateral ridge region of EDIII. We found that both mutations are needed to alter neutralization by mAb 8A1. Study of antibody-antigen affinity of whole virus suggested the distinct neutralization sensitivity is mainly attributed to binding affinity differences between different residues at these two positions. Using SPR and recombinant EDIII protein, we further found that binding differences are determined mainly by the dissociation rate and mutation at site 301 alone affects the dissociation rate.

Our finding that only two residues are responsible for differential neutralization between DENV-3 genotypes I, II, III and IV sheds light on our understanding of how neutralization is modulated by viral genetic variations. Consistent with previous research, our ELISA data suggests that binding affinity differences cause neutralization sensitivity variation (Gromowski and Barrett 2007; Lisova, Hardy et al. 2007; Gromowski, Barrett et al. 2008; Gromowski, Roehrig et al. 2010). However, mutant T301L restores fully binding affinity but it restores only partially the neutralization sensitivity. This suggests that site 383 also contributes to 8A1 neutralization sensitivity via a mechanism other than binding affinity. The evidence is that mutant N383K increased neutralization sensitivity about 4 times, without increasing binding affinity (Figure 4) or half-life of binding (Table 1). It is even more interesting to find that after introducing mutation N383K into the mutant virus T301L to make it double mutant/genotype I or II, the neutralization sensitivity increased about 3–5 times from mutant T301L, comparable to the effect of introducing N383K into the parental genotype III (Table 1). We can find a similar pattern for mutation T301L as introducing this mutation to genotype III increased sensitivity for about 10 times and introducing this

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mutation into mutant N383K also increased sensitivity for about the same extent. This pattern strongly argues that both sites contribute to 8A1 neutralization sensitivity through different and independent mechanisms. We speculate that N383K could alter neutralization by affecting virus structural dynamics so that each 8A1 antibody binding may exert more constraints on virus post-attachment steps such as conformational change for fusion, as mAb 8A1 neutralizes virus by blocking post-attachment steps (2unpublished data). It has been shown that flavivirus dynamic equilibrium (breathing) can regulate antibody neutralization (Lok, Kostyuchenko et al. 2008; Dowd, Jost et al. 2011; Cockburn, Navarro Sanchez et al. 2012). It is also possible that the 383 mutation affects binding when the virion is in alternate conformation which can’t be detected with current ELISA methods.

SPR has been proven a powerful tool in studying and viewing antigen-antibody interactions from a kinetic and structure perspective (Bedouelle, Belkadi et al. 2006). The correlation between the virus binding affinity data (Figure 4) and the half-life data (Table 1) suggests that 8A1 binding affinity is determined by off-rate. We further found that both residues 301 and 383 can affect association rate as mutation at either residue changed the association rate (Table 1). However, only residue 301, not residue 383, affects dissociation rate, and also half-life (Table 1). These data are consistent with previous mapping data of 8A1 showing that mutation of residue 383 did not affect 8A1 binding (Wahala, Donaldson et al. 2010).

The association rate of genotype II is aberrant and extremely low, consequently the resulted unexpectedly high KD value contradicts with both genotype II EC50 and 8A1 neutralization titer (Table 1). This anomaly could be either due to a) instability of this particular construct or b) non-specific interaction between the DIII and MBP fusion partner that occludes recognition of the 8A1 epitope. Another factor we must take into consideration is that there is essential difference between the affinity measured using EDIII-MBP fusion protein against immobilized 8A1 antibody (SPR) and the avidity measured using immobilized whole virus and 8A1 antibody (EC50). The two values may not necessarily corroborate each other. Thus, caution must be taken when using the protein-antibody SPR results to interpret virus binding and neutralization.

In sum, these data established a causal relationship between binding kinetics, binding affinity and neutralization. Site 301 determines dissociation rate to 8A1 between the genotypes, which dominates binding kinetics difference, and subsequently determines binding affinity variation, and contributes 2/3 of neutralization differences. The other 1/3 is attributed to mutation at site 383, through a neutralization mechanism different and independent from binding affinity variation. Structural investigation is underway to resolve this problem.

Our work demonstrates how two naturally occurring mutations altered binding kinetics, leading to change of affinity and then neutralization sensitivity. Understanding how single or multiple mutations alter the viral neutralization profile is critical for further understanding of how different virus strains elicit immunity after infection, and how potentially protective antibodies differentially interact with virus infection in a secondary infection with a homologous serotype. More importantly, for a vaccine to elicit immunity that fully neutralize all DENV-3 genotypes with minimal risk of ADE, studies like this one will help to understand which epitopes among the inter-genotype variable sites should be targeted. This knowledge is especially helpful considering the high diversity in the lateral ridge in DENV-3 and other serotypes. Our results identifying how mutations in this region lead to neutralization alteration through changing association and dissociation rates will help identify the most critical neutralization epitope of dengue virus. Future studies of antibodies with intra-serotype or intra-genotype neutralization variation employing this approach

should define additional important neutralization epitopes and the mechanism of differential neutralization.

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Highlights

1. we mapped the 8A1 differential neutralization to two amino acids at site 301 and 383.
2. we found that binding difference is responsible for 2/3 of the neutralization difference between DENV3 genotypes.
3. we found that dissociation rate determines the binding difference between 8A1 and DENV3 viruses.
4. we found site 301 determines the dissociation rate of 8A1 binding to DENV3 viruses.

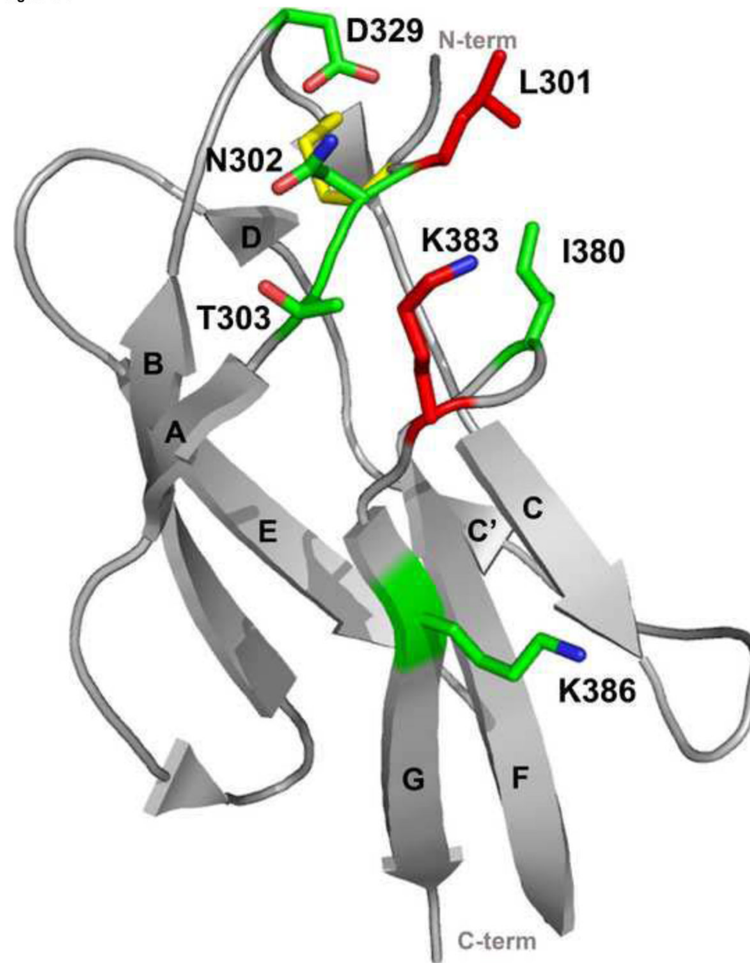

Figure 1B

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                     N-term        A-strand            B-strand          C-strand
                  295    300           310                 320              330                340   346
Indonesia '82   (gI)   MSYAMCLNAFVLKKEVSETQHGTILIKVEYKGENAPCKIPFSTEDGQGKAHN
Thailand '95    (gII)  MSYAMCLNTFVLKKEVSETQHGTILIKVEYKGEDAPCKIPFSTEDGQGKAHN
Cuba '02        (gIII) MSYAMCTNTFVLKKEVSETQHGTILIKVEYKGEDAPCKIPFSTEDGQGKAHN
Sri Lanka '89   (gIII) MSYAMCTNTFVLKKEVSETQHGTILIKVEYKGEDAPCKIPFSTEDGQGKAHN
Puerto Rico '77 (gIV)  MSYAMCSGTFVLKKEVSETQHGTILIKIEYKGEDAPCKIPFSTEDAQGKAHN

                     C'-strand   D-strand      E-strand        F-strand        G-strand
                  347 350           360                370                380                390      398
Indonesia '82   (gI)   GRLITANPVVTKKEEPVNIEAEPPFGESNIVIGIGDKALKINWYKKGSSIGK
Thailand '95    (gII)  GRLITANPVVTKKEEPVNIEAEPPFGESNIVIGIGDKALKINWYKKGSSIGK
Cuba '02        (gIII) GRLITANPVVTKKEEPVNIEAEPPFGESNIVIGIGDNALKINWYKKGSSIGK
Sri Lanka '89   (gIII) GRLITANPVVTKKEEPVNIEAEPPFGESNIVIGIGDNALKINWYKKGSSIGK
Puerto Rico '77 (gIV)  GRLITANPVVTKKEEPVNIEAEPPFGESNIVIGTGDKALRINWYKKGSSIGK
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Figure 1.

A) Cartoon of DENV3 EDIII. Lateral ridge EDIII variable sites for the recombinant clones are color labeled: 301 and 383 (red); 302, 303, 329, 380 and 386 (green). Secondary structure features are labeled. PDB ID 3VTT. B) Alignment of EDIII amino acid sequences. Beta-strands are labeled as arrows and genotypic variation is highlighted according to Fig 1A.

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Figure 2A

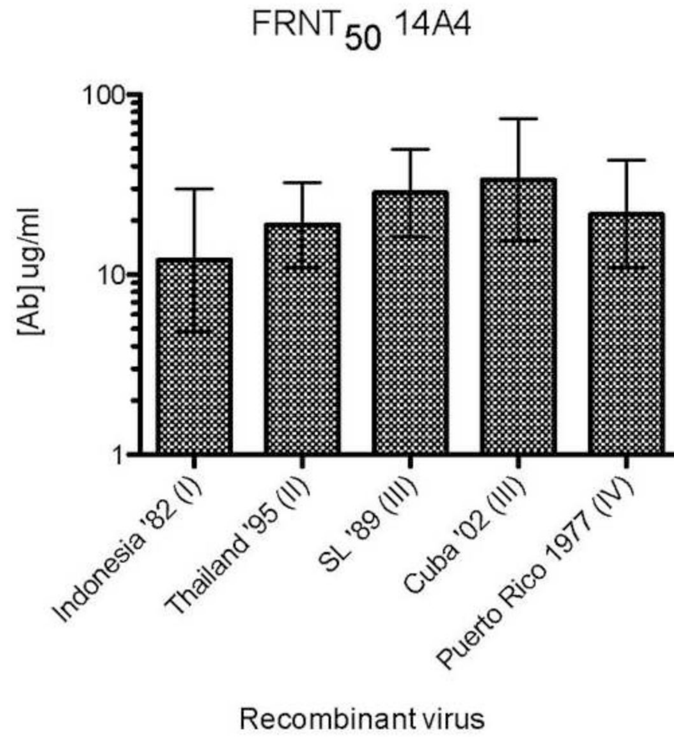

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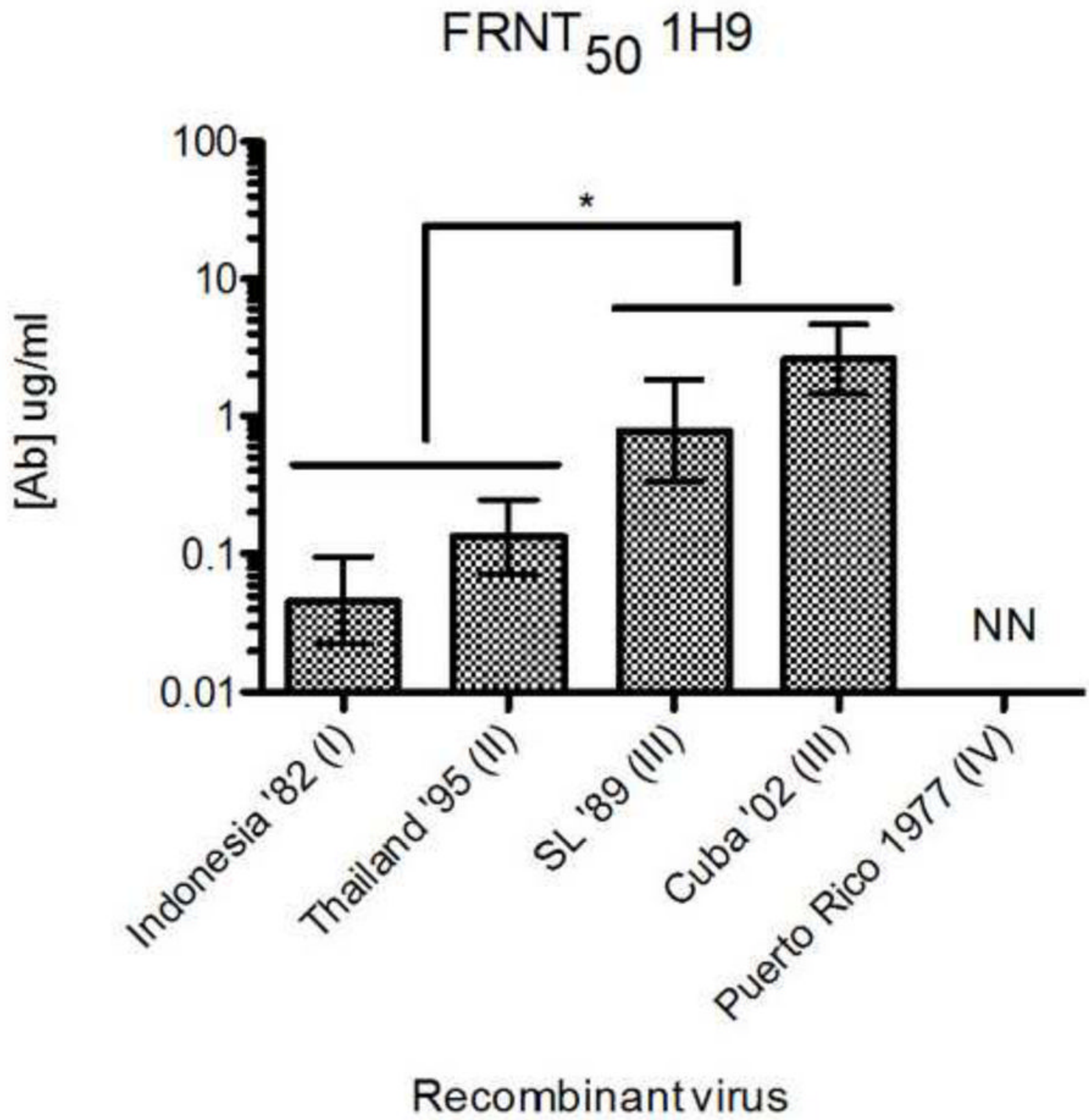
Figure 2B



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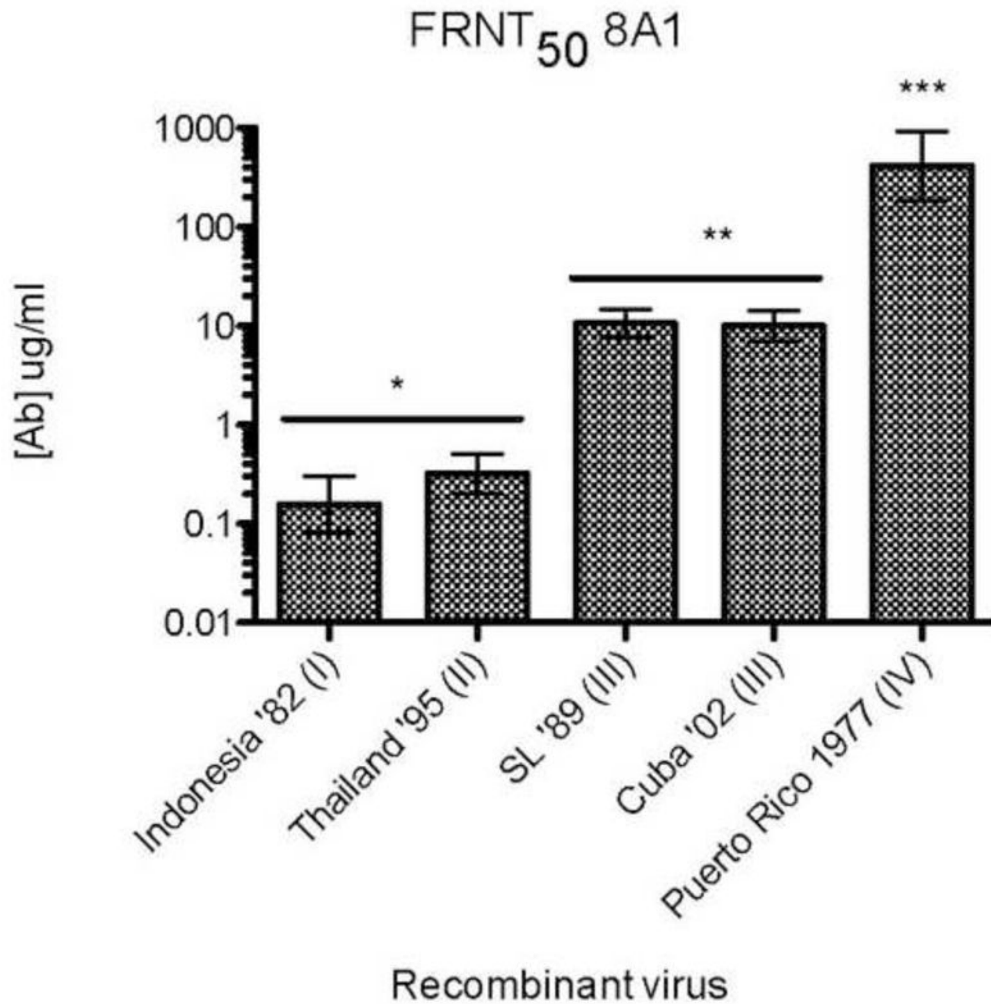

Figure 2.

Calculated $FRNT_{50}$ values for mouse mAbs against the recombinant virus clones. Error bars show 95% confidence intervals. A) Calculated $FRNT_{50}$ values for 14A4 did not differ (P >0.05 ANOVA). B) Calculated $FRNT_{50}$ values for 1H9 against Indonesia '82 (I) and Thailand '95 (II) were significantly lower than SL '89 (III) and Cuba '02 (III) (P<0.05 ANOVA followed by Tukey's HSD). NN = not neutralized. C) Calculated $FRNT_{50}$ values for 8A1 against Indonesia '82 (I) and Thailand '95(II) were significantly lower than SL '89 (III) and Cuba '02 (III) and Puerto Rico 1977 $FRNT_{50}$ was significantly greater than against the other clones (P <0.05, ANOVA followed by Tukey's HSD).

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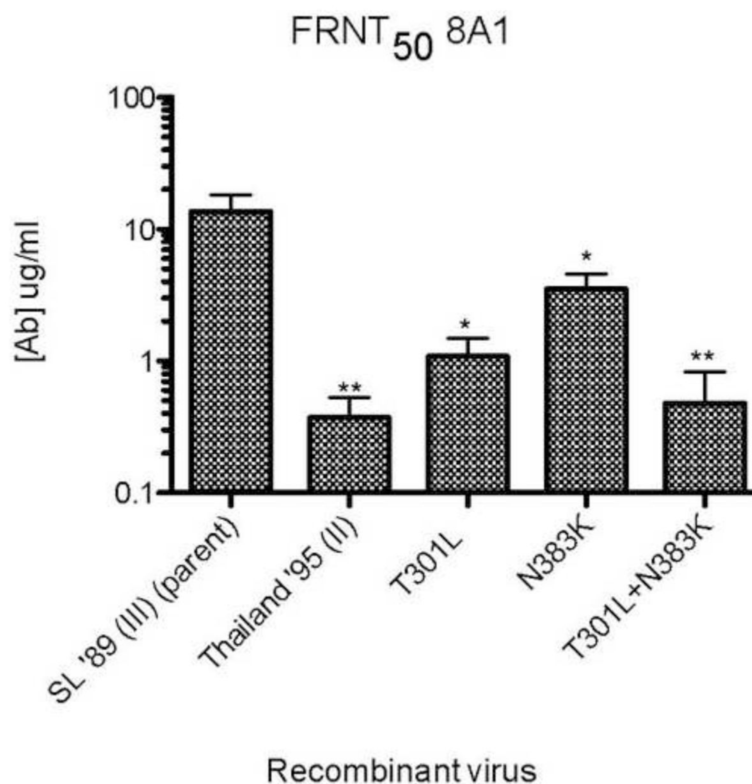

Figure 3.

Calculated $FRNT_{50}$ values for mAb 8A1 against the parent SL'89 clone, Thailand '95 clone and point mutant clones T301L, N383K and the combined mutant T301L+N383K clone. Error bars show 95% confidence intervals. Calculated $FRNT_{50}$ for 8A1 against Thailand '95 (II), mutant clones T301L, N383K and combined mutant T301L+N383K clone were significantly lower than parent SL '89 clone (III) ($P<0.05$ ANOVA followed by Tukey's HSD). Calculated $FRNT_{50}$ against Thailand '95 clone (II) do not differ significantly from combined mutant T301L + N383K clone ($P>0.05$ ANOVA followed by Tukey's HSD).

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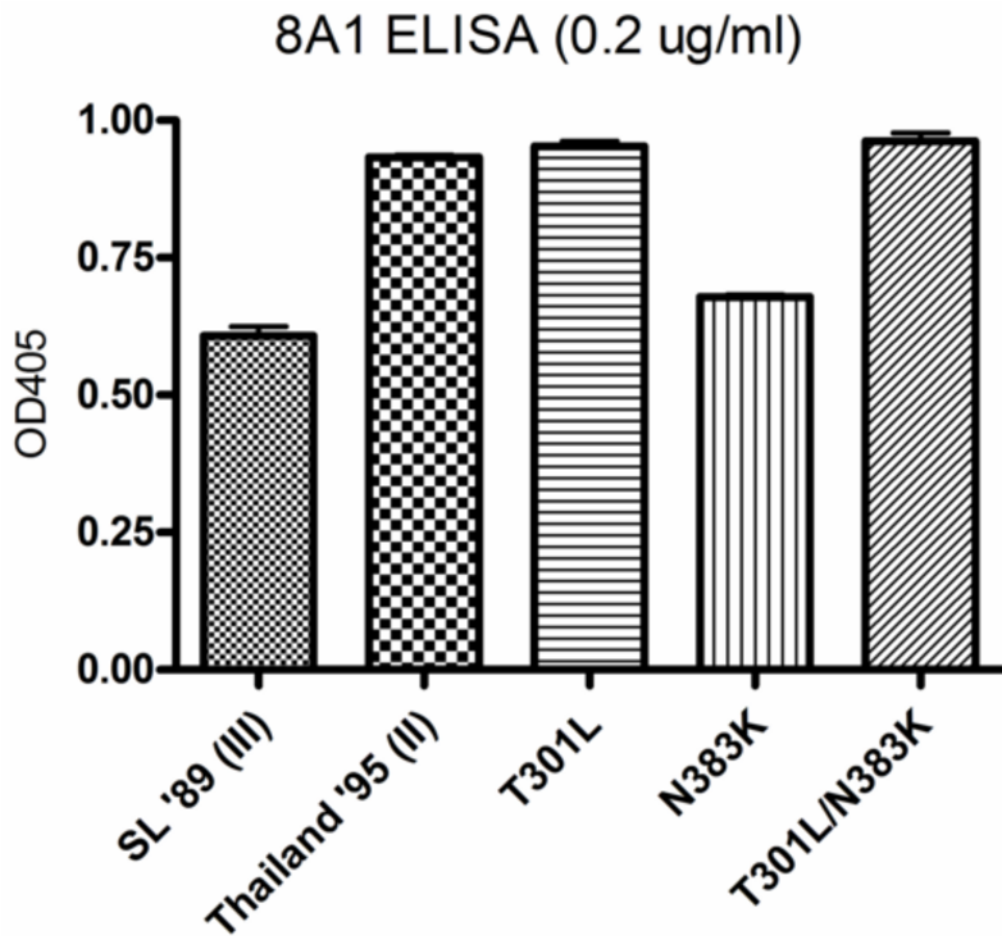

Figure 4.
Results of ELISA using mouse mAb 8A1 (0.2 ug/ml) against captured whole parent virus SL ’89, recombinant clone Thailand ’95, and mutant clones T301L, N383K and T301L +N383K. Error bars show 95% confidence intervals. Whole virus was captured with human mAb 1M19, a cross-reactive EDIII binding antibody. The virus captured were quantified to be equal amount using 12C1 (cross-reactive EDIII binding mouse mAb) as control.

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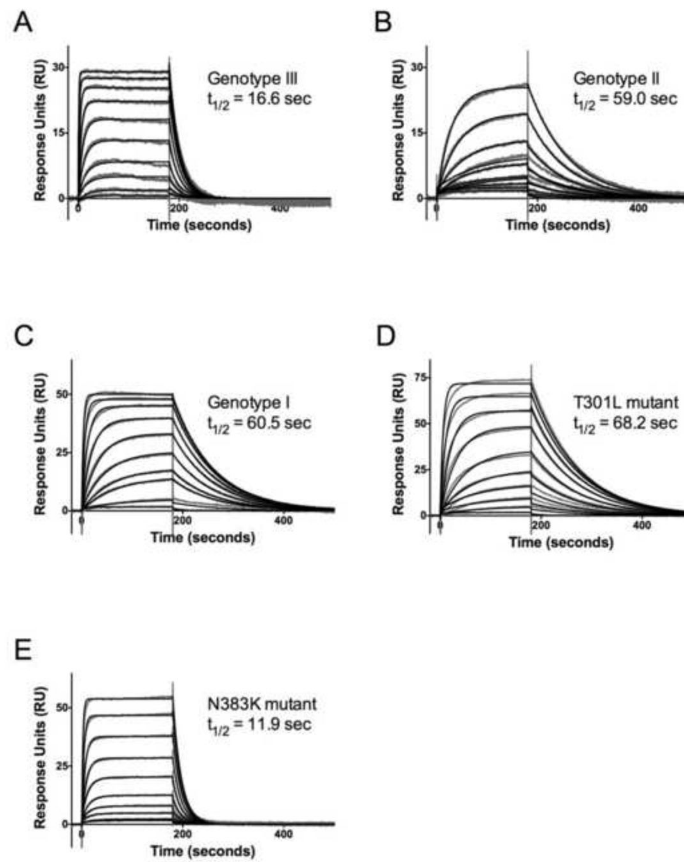

Figure 5.

Kinetic analysis of 8A1 interaction with DENV-3 EDIII-MBP genotypes and mutants. mAb 8A1 was captured and recombinant EDIII-MBP proteins of genotype I, II, III or mutants were flowed over at two fold serial diluted concentrations to measure the kinetic parameters of the interaction. SPR sensorgrams and traces are presented as mAb 8A1 interacting with A) genotype III, B) genotype II, C) genotype I, D) mutant T301L, E) mutant N383K.

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Table 1

Kinetics of DENV-3 DIII-MPB variants with 8A1.

DIII Variant	301	383	ka ($10^5 M^{-1} s^{-1}$)	kd ($10^{-3} s^{-1}$)	*KD* (nM)	*t* ½ (sec)	$FRNT_{50}$ (ug/ml)
III	T	N	2.176	43.51	200.9	16.6	13.5*
I	L	K	1.124	11.51	103.2	60.5	0.20
II	L	K	0.268	12.62	470.9	59.0	0.37
T301L	L	N	0.6892	10.15	155.3	68.2	1.08
N383K	T	K	0.823	59.8	718.2	11.9	3.50
IV	S	K	NA	NA	NA	NA	413.1

*There are two genotype III virus clones: SL '89 and Cuba '02. $FRNT_{50}$ of SL '89 (III) was indicated in this table and $FRNT_{50}$ of Cuba '02 (III) is 9.9 ug/ml. 8A1 binding was not detected for genotype IV DIII-MBP at concentrations of 5000 nM.