THESIS

# MOLECULAR DETERMINANTS OF DENGUE VIRUS TYPE-2 CRITICAL FOR EARLY EVENTS IN ANTIBODY-DEPENDENT ENHANCEMENT OF INFECTION

Submitted by

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In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Summer 2013

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ABSTRACT

MOLECULAR DETERMINANTS OF DENGUE VIRUS TYPE-2 CRITICAL FOR EARLY EVENTS IN ANTIBODY-DEPENDENT ENHANCEMENT OF INFECTION

Antibody-dependent enhancement (ADE) of infection might be one of the major factors in the development of more severe forms of dengue disease in patients undergoing a secondary infection of dengue virus (DENV). The ADE is caused by cross-reactivity of subneutralizing or non-neutralizing antibodies (Abs), which form virus-Ab complexes and enhance virus infection by binding to the Fcγ receptors (FcγR) on FcγR-bearing cells.

The early events in non-ADE infection have been previously studied, but the virus entry pathway and the DENV molecular determinants involved in ADE are still largely unclear. There are two hypotheses for the early entry pathway of ADE infection: (1) Ab opsonized DENV binds to FcγR and directly enters cells through phagocytic pathway; (2) FcγR plays an auxiliary role in concentrating the opsonized virus to the cell surface, but other cellular receptors are still required for virus entry into the endocytosis pathway. Herein, we investigated contributions of the DENV2 E proteins to ADE infection, as well as the role of FcγRIIA in the enhancement of infection and possible entry route of the Ab-opsonized virion.

ADE of wild type (WT) DENV2 infection can be promoted in FcγRIIA-bearing K562 cells by using subneutralizing cross-reactive flaviviral monoclonal antibody (MAb), subcomplex DENV MAb or serotype-specific DENV2 MAb against the E protein, as well as non-neutralizing anti-DENV-prM MAb. The enhancement of infection was analyzed by comparing DENV2 infection under ADE conditions with DENV-Ab complex and non-ADE conditions with DENV

alone. Numbers of DENV infected cells were determined by flow cytometry of infected cells stained with labeled MAb 2H2-AlexaFluor-488, while increases in viral output were quantified by qRT-PCR of viral genomes.

We investigated multiple DENV mutants generated by reverse genetic technology to identify molecular determinants in the envelope protein (E) of DENV-2 that are critical for DENV attachment and viral-endosomal membrane fusion in both non-ADE and ADE infection of the virus in K562 cells. We determined that binding of virus-Ab complex with FcγRIIA alone is not sufficient for virus entry during ADE infection. Furthermore, the molecular determinants of E protein critical for virus entry and virus-mediated endosomal membrane fusion involved in non-ADE infection were also required for ADE infection.

ADE was also tested in FcγRIIA-transfected CV-1 cells (CV-1-FcγRIIA). Surprisingly, no enhancement occurred with any of the tested MAb in this cell type. Numbers of cells expressing FcγRIIA and density of FcγRIIA molecules expressed on CV-1-FcγRIIA cell surface were similar to those of K562 cells. These results supported our findings that FcγRIIA alone was not sufficient to enhance viral infection. The results also suggested that cellular components that are present in K562 cells but absent in CV-1-FcγRIIA cells play a major role in ADE of infection.

# ACKNOWLEDGEMENTS

Thanks to my advisor, Dr. Carol Blair for her valuable guidance and support she provided me throughout my graduate school career. Extremely thanks to my co-advisor, Dr. Claire Y.-H. Huang (Centers for Disease Control and Prevention; CDC) who always opens the door for me, provides indispensable advice and troubleshooting in the research. Without her immense dedications, this work cannot be accomplished. I would also like to thank Dr. Chaoping Chen, who served on my committee. Critical comments she provided were very helpful to improve my research project.

I would like to extend my gratitude to Dr. John Roehrig (CDC) who provided great discussion regarding my project and advice concerning dengue antibodies and dengue E protein. Thanks to Dr. Jacob Schlesinger (University of Rochester Medical Center) who provides the CV-1-FcγRIIA for this study, and I also thank Dr. Richard Kinney who helped me improve my writing. His lifelong experience in scientific writing and knowledge about dengue virus are great examples for me to learn.

This work cannot be completed without great helps from the members from Huang's lab (CDC): Dr. Siritorn Butrapet for the mutant viruses, Karen Boroughs, Jannae Stovell, Betty Luy, Andrei Gurau, and John Shannon for their helps in the lab, members from Roehrig's lab (CDC): Dr. Amanda Calvert, Susie Bennet and Kandice Dixon for monoclonal antibodies used in this study, and lastly, Dr. Amy Ullmann for the help with flow cytometry machine.

I also would like to thank Fulbright scholarship and Thailand-United States Educational Foundation (TUSEF) for selecting me as a scholar in this prestige program and supporting my graduate study here in the US.

Last but not least, I am greatly thankful for my family and my friends from Colorado State University and CDC for their constant support and encouragement.

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# CHAPTER 1

# LITERATURE REVIEW

## *Dengue virus*

Dengue virus (DENV) is a member of genus *Flavivirus* in the family *Flaviviridae,* sharing genetic and structural similarities with other human pathogenic viruses, including West Nile virus (WNV), Japanese encephalitis virus (JEV), yellow fever virus (YFV), and tick-borne encephalitis virus (TBEV). DENV is a small spherical-shaped, enveloped virus containing a single-stranded positive sense RNA genome (Lindenbach et al., 2007). It is a mosquito-borne virus transmitted by *Aedes* mosquitoes, principally urban-adapted *Aedes aegypti*. There are four serotypes of DENV (DENV1, DENV2, DENV3 and DENV4) sharing 70-80% amino acid sequence homology (Flipse et al., 2013). All four serotypes are causative agents of dengue fever (DF) and the more severe forms of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS). Infection by one serotype does not elicit long-term immune protection against other serotypes (Halstead, 1988).

The four serotypes co-circulate throughout the tropical and subtropical countries around the world, especially in Southeast Asia, Africa, Western Pacific, and South and Central America, where over 40% of the world population resides. To date, DENV continues to spread and emerge in new areas. Changes in human demographics and behaviors such as increasing population density, urbanization, migration, and international travelling could introduce the vectors and the

viruses into new suitable niches. Moreover, climate change may also contribute to sustainability of virus transmission. For instance, temperature increases could expand the territory of the vector into areas where the human population is naïve to the infection, increasing or decreasing rain fall could impact the breeding of the mosquitoes; finally, drought, crop failures and famine could contribute to host susceptibility to dengue infection (Khasnis and Nettleman, 2005; Morse, 1995). A recent study estimated that 390 million DENV infections with 96 million apparent illnesses occurred in 2010 (Bhatt et al., 2013).

## *Clinical signs and symptoms of DENV infection*

Most cases of DENV infection are asymptomatic or mildly symptomatic. Among symptomatic patients, the majority of the infected individuals display clinical signs of DF and a small percentage develop life-threatening severe DHF and/or DSS. The incubation period of dengue ranges from 3-14 days, but generally 4-7 days before patients start to show signs and symptoms. Clinical manifestations of DF are characterized by flu-like symptoms including onset high fever, headache, nausea, together with retro-orbital pain, myalgia and arthralgia, and rash which usually lasts up to 10 days. While DF is self-limited, the severe DHF/DSS without proper medical care can result in mortality rates up to 20%. Severe dengue disease usually exhibits classical DF at its initial stage. During the defervescence period, patients start to develop the DHF/DSS characteristics of thrombocytopenia (<100,000 platelets/$mm^3$), fatigue, capillary fragility, petechiae, ecchymosis, severe abdominal pain, persistent vomiting, restlessness, mucosal and gastrointestinal hemorrhage, vascular leaking, multiple organ failure and hypovolemic shock. This critical stage usually lasts for 24-48 h. Medical care by replacing lost

fluid (crystalloids) and maintaining the patient's body fluid volume can significantly decrease the mortality rate to less than 1% (reviewed in Gubler, 1998; Guzman et al., 2010; WHO, 2009).

## *DENV genome and proteins*

The DENV genome, as with other flaviviruses, is a single-stranded positive sense RNA approximately 11 kb in length. The genome consists of a 5' untranslated region (UTR) that has a 7-methylguanosine cap, a single long open reading frame (ORF), followed by a 3'UTR that lacks a polyadenylated tail (Rice et al., 1985). The sequence of the 5' UTR is not highly conserved but common secondary structures in the 5'UTR play critical roles in viral replication and pathogenicity. Substitution or deletion of single to multiple nucleotides within this region often disrupts RNA structure, causing attenuation or lethality of the virus (Sirigulpanit et al., 2007). The 5'UTR is involved in initiating RNA replication, as antisense molecules binding to the 5'stem-loop structure (5'SL) can inhibit both viral replication and translation (Holden et al., 2006). Likewise, flaviviral 3' UTR sequences are greatly diverse, but contain secondary RNA stem-loop structures (3'SL) as common features (Bryant et al., 2005). The 3'UTR is also essential for viral replication, as it contains a highly conserved 25-nucleotide 3'SL that serves as a 3' cyclization sequence (3'CS) that is complementary to a sequence at the 5'end of the capsid gene, suggesting cyclization of the viral RNA (Hahn et al., 1987; You et al., 2001). Additionally, the 3'SL serves as recognition site for RNA dependent RNA polymerase (RdRp) and helicase binding, indicating roles in viral RNA (vRNA) synthesis (Chen et al., 1997a).

Translation of the single ORF results in a single long polyprotein which is subsequently cleaved by viral and cellular proteases into three structural proteins: capsid (C), pre-membrane

(prM) and envelope (E), and seven non-structural (NS) proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Lindenbach et al., 2007).

C is the first protein to be translated, which occurs in the cytoplasm. The carboxy terminus of C protein is a hydrophobic region that serves as a signal peptide for translocation of the rest of the polyprotein into the endoplasmic reticulum (ER) lumen (reviewed in Perera and Kuhn, 2008). The signal peptide is cleaved from mature C by NS2B/NS3 viral serine protease (Amberg and Rice, 1999). C protein naturally forms dimers. The tertiary structure of C protein is composed of four alpha helix domains (α1-α4).The distribution of basic charges are concentrated in the α4 domain, which is believed to bind with vRNA, while the distribution of hydrophobic amino acids (AA) concentrate in the α2 domain, which is believed to interact with the viral membrane (Ma et al., 2004).

prM is a glycoprotein precursor of membrane (M) protein and prevents inappropriate fusion of E in immature virions to cellular membranes at acidic environment during viral budding. Irreversible proteolytic cleavage of prM to M by host furin protease occurs within the trans-Golgi network (TGN) during the exocytotic pathway. Pr peptide dissociates from the M protein when the mature particle is released out of the cell into neutral pH environment (Yu et al., 2008).

E protein is the major glycoprotein on the viral surface. DENV E shares about 40% of AA identity with other flaviviruses (reviewed in Perera and Kuhn, 2008). At low temperature and physiological pH, 180 copies of E protein form 90 homodimers lying smoothly on the mature virion surface of ~500Å (Figure 1.1C). At 37°C the virion surface becomes “bumpy” and the envelope shell expends to ~550Å. The rough form of virion at 37°C reveals certain epitopes

of E protein that were previously found cryptic at lower temperature, and the exposure of these epitopes may contribute to the higher infectivity of the rough virions to the mammalian cells than the smooth virions (Fibriansah et al., 2013; Zhang et al., 2013b).

E protein binds to the cellular receptor to induce the sequence of entry events, as well as serves as the main target of neutralizing antibodies (Ab). Structures of several flaviviral E proteins have revealed the E homodimer structure (Modis et al., 2003, 2005; Mukhopadhyay et al., 2003; Rey et al., 1995; Zhang et al., 2013a; Zhang et al., 2004). It is composed of 3 discontinuous β-barrel domains designated as domain (D) I, II and III (shown in red, yellow and blue, respectively, in Figure 1.1). DI is located at the center of the protein, and is composed of 120 AA (based on DENV-2; AA 1-52,132-192 and 280-295). The single peptide strand connecting DI to DIII and four peptide strands connecting DI to DII (arrows, Figure 1.1B) are molecular hinge regions functioning in conformational reorganizations during transitions between immature, mature and fusion-active states. The conformational changes are triggered by acidic pH (Modis et al., 2004; Zhang et al., 2013a; Zhang et al., 2004).

DII (AA 53-131 and 193-279) is an elongate finger-like structure that mediates dimerization of E proteins. It contains a conserved fusion peptide (CD loop, AA98-111) at the distal end of the domain that is essential for endosomal membrane fusion (Modis et al., 2004). E protein contains two N-linked glycosylation motifs at residues N153 (in DI), which is conserved

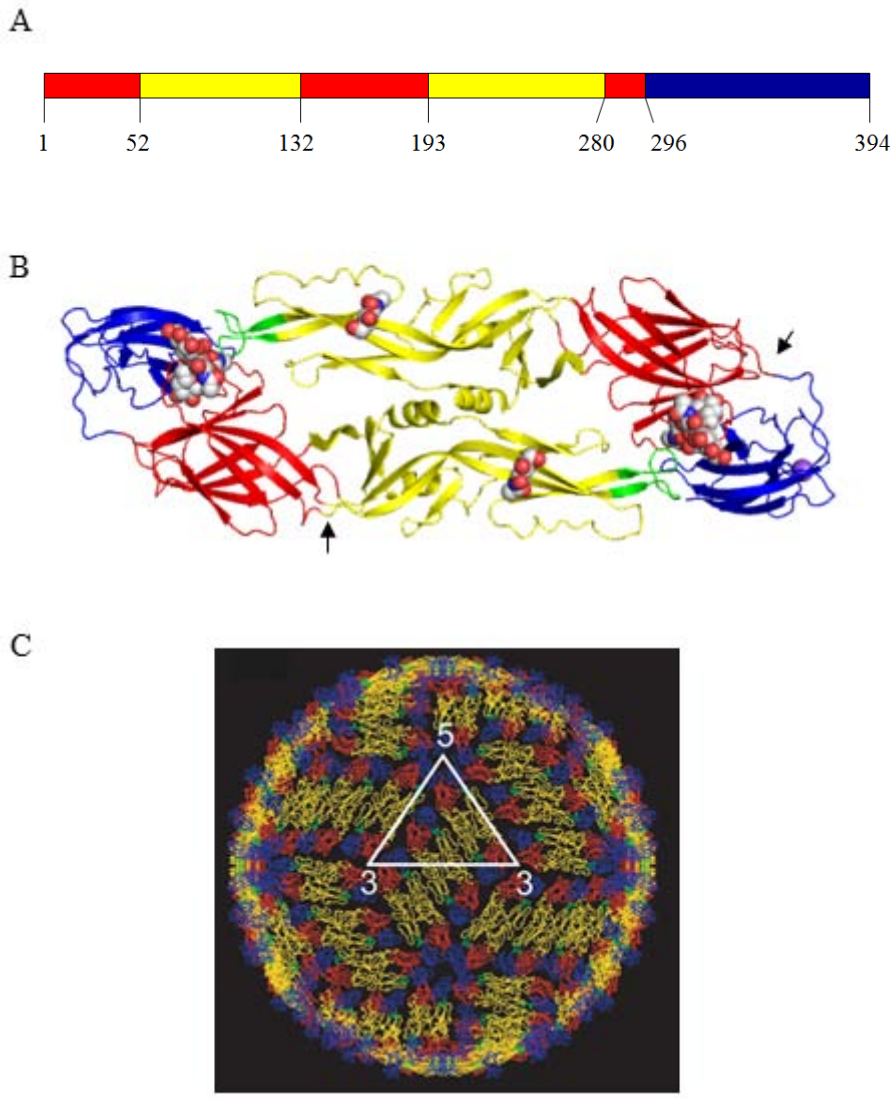


Figure 1.1 Molecular structure of DENV E protein. (A) Linear AA positions of E monomer ectodomains. DI is shown in red, DII is shown in yellow and DIII is shown in blue. (B) Top view of homodimer arrangement of E proteins on virion at physiological pH. Green color indicates fusion loops (DII-CD loop AA 98-111), arrow indicates hinge region and sphere represents glycans ligands. (C) Ninety copies of E protein homodimers form a herringbone on the virion surface, and the triangle indicates 3-fold and 5-fold axes. A and B are based on Modis et al., 2004, and C is from Kuhn et. al., 2002. E protein dimers were obtained from the protein database bank [PDB ID:1OAN], and were rendered in Polyview-3D (Porollo et al., 2004).

among flaviviruses, and N67 (in DII), which is unique in DENV (Rey, 2003). Both N-linked carbohydrates can bind to dendritic cell-specific ICAM3-grabbing non-integrin (DC-SIGN) expressed on monocyte-derived dendritic cells (DC), suggesting a function in internalization of DENV (Rey, 2003).

DIII (AA 296-394) is located before the carboxy-terminal membrane domain of the E protein. DIII is believed responsible for cell receptor binding due to its immunoglobulin-like structure, which is a common structure of cell-adhesion proteins (Rey et al., 1995). In addition, several neutralizing MAbs recognize epitopes residing in DIII (Crill and Roehrig, 2001; Hiramatsu et al., 1996; Roehrig et al., 1998; Sukupolvi-Petty et al., 2007). DIII of all mosquito-borne flaviviruses, including DENV, contains a 4-AA extended FG loop on the lateral ridge, whereas this loop is absent in tick-borne flaviviruses. Consequently, the presence of this loop may determine host range and host tropism of mosquito-borne flaviviruses (Erb et al., 2010; Hung et al., 2004; Rey et al., 1995). The role of E protein in cell entry will be emphasized in the next section.

Functions of the NS proteins include RNA replication, polyprotein processing and immune evasion. NS3 and NS5 play important roles in viral replication. NS3 contains helicase and RNA triphosphatase activities (Issur et al., 2009; Natarajan, 2010), while NS5 has highly conserved domains with RNA-dependent RNA polymerase (RdRp) and 5' methyl transferase activities (Issur et al., 2009; Van Slyke et al., 2012). In addition, NS1 and NS4A were found to co-localize at viral replication sites and are required in vRNA replication, yet the roles of these proteins in RNA replication is unclear (Lindenbach et al., 2007). The role in viral polyprotein processing is performed by the serine protease domain located at the amino-terminus of the NS3 protein, which forms a complex with NS2B as a co-factor. This NS3/NS2B serine protease

cleaves the viral polyprotein within C, NS2A, NS3 and NS4A and at the NS2A/NS2B, NS2B/NS3, NS3/NS4A and NS4B/NS5 junctions (reviewed in Falgout et al., 1991; Natarajan, 2010). Lastly, NS2A, NS4A and NS4B play important roles in DENV immune evasion. These proteins, especially NS4B show strong inhibitory effects and act as interferon β (IFN-β) antagonists by interfering with phosphorylation of STAT1 (Munoz-Jordan et al., 2003).

## *Roles of DENV E protein in the early events of cell entry during primary infection*

The first major role of DENV E protein in viral entry is viral attachment to the host cell receptor. Several cellular molecular candidates such as sulfated glycosaminoglycans (GAGs) (Vero and BHK-21)(Chen et al., 1997b; Kato et al., 2010), DC-SIGN (dendritic cells, DC) (Navarro-Sanchez et al., 2003; Pokidysheva et al., 2006; Tassaneetrithep et al., 2003), mannose receptor (macrophages) (Miller et al., 2008), heat shock proteins (HepG2, U937) (Reyes-Del Valle et al., 2005), stress response protein Grp78 (HepG2) (Jindadamrongwech et al., 2004) and other unidentified proteins have been demonstrated to be capable of binding to DENV E protein (reviewed in Hidari and Suzuki, 2011). Negative charge-rich heparan sulfate, an example of GAGs, is believed to bind to positive charge-rich regions on the E protein in a non-specific manner and acts as a co-receptor molecule facilitating viral entry (Chen et al., 1997b; Kato et al., 2010). DC-SIGN is capable of mediating infection by all four DENV serotypes by interacting with the carbohydrate moiety at N67 (Rey, 2003; Tassaneetrithep et al., 2003). Studies of DENV resistant cell lines, Raji and THP-1, demonstrated that these cell lines became susceptible to DENV infection when they ectogenetically expressed DC-SIGN on the surface (Boonnak et al.,

2008; Tassaneetrithep et al., 2003). Furthermore, Ab against DC-SIGN or soluble DC-SIGN can inhibit DENV infection (Navarro-Sanchez et al., 2003; Tassaneetrithep et al., 2003). However, whether or not DC-SIGN functions as a DENV-specific receptor is still under debate. Some studies have demonstrated that the internalization of DC-SIGN was not essential for DENV infection, and the cell surface DC-SIGN does not constitutively internalize into cells (Lozach et al., 2005; Perera and Kuhn, 2008). In summary, a number of studies have demonstrated that DENV can interact with various cell surface molecules. These observations suggested that DENV might utilize multiple receptors or form complexes with various receptors prior to internalization.

Recent studies have demonstrated that DENV, as well as other flaviviruses including JEV and WNV, enters mammalian cells via cell-mediated clathrin-dependent endocytosis (Chu and Ng, 2004; Kalia et al., 2013; Krishnan et al., 2007; van der Schaar et al., 2008). By treating the cells with chlorpromazine, a drug inhibiting formation of clathrin-coated pits, viral infectivity was greatly reduced (Kalia et al., 2013; Krishnan et al., 2007; van der Schaar et al., 2008). Moreover, a study of DENV single-particle tracking showed that DENV moved along cell surfaces in a diffusive manner. The mobility of viral particles was reduced when it colocalized with a clathrin-coated pit, suggesting that DENV might roll over attachment factors until it binds to the entry receptor and is captured by a pre-existing clathrin-coated pit (van der Schaar et al., 2008). After virus binding, the clathrin-coated vesicle pinches off into the cell cytoplasm and delivers the virion into a Rab5-expressing early endosome. Eventually, the early endosome becomes more acidic and gradually matures to a late endosome either by gradually accumulating Rab7 or by fusing with a late endosome expressing Rab7 and losing Rab5. The study by van der Schaar and colleagues showed that the fusion process of DENV2 strain S1 took place in the late

endosome (van der Schaar et al., 2008), while the study by Krishnan et al. demonstrated that DENV2 strain New Guinea C fused with the early endosome (Krishnan et al., 2007). These contradictory results might reflect different characteristics of each strain, which might require different pH thresholds for triggering the endosomal fusion process (Butrapet et al., 2011; Huang et al., 2010; van der Schaar et al., 2008).

The hinge region located between DI and DII was proposed to be responsible for the movement of E protein during conformational reorganization. Previous studies showed that mutations within this region could alter the pH threshold requirement for fusion (Butrapet et al., 2011), and protonation of two conserved histidines (AA 146 and 323) in TBEV has been shown to act as a pH sensor that is critical for triggering E protein conformational change (Fritz et al., 2008). In contrast, mutagenesis of WNV showed that these histidine residues were not required for initiating conformational rearrangement for membrane fusion (Nelson et al., 2009). Once the pH inside the endosome becomes more acidic, the E protein structure undergoes dramatic changes. Dissociation of homodimers into monomers leads to outward projection of DII, exposing the fusion loop on the tip and inserting the hydrophobic side chains of Trp 101 and Phe 108 into the endosomal membrane. Subsequently, three copies of E protein form homotrimers and undergo another conformational change by rotating DIII back against the fusion loop. This E protein rearrangement releases free energy, which subsequently results in formation of a hemifusion intermediate structure, in which the inner leaflet of the endosomal membrane fuses to the viral membrane while the distal leaflet of the membrane remains unfused. Eventually, a fusion pore is formed, and the viral nucleocapsid is released into the cytoplasm for further viral genome replication and progeny viral assembly (Modis et al., 2004).

## *Neutralization and antibody-dependent enhancement of DENV infection*

The major role of the mammalian Ab response to viral infection is neutralization. Neutralizing Ab produced against DENV infection provides lifelong protection against the infecting serotype, but provides only a short period of neutralization capability against heterologous serotypes. Work by Sabin performed during World War II (WWII) demonstrated that naïve human volunteers who were inoculated with DENV1 or DENV2 and challenged with the same serotype were protected from the secondary infection for at least eighteen months. In contrast, the subjects who were challenged with the heterologous serotype were cross-protected for 2-3 months post primary infection, but developed clinical illness and significant viremia after the protective period ended (Sabin, 1952). Veterans who acquired dengue fever-like illness more than 60 years earlier during WWII had serum Ab neutralizing titers of ≥160) against DENV1 (Imrie et al., 2007).

Most MAb studies of neutralization and enhancement of DENV infection have been done with mouse MAbs. The strongest neutralizing MAbs are serotype-specific MAbs recognizing epitopes on DIII of the E protein at the A strand or lateral ridge (reviewed in Wahala and de Silva, 2011). There are two well-accepted mechanisms by which MAb neutralizes DENV in cell culture. One mechanism is that the MAb neutralizes virus by preventing viral attachment to cell receptors, while the other mechanism involves blocking the virus at post-attachment step, presumably during endosomal membrane fusion (Crill and Roehrig, 2001; Kaufmann et al., 2006). There are 180 E monomer potential binding sites on the surface of the virion. However, it is unlikely that MAb will bind to all epitopes, due to steric hindrance and the limited accessibility of some epitopes. For example, 1A1D-2 MAb can only bind to DENV at 37$^{o}$C but

not 4$^{o}$C; thus the accessibility of this epitope depends on the dynamic movement of the E protein (Lok et al., 2008). Neutralization of flaviviruses requires binding of multiple Abs. Pierson and colleagues demonstrated that to effectively neutralize WNV, only about 30 molecules of E16 anti-WNV DIII MAb were required. They proposed that flaviviruses can be neutralized when the number of Ab molecules bound to the virion exceed a certain threshold, which varies depending on the affinity of the Ab and the accessibility of the epitopes (Pierson et al., 2007).

While Ab can neutralize the infection of the homologous DENV serotype during secondary infection, it, unfortunately, may enhance infection by a heterologous DENV serotype. Cross-protection against heterologous serotypes lasts only a short time (Halstead, 1970, 1988). After this period, low-level subneutralizing or non-neutralizing Ab produced from the previous infection cross-react to the newly infecting heterologous serotype and facilitate the infection of mononuclear phagocytic cells via Fc gamma receptors (Fc$\gamma$R) (Halstead, 1970). This phenomenon is called antibody-dependent enhancement (ADE). The consequences of ADE are an increased number of infected cells and virus replication, which results in development of more severe clinical manifestations(Halstead et al., 1970).

The ADE hypothesis was proposed by Halstead in 1970, about 15 years after dengue outbreaks in the Philippines (1954) and Thailand (1959) where large numbers of DHF and DSS patients were recognized for the first time (Kuno, 2007). Halstead and his colleagues found a strong correlation between the severe forms of the disease and the immune status of the patients. Two pathologically similar but immunologically distinct groups of severe dengue patients were observed. The first group was children whose ages were more than one year old who produced Ab against the secondary infection; whereas the second group involved less than one year old infants who produced Ab against the primary infection (Halstead et al., 1970; Kliks et al., 1988).

The development of severe dengue in children more than one year old can be explained by virus enhancement due to pre-existing Ab against a heterologous DENV serotype. It was proposed that enhancement of infection in infants was due to the infants acquiring transplacentally transferred cross-reactive anti-DENV Abs from the mother. The concentration of this anti-DENV Ab remains at a protective level for only few months after birth, then declines below a protective level, which the subneutralizing level of Ab inversely enhances the infection (Halstead, 1970). This hypothesis is supported by the observations that the majority of severe dengue cases in infants occurred in six to eight month-old children, while only few cases occurred in infants younger than three months. Moreover, the actual age of infants who developed severe dengue correlated with the neutralizing titer of anti-DENV Ab in the mothers and the age at which maximum enhancement activity elicited in mononuclear phagocytes was predicted (Halstead et al., 2002; Kliks et al., 1988).

Observations during Cuban dengue outbreaks in 1977 and 1981 further supported the ADE hypothesis. In 1977, there was a major DENV1 outbreak resulting in about 0.5 million reported cases or about 44.5% of the urban population. This population developed mild disease and then recovered from the infection. In 1981, an outbreak of DENV2 occurred in Cuba, resulting in many infections, including 10,000 severe dengue cases. The study of 126 children who were hospitalized revealed that there was no severe dengue case in 1-2 year old children who were born after the period of the DENV1 outbreak and had only been exposed to DENV2. (Halstead, 1988; Kouri et al., 1989).

*In vitro* experiments have been performed supporting the ADE hypothesis. Infection of cultured mononuclear peripheral blood leukocytes with DENV complexed with high concentrations of homologus anti-DENV IgG resulted in viral neutralization; whereas infection

with DENV complexed with the same Ab at lower concentration showed an enhancement effect. Additionally, *in vivo* studies in Rhesus monkeys (Halstead, 1979) and IFN receptor-deficient AG129 mice (Balsitis et al., 2010) showed that animals that were passively administered anti-DENV Ab prior to DENV challenge exhibited higher viremia and more severe disease than the control group.

To date, considerable effort has been invested to better understand the mechanism of ADE and its association with the severe form of the dengue disease. Pathways and mechanisms utilized by ADE to enhance the number of infected cells (extrinsic ADE) and to modulate the innate immune response leading to higher viral production per cell (intrinsic ADE) have been investigated previously, yet many answers remained elusive. It is apparent that all Abs against the E protein have the potential to enhance the viral infection when the Ab concentration is diluted below the neutralizing threshold (subneutralizing level) (Pierson et al., 2007). Anti-prM Ab, which is highly cross-reactive to all DENV serotypes but poorly neutralizing (Dejnirattisai et al., 2010; Rodenhuis-Zybert et al., 2010; Rodenhuis-Zybert et al., 2011), may facilitate cell infection by immature viruses, which are noninfectious in the absence of anti-prM MAb (Rodenhuis-Zybert et al., 2010).

Ab facilitates infection by DENV through binding the Fc portion of IgG subclass Ab to Fcγ receptors (FcγR) displayed on monocytes, macrophages and DC. $F(ab)'_2$ fragment only or IgG containing mutations at FcγR binding site failed to enhance infection of DENV both *in vitro* and *in vivo*, although the ability of the molecules to bind to DENV E protein in ELISA was not different from the intact Ab (Balsitis et al., 2010; Halstead and O'Rourke, 1977). DENV-Ab complexes bind to FcγRIA, and more readily to FcγRIIA (Rodrigo et al., 2006; Rodrigo et al.,

2009b). However, Boonnak et al. demonstrated that the level of FcγRIIA is similar on both mature and immature DC surfaces, but only mature DC, not immature DC, support ADE of infection (Boonnak et al., 2008). Disruption of the cytoplasmic tail of FcγRI or FcγRIIA was found to abolish ADE infection (Rodrigo et al., 2006).

Another mechanism of ADE, intrinsic ADE, was recently described to modulate the innate immune response in infected cells (Chareonsirisuthigul et al., 2007). Upon infection with DENV-Ab complex, the immune response is altered by down-regulating toll-like receptor (TLR) expression, nitric oxide (NO) production, and type I IFN expression to facilitate viral replication in host cells. These activities suppress the induction of an antiviral state, resulting in prolonged survival of infected cells and higher level of total viral production (Chareonsirisuthigul et al., 2007; Ubol et al., 2010)

Factors and mechanisms influencing the development of severe dengue remain to be studied. Host gender, age, genetics, as well as DENV strain seem to be involved in the development of severe dengue (Halstead, 1970; Halstead et al., 2002; Stephens et al., 2002). No satisfactory explanation indicates why DHF/DSS also occurs in patients suffering primary DENV infection (Halstead et al., 2002). Lastly, cell-mediated immune responses may also play roles in developing severe dengue disease (reviewed in Murphy and Whitehead, 2011)

ADE is a major challenge for dengue vaccine development. A tetravalent vaccine against all four serotypes of DENV is needed to induce long-term protective immunity against all serotypes. Otherwise, vaccine recipients might be at risk of severe dengue following infection with wild-type DENV, if the neutralizing titer against one or more serotypes decreases over time (reviewed in Whitehead et al., 2007).

## *Aims of the present study*

Detailed knowledge of ADE of DENV infection remains to be explored, including the early events of the entry pathway of the DENV-Ab complex upon binding to the FcγR. Two entry models were proposed: (1) the Ab opsonized DENV may directly be internalized by the cells by the phagocytic pathway through binding with FcγR; (2) the FcγR may only play an indirect role in concentrating DENV-Ab complexes to the cell surface, but other cellular receptors are required for successful complex internalization (Figure 1.2). In previous mutagenesis studies, we have identified critical molecular determinants of DENV2 E protein for the early events of non-ADE virus entry in non-FcγR cells (Butrapet et al., 2011; Erb et al., 2010; Huang et al., 2010; Roehrig et al., 2013). In this study, we used some of these DENV2 E mutants to further understand the roles of these molecular determinants in both ADE and non-ADE of DENV infection in the FcγR-bearing cells. Through identification of the DENV E protein determinants for both types of infection, we can elucidate the virus-Ab complex entry pathway.

The study included three specific aims: (1) to establish methods for inducing and detecting ADE of infection; (2) to identify critical determinants on DENV2 E protein in viral-cell attachment and virus-mediated endosomal membrane fusion in both ADE and non-ADE infections using a panel of previously engineered DENV2 mutants; (3) to further investigate the involvement of intrinsic and extrinsic ADE mechanisms. We compared the ADE infectivities of DENV2 between K526 cells (a natural FcγRIIA- expressing cell line) and CV-1-FcγRIIA (an ectogenetically FcγRIIA-transfected cell line). Understanding of the viral molecular

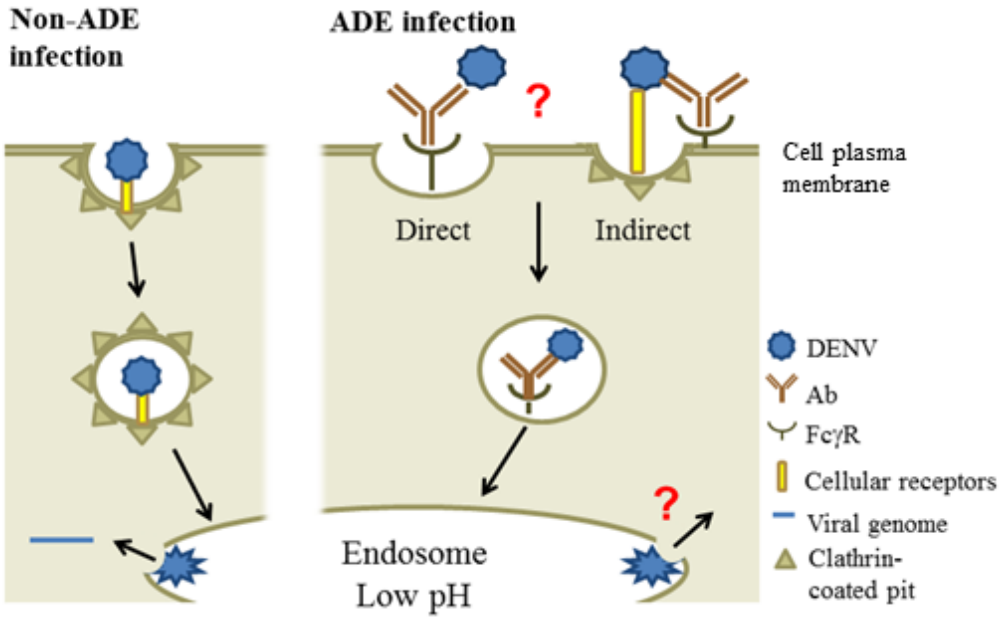


Figure 1.2 The early events of DENV infection pathway: regular (non-ADE) infection and ADE infection through FcγR.

determinants and cellular components involved in early events of ADE of infection will greatly facilitate rational designs of anti-DENV therapeutics and vaccines against the more severe form of dengue disease associated with ADE.

# CHAPTER 2

# MATERIALS AND METHODS

## *Cell Cultures*

The human myelogenous leukemia cell line, K562 (ATCC, CCL-243), was cultured in Iscove's Modified Dulbecco's Medium (IMDM). Fc gamma receptor IIA (FcγRIIA or CD32) - transfected Grivet monkey kidney CV-1 cells (CV-1- FcγRIIA) (Rodrigo et al., 2009) were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 50 mg/mL Hygromycin B (Roach). Both cell lines were cultured in medium supplemented with 10% fetal bovine serum (FBS) and 10 units/mL Penicillin and 10 µg/mL Streptomycin (Life Technologies), and were incubated at $37^{o}$C with 5% $CO_2$.

## *Viruses*

Viruses used in this study were recombinant viruses derived from infectious cDNA clones based on pD2IC-30P-NBX, a modified clone of pD2IC-30P-A that was engineered with the genome cDNA of DENV2 strain 16681(Huang et al., 2010; Kinney et al., 1997). Site-directed mutagenesis of the WT cDNA clone permitted engineering of the putative receptor binding mutants VEPGΔ (Erb et al., 2010) and KKK305/307/310EEE (Roehrig et al., 2013), and the fusion-defective temperature-sensitive mutants G104S (Huang et al., 2010) and L135G

(Butrapet et al., 2011). Due to instability of all the mutants in Vero cells, all viruses including WT virus were derived from C6/36 cells and passaged once prior to infecting K562 cells.

## *Antibodies*

Mouse monoclonal antibodies (MAbs), 4G2 (IgG2a anti-flavivirus E protein), 1A1D-2 (IgG2a anti-DENV-1, 2, and 3 E), 3H5 (IgG1 anti-DENV-2 E), and 2H2 (IgG2a anti-DENV prM) used in this study were available at CDC, DVBD, Fort Collins, CO. The 2H2 was fluorescently labeled with Alexa-Flour 488 using a kit (Invitrogen) according to manufacturer's protocol. Briefly, the MAb 2H2 (adjusted to pH 8.3 with sodium bicarbonate) was incubated with Alexa Fluor 488 reactive dye for 1 h at room temperature. The labeled 2H2-AlexaFluor-488 Ab was purified from unlabeled Ab and unincorporated dye by using size exclusion column chromatography and was eluted in PBS. The labeled 2H2-AlexaFluor-488 was used in cytometry analysis to stain *de novo* prM proteins synthesized within infected cells.

## *ADE assay*

The *in vitro* ADE assay was conducted by infecting FcγR-bearing cells with virus-Ab complex. The WT DENV2 30P-NBX or mutant viruses at MOI of 0.01 to 1 (indicated in each experiment) were incubated with MAb to form DENV-Ab complexes at 37$^{o}$C for 1 h in total volume of 125 µL prior to adsorbing to the cells.

To prepare cells for infection, 2-3 day-old K562 cells were washed twice with PBS by centrifugation at 220 ×g for 5 min with gentle deceleration. After virus and enhancing Ab were allowed to form the complex for 1 h, the complex was mixed with a cell suspension at final cell concentration of $6\times10^6$ cells/mL in 250 µL in a microcentrifuge tube. This mixture was incubated at 37$^o$C and shaken every 20 min for 2 h. Following the adsorption period, the infected cells were washed twice with IMDM medium without FBS and centrifuged at 130×g for 5 min to remove excess Ab and viral particles. After washing, the infected cells were resuspended in 3 mL of IMDM supplemented with 5% FBS and incubated in 5% $CO_2$ incubator at 37$^o$C for 2-5 days depending on MOI. For temperature-sensitive mutants, the pre-incubation of virus and Ab and cell infection were conducted at both 28 and 37°C.

The method for ADE infection in attached CV-1- FcγRIIA cells was slightly different from the method used to infect suspensions of K562 cells. Briefly, the infection was performed in two to three-day-old 90-100% confluent cells cultured in 6-well plates. Cells were washed twice with PBS and inoculated with 100 µL of virus-Ab complex. The adsorption inoculum was incubated at 37$^o$C for 2 h, and the plates were shaken every 10-15 min to prevent drying of the cell sheets. After adsorption, cells were washed twice by FBS-free DMEM before culturing in 4 mL of DMEM supplemented with 5% FBS and 50 mg/mL Hygromycin B.

Culture medium and infected cells were harvested on the day of peak enhancement, which was 2-4 days post infection (pi) depending on the MOI of each experiment. Culture media containing released viral particles were collected, supplemented with FBS to final concentration of 20%, and stored at -70°C until further viral RNA (vRNA) quantification by quantitative RT-PCR (qRT-PCR). Infected cells were harvested (see below) and subjected to flow cytometry or cellometer analysis to measure the percentage of cells infected with DENV2.

The ADE infection was compared side-by-side with the infection by virus without Ab (non-ADE infection). ADE and non-ADE infections of DENV2 30P-NBX were included as WT virus controls. Mock infection was included as a negative control for each experiment and used for standardizing experiments.

## *Analysis of DENV infection by flow cytometry or Cellometer*

To harvest K562 cells, the cells were washed twice with PBS and centrifuged at 130 ×g for 5 min. The infected CV-1-FcγRIIA cells were harvested by trypsinization to detach cells from the plates. The trypsinized cells were dispersed by pipetting up and down several times, and then washed twice with PBS and centrifuged at 240 ×g for 5 min.

After washing, cells were fixed with 250 μL of BD fixation/permeabilization solution (BD Biosciences) at 4$^{o}$C and protected from light for 20 min. Cells were then washed with BD perm/wash buffer (BD Biosciences) twice to remove the fixative by spinning at 240 ×g for 5 min. The fixed cells were first incubated with 20 μL of 1:20 human FcR binding inhibitor (eBioscience) at 4$^{o}$C for 20 min to prevent binding between Fc portions of the staining Ab (2H2) to the FcR on the cell surface, and then were stained with 2H2-AlexaFluor-488 at a final volume of 50 μL for 1 h at 37$^{o}$C. The stained cells were protected from light until results were acquired. After incubation and washing with BD perm/wash buffer, the cells were resuspended with 0.5-1.5 mL of BD perm/wash buffer and were subjected to FACSCalibur flow cytometry (BD Biosciences) or Cellometer (Nexcelom Bioscience) analysis.

For the FACSCalibur analysis, fluorescence emitted from the stained cells was read in fluorescence intensity channel 1 (FL-1), and at least 20,000 events were acquired for each sample and reported in a dot plot between FL-1 and size scatter (SSC). The percent of cells infected with DENV2 was analyzed by Cell Quest Pro software (BD Biosciences). The readout of the mock infection sample was used to set proper gating parameters for each experiment, so that the cell auto-fluorescent background was below 1% in each experiment. All the percent infection results were further normalized by subtracting the background percent of auto-fluorescence (based on mock infection) from the percent of positive cells in each sample.

To measure infected cells by Cellometer, 20 μL of the cells (at a concentration of 2-7x$10^6$ cells/mL) stained with 2H2-AlexaFluor-488 were loaded into the Cellometer cell counting chambers. Images of cells were captured in both bright field and fluorescence field by Cellometer Vision CBA software (Nexcelom Bioscience), and the percent of infected cells was analyzed by FCS Express 4 Flow Research Edition software (Nexcelom Bioscience). Similar to the analysis by FACSCalibur, the fluorescence data of each cell type was plotted between fluorescence intensity (x-axis) and cell size (y-axis). The gate indicating positive population was set to exclude the auto-fluorescence from mock infection. Percent of cells located within the gate was reported as percent DENV2 infection.

## *Quantification of viral RNA*

Total intracellular RNA was extracted from infected cells using TRIzol® reagent (Invitrogen) by following the manufacturer's protocol, whereas extracellular vRNA was extracted from supernatant using the QIAamp Viral RNA kit (Qiagen). Viral genomic

equivalents were quantified by one-step qRT-PCR with Quantitect virus kit (Qiagen) previously described using the CFX 96 Touch$^{TM}$ Real-Time PCR Detection System (BioRad) (Butrapet et al., 2011; Roehrig et al., 2013). Briefly, 5 μL of extracted RNA was mixed with 10 μM universal DENV primers complementary to 3' NCR (forward: DEN-10578 – AAGGACTAGAGGTTAGAGGAGACCC, reverse: cDEN-10687-GGCGTTCTGTGCTGGAATGATG), 5 μM of probe (D24-10616P FAM probe – AACAGCATATTGACGCTGGGAAAGACC), 5 μL of 5x QIAGEN virus NR/master mix buffer and 0.25μL of QIAGEN Quantitect Virus RT mix (QIAGEN) in a final 25 μl reaction. Quantitative RT-PCR cycling was performed as follows: 1 step of 50$^{o}$C, 20 min and 95$^{o}$C, 5 min followed by 50 cycles of 95$^{o}$C, 15 sec and 57$^{o}$C, 1 min 15 sec. Standard RNA was *in vitro* transcribed from plasmid containing the 3' NCR region, including primer and probe binding sites. Serial dilutions of RNA standard were used to generate a standard curve for quantification of RNA samples. Each qRT-PCR sample was tested in 2-3 replicates, and the average number of vRNA copies per mL was reported.

## *Determination of FcγRIIA on cell surface by FACS*

The percent of cells bearing FcγRIIA (CD32) and the intensity of FcγRIIA expression on the K562 and CV-1- FcγRIIA cell surfaces were compared. Uninfected cells were washed and fixed using the same method as described above. After fixation, cells were washed twice with staining buffer (PBS with 1% FBS) and were separated into 2 tubes. Cells in the first tube were stained with mouse anti-human CD32 conjugated with phycoerythrin (PE) (anti-CD32-PE Ab, eBioscience) for FcγRIIA staining, while the cells in the second tube were stained with mouse

anti-IgG1 isotype control-PE Ab (eBioscience) for non-specific binding control. Fluorescence was detected in the FL-2 channel of the FACS machine.

The fluorescence intensities from stained FcγRIIA on K562 and CV-1-FcγRIIA cell surfaces were compared in histograms. Distribution of fluorescence signal detected in the IgG isotype control-PE-staining sample was set to the left border of the histogram yielding geometric mean fluorescence intensity (GMFI) of about 6.5 by adjusting voltage gain parameter. This setup parameter was used to acquire anti-CD32-PE staining samples. The results were reported by overlaying the anti-CD32-PE staining histogram onto the IgG isotype control-PE-staining histogram of the same cell type. The average GMFI±SD of anti-CD32-PE stained samples were calculated from 3 separate experiments. The percent of cells expressing FcγRIIA (or CD32) was calculated by subtracting the percent of anti-IgG isotype control-PE-staining background from the percent of anti-CD32-PE for each cell type. The final percentage was the average of 3 separate experiments.

## *Statistics*

The Student t-test was used to compare percent of DENV2 infected cells between infection with virus only and infection with virus-Ab complex. Significant differences were accepted at $p<0.05$ and were calculated from single experiments with triplicate samples or multiple experiments as specified in the figure legends.

# CHAPTER 3

# RESULTS

## *Optimization of in vitro ADE assay*

### A. Selection of MAb for ADE assay

To study the ADE phenomenon *in vitro*, we established an assay to induce and detect ADE of infection in K562 cells with DENV2-Ab complex. To select a MAb and determine the proper enhancing Ab amount for inducing ADE infection by DENV2, serial dilutions of 4 different MAbs were tested. The vRNA output from infected cells was analyzed by qRT-PCR to determine peak ADE viral titers after treatment with each DENV2-Ab complex. Figure 3.1A shows results from 4-fold serial dilutions of the anti-flavivirus MAb, 4G2 (total protein concentration of unpurified mouse ascitic fluid = 67.4 mg/ml). Both intracellular and extracellular vRNA levels were higher (enhancement) at 1:1,000-1:4,000 dilutions of 4G2 than the vRNA levels of the non-ADE infection control, and both levels were abrogated (virus neutralization) at a 1:100 dilution of 4G2. Dilutions of 4G2 equal to or greater than 1:16,000 resulted in vRNA levels that were similar to those of the non-ADE infection control. We further refined the screening for the enhancement criteria for 4G2 by testing 2-fold serial dilutions of the Ab with virus input at MOI of 1 and 0.2. Surprisingly, the enhancement of infection using either MOI resulted in similar titration curves, with enhancement of vRNA output occurring at 1:2,000-1:4,000 dilutions of the 4G2 (Figure 3.1B). In addition, we tested 3 other MAbs, 2H2, 3H5, and

1A1D-2 in the ADE assays (Figure 3.1C and D). The extracellular vRNA results showed that 2H2 lacked virus neutralization ability and had minimal enhancement capacity at the titers tested. The DENV2-E-DIII-specific 3H5 and DENV subcomplex-E-specific 1A1D-2 MAbs exhibited high peaks of ADE at 1:1,000 dilution and 1:10,000 dilution, respectively. Because MAbs 3H5 and 1A1D-2 do not bind to some of the mutant viruses used in this study (Erb, 2011; Gromowski and Barrett, 2007; Sukupolvi-Petty et al., 2007) and enhancement by 2H2 was minimal, we chose to use the 1:4,000 dilution (about 16.9 µg/ml of protein from mouse ascitic fluid) of MAb 4G2 for the ADE assay in the study.

**B. Detection of ADE and non-ADE of DENV2 infection by FACS and Cellometer**

To measure infected cells numbers, we evaluated 2 detection systems, FACS and Cellometer. Both systems were optimized to analyze the percentage of WT DENV2-infected cells based on fluorescence staining of nascent intracellular prM proteins with 2H2-AlexaFluor-488 conjugate. Although both systems can be successfully used for detecting ADE and non-ADE of DENV2-infection of K562 cells (Figure 3.2 A and B), we found that the FACS was usually more sensitive and accurate than the Cellometer system. The small benchtop Cellometer is a fluorescent image-based system useful for small sample size (up to 6,000 cells are captured and analyzed per counting chamber of 20 µl) assay, while the FACS can analyze more than 20,000 cells (events) for each sample. Since our sample size was appropriate for FACS detection, we chose to use FACS system for the following study.

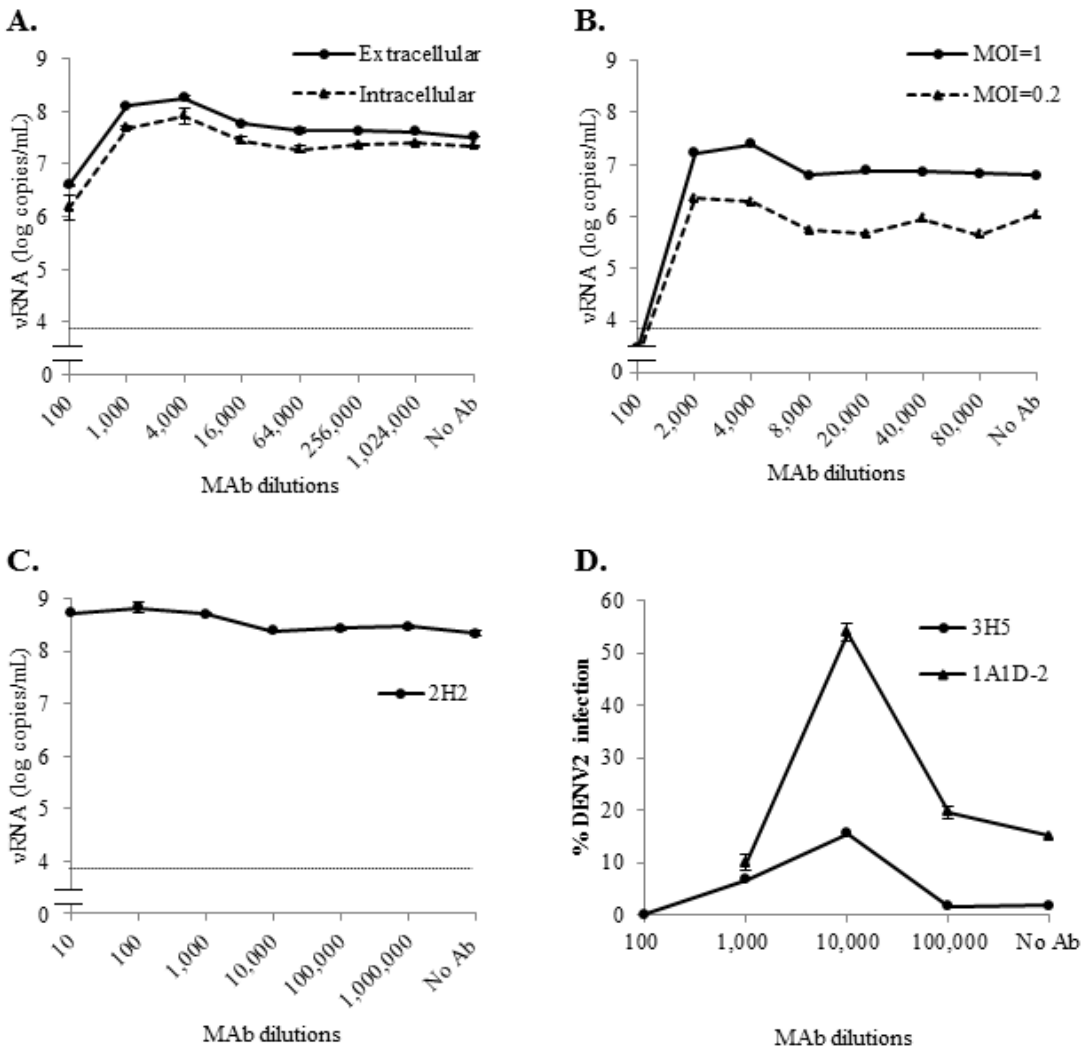


Figure 3.1 Titrations of MAbs for ADE of infection in K562 cells. Quantification of RNA outputs by qRT-PCR from cells infected with (A) WT DENV2 (MOI=1) pre-incubated with 4-fold serial dilutions of 4G2, (B) WT DENV2 (MOI=1 or 0.2) with 2-fold serial dilutions of 4G2 (extracellular vRNA) and (C) WT DENV2 (MOI=1) with 10-fold serial dilutions of 2H2. Dash lines show detection limit of qRT-PCR. (D) FACS analysis of cells infected with WT DENV2 (MOI=0.5) with 3H5 or 1A1D-2.

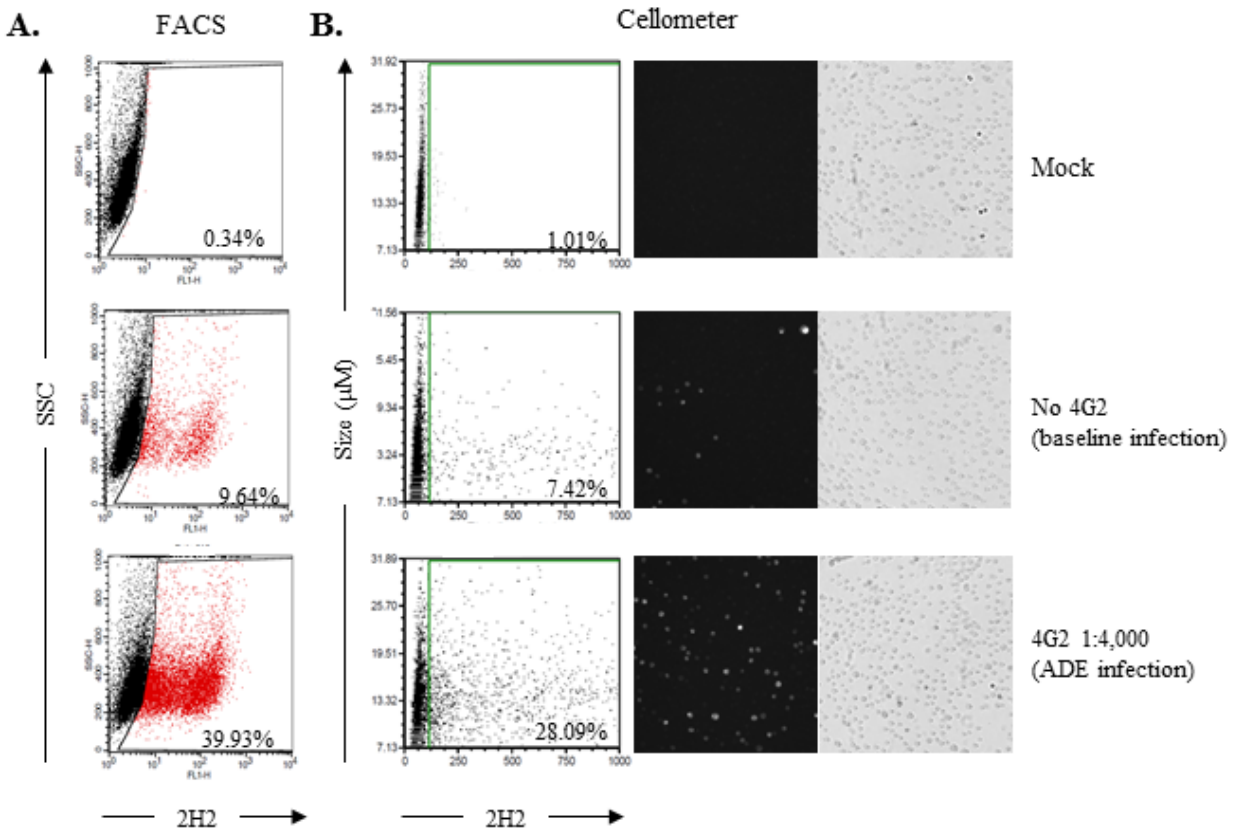


Figure 3.2 Detection of ADE by fluorescence based methods. WT DENV2 (MOI=0.5) infected K562 cells were harvested and stained with 2H2-AlexaFluor-488 on day 2 pi. (A) Dot plot analysis from FACS and (B) dot plot analysis and cell images from Cellometer (left: green fluorescence; right: bright field). Numbers indicate percentage of infected cells (MOI=0.5)

## C. Optimization and verification of the ADE assay

On day 2 pi, K562 cells infected with 4G2-opsonized WT DENV2 (MOI=0.5) showed significantly higher percentage (39.39%) of infected cells than non-ADE infection control (9.64%) (Figure 3.2A). Experiments with various MOI showed that 4G2-opsonized WT DENV2 usually had 1.2 to 4-fold increased infection rates compared to non-opsonized WT DENV2, and these differences were statistically significant.

Although the enhancing titers of 4G2 were similar in experiments with MOI of 1 and 0.2 (Figure 3.1B), the day of highest enhancement effect (peak enhancement day) was delayed in the experiment with lower MOI (Figure 3.3A). The peak enhancement days of infection using the WT virus at MOI of 0.5, 0.1 and 0.01 were day 2, 3 and 4 pi, respectively, and enhancement became undistinguishable from the non-ADE infection control quickly after the peak day. As a result, we harvested cells on the peak enhancement days based on the MOI of the WT DENV2 in each experiment. To compensate for the slower replication characteristics of the DENV2 E mutant viruses, we also extended the harvest days to ensure measurements of potentially delayed enhancement kinetics of these mutants.

To confirm that the enhanced infection was Ab-dependent, we tested a 4G2-epitope knockout mutant, DENV2 G106L, in the ADE assay. The G106L mutant was previously engineered with the mutation of G to L at residue 106 of the E protein fusion peptide region, and although the mutant is still fusion competent and infectious, it lost the capacity to bind to the MAb 4G2 (Huang et al., 2010). Unlike the 4G2-opsonized WT virus showing enhancement effect on day 2 and 3 pi, preincubation of the G106L with 4G2 did not result in enhancement of cell infection (Figure 3.3B) between 2-5 days pi, confirming that the enhanced infection measured in the experiment was through the binding of 4G2 to DENV2.

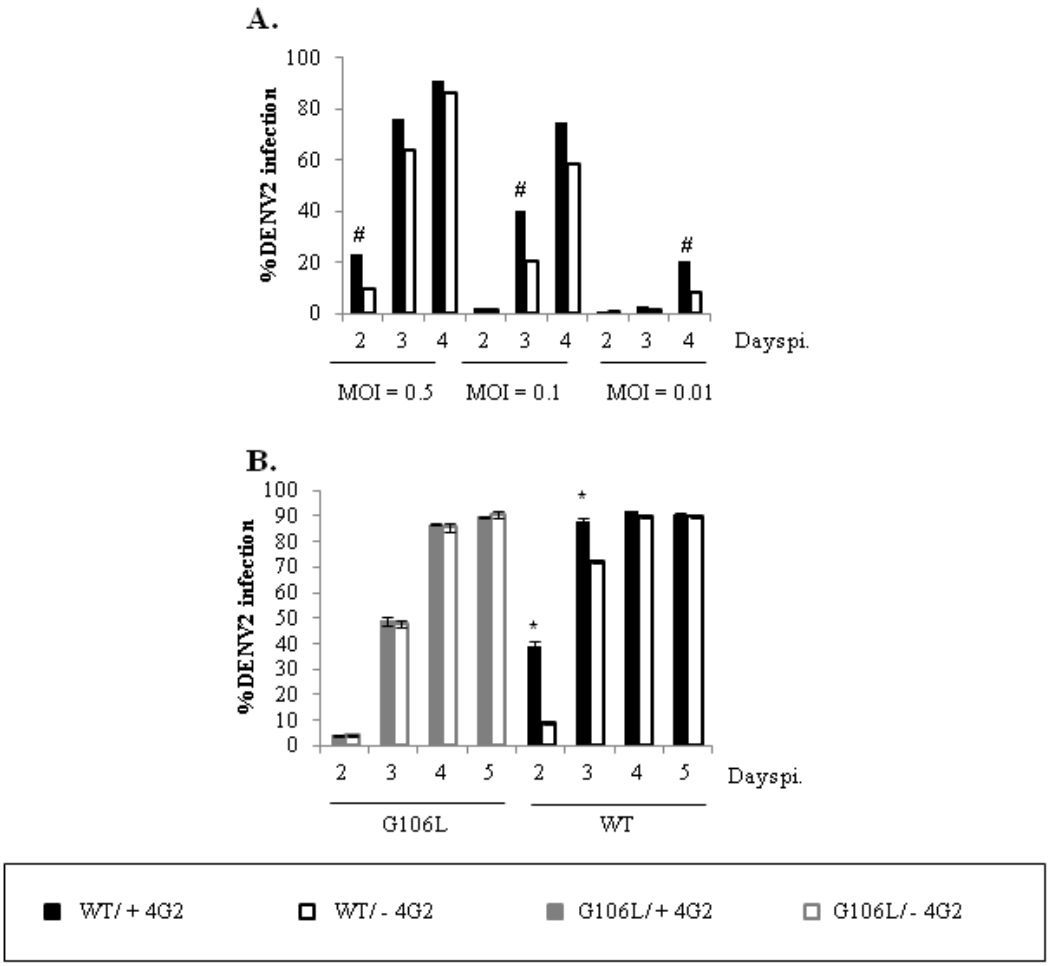


Figure 3.3 Optimization and verification of ADE assay. (A) Percentage of K562 cells infected with WT DENV2 at different MOI with 4G2 (solid bars) or without 4G2 (open bars). The day pi showing the highest enhancement of each MOI was marked with #. (B) DENV2 WT virus (black) and G106L mutant virus (gray) were used as positive and negative ADE controls for ADE infection, respectively. Significant differences (*, $p$ <0.05) between infection with and without 4G2 were calculated by Student's t-test. The results in both figures were detected by FACS.

## *Molecular determinants of DENV2 E protein involved in early events of ADE infection in K562 cells*

Previously we engineered a panel of DENV2 mutants to study important functional domains of the viral E protein in the early events of non-ADE virus infection in mammalian and mosquito cells (Butrapet et al., 2011; Erb et al., 2010; Huang et al., 2010; Roehrig et al., 2013). In this report, we chose 4 of these mutant viruses for studying the early events in ADE of DENV2 infection. These mutants bear mutations at critical motifs involved in receptor binding (VEPGΔ and KKK305/307/310EEE mutants) or virus-mediated membrane fusion (G104S and L135G mutants) during non-ADE of DENV2 infection (Figure 3.4).

### A. Putative receptor binding mutants

#### *(1) DENV2 VEPGΔ mutant: Deletion of DIII FG loop*

The FG loop is an extended loop between strands F and G of the dengue E protein. The loop is composed of 4 AA (382VEPG385, for DENV2) located on the lateral ridge of the E DIII. The structure of this loop is conserved among mosquito-borne flaviviruses, but absent in tick-borne flaviviruses (Rey et al., 1995). Although the loop was thought to be involved in binding to mosquito C6/36 cells (Hung et al., 2004), previous work in our lab has shown that this loop is dispensable in DENV2 infection of C6/36 cells (Erb et al., 2010). However, deletion of the FG loop resulted in significantly lower viral replication efficiency in Vero cells and in mosquito midguts (Erb et al., 2010).

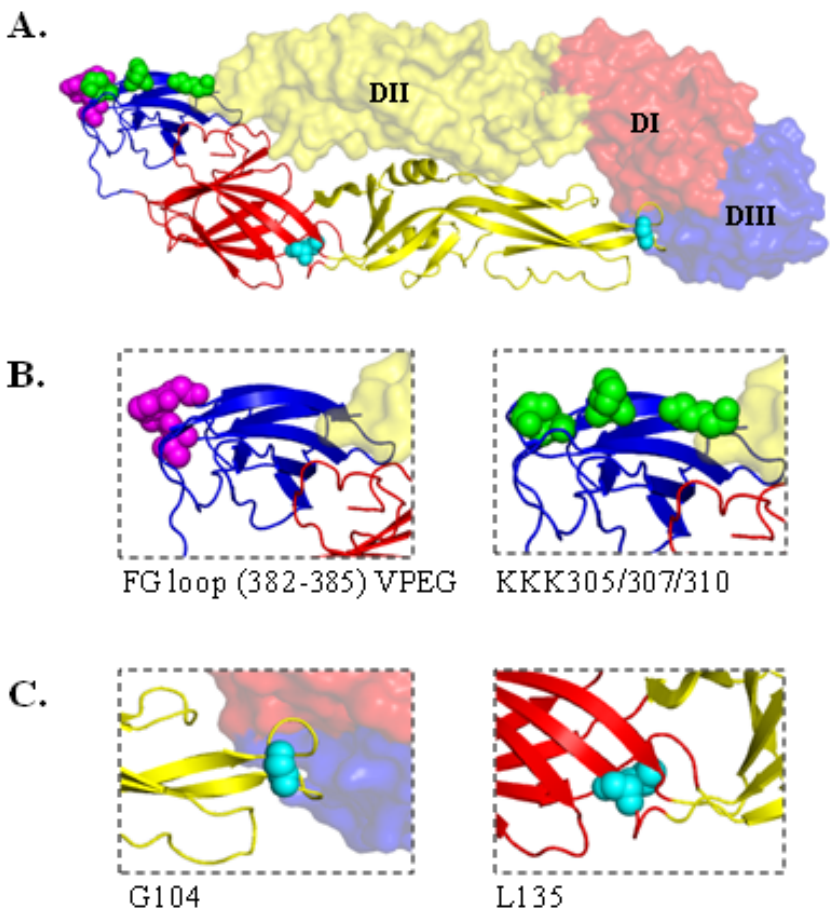


Figure 3.4 Location of mutations introduced in DENV2 E protein. (A) Top-view of E homodimer [PDB ID: 1OAN] on mature virion was acquired from Protein Data Bank (PBD) and rendered by Polyview-3D (Porollo and Meller, 2007). E protein domains I, II and III are shown in red, yellow and blue, respectively. The glycosyl chains are not shown. Locations of mutated AA residues were highlighted on WT DEN2 E protein (A) as well as shown in the zoom-in images (B and C). (B) Target residues for putative receptor binding mutants, FG loop (382-385 VEPG) is shown in magenta and KKK305/307/310, is shown in green. (C) Target residues for fusion defective mutants, G104S and L135G, are shown in cyan.

In this study, we infected K562 cells with the FG loop deletion mutant, VEPGΔ, at 0.5 MOI with or without enhancing 4G2, and compared this mutant side-by-side with the infection by WT virus under the same conditions (Figure 3.5A). The results showed that without enhancing 4G2, the percentage of VEPGΔ-infected cells was similar to the WT virus-infected

cells on day 2 pi, but the mutant showed a somewhat lower infection rate than the WT virus on day 3 pi (Figure 3.5A). This result was consistent with the previous observation that deletion of the FG loop impaired the mutant virus replication efficiency in mammalian cells (Erb et al., 2010). When VEPGΔ was pre-opsonized with enhancing 4G2, a significant increase of infected cells compared to the non-4G2 infections was observed on both days 2 and 3. These ADE results were confirmed by measuring vRNA output released into culture medium on day 3 pi (Figure 3.5B). Although day 3 pi was beyond the optimal time point to measure the ADE of WT virus infection by qRT-PCR, we were able to detect the enhancement of vRNA output in cells infected by 4G2-opsonized VEPGΔ on day 3 pi due to its slower viral replication kinetics. Together, these results indicated that the deletion of the FG loop did not impair enhancement of DENV2 infection in K562 cells.

### *(2) KKK305/307/310EEE mutant at DIII*

The lysine cluster at residues 305, 307, and 310 on a lateral ridge of E-DIII is proposed to be important for heparan sulfate binding (Chen et al., 1997b; Hung et al., 2004). They are also part of the MAb- binding epitope for several neutralizing MAbs, including 3H5 and 1A1D-2 (Gromowski and Barrett, 2007; Hiramatsu et al., 1996). Because these MAbs have been shown to block attachment of virus to Vero cells (Crill and Roehrig, 2001), it is speculated that this positivly-charged motif is important for binding to cell receptors. Previous study has shown the KKK305/307/310EEE triple mutation is lethal for virus infection in Vero cells, but the mutant can infect C6/36 cells (Roehrig et al., 2013). However, the infectivity of the mutant in C6/36 cells was significantly less than that of the WT DENV2.

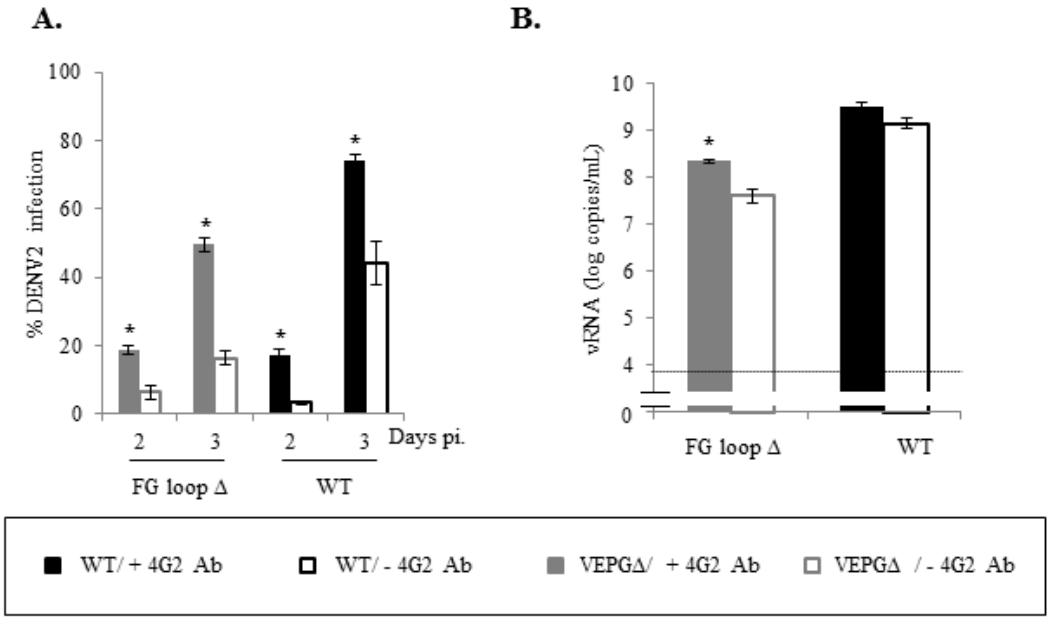


Figure 3.5 Comparison of VEPGΔ and WT infection with and without enhancing 4G2 in K562 cells. (A) Percentage of infected cells, measured by FACS, of VEPGΔ mutant with (gray) or without (white) 4G2 (1:4000) were compared with the WT with (black) or without 4G2 (white) at MOI=0.5. (B) vRNA output of VEPGΔ and WT infection with or without 4G2 on day 3 pi. Dash lines show detection limit of qRT-PCR. Significant differences (*, $p<0.05$) between infection with and without 4G2 were calculated by Student's t-test.

In this study, we conducted ADE and non-ADE infection by the mutant in K562 cells to investigate whether binding of the 4G2-opsonized virus to FcγR will rescue the infectivity of the triple mutant in mammalian cells. Without 4G2, the extracellular vRNA level of the mutant virus produced from K562 cells did not increase between day 0 and day 3 pi, suggesting that the mutant is also lethal in K562 cells in non-ADE infection (Figure 3.6A). FACS results of the ADE assay at MOI of 0.01 on day 3-5 pi also showed that the 4G2-opsonization failed to rescue infectivity of the mutant (Figure 3.6B). The extracellular vRNA level on day 4 pi showed a small amount of viral RNA in the culture medium, which was likely from the vRNA introduced by the mutant virus inoculum itself. The amount of mutant vRNA was about 3,000 and 150-fold less

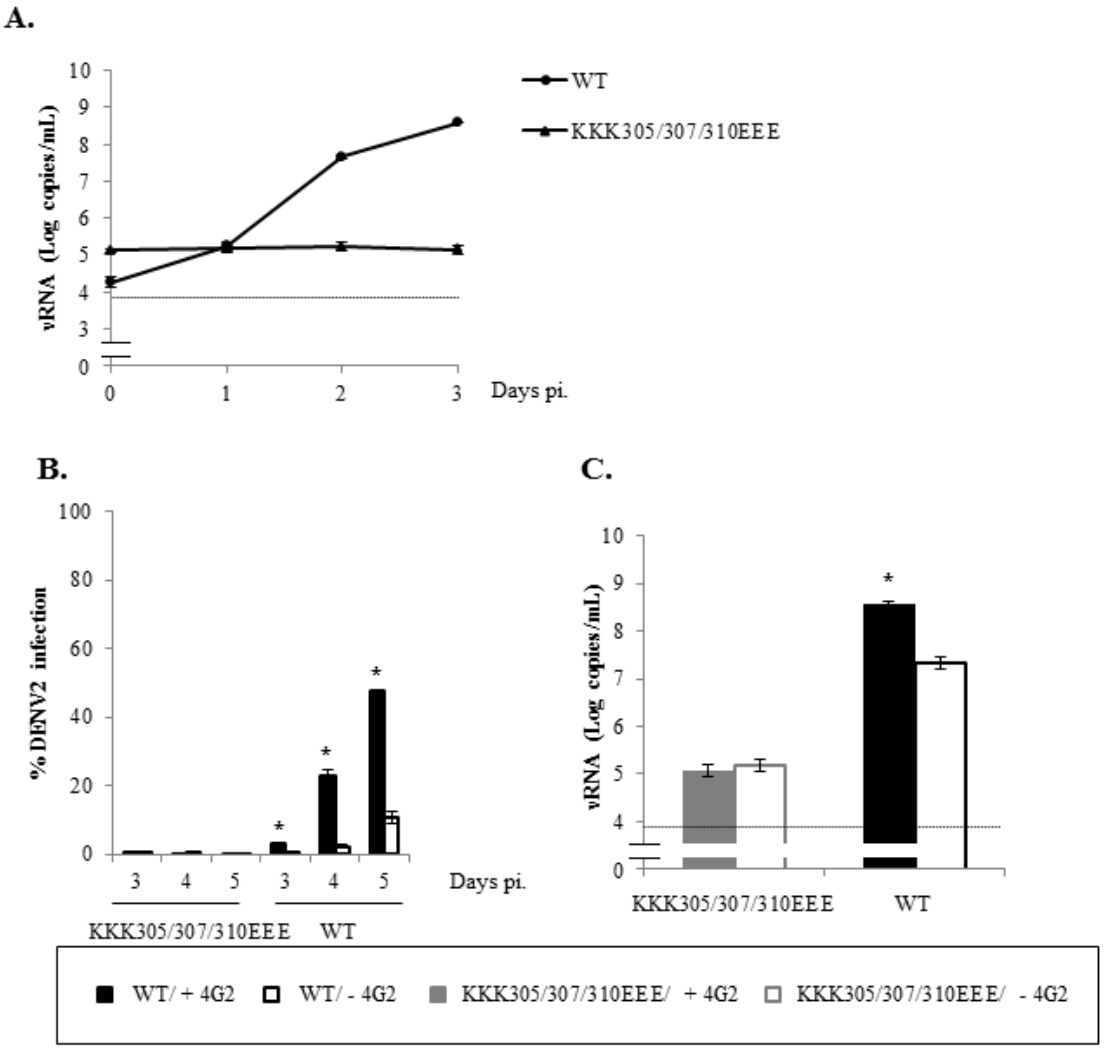


Figure 3.6 Comparison of KKK305/307/310EEE and WT infection with and without enhancing 4G2 in K562 cells. (A) DENV2 vRNA in culture medium produced from infected K562 cells (at MOI=0.03) without 4G2 and quantified by qRT-PCR at day 0-3 pi. (B) and (C) Cells were infected by virus at MOI=0.01 with or without 4G2 (1:4,000). (B) Percentage of infected cells measured by FACS. (C) Comparison of vRNA harvested from culture medium on day 4 pi. Dash-lines show detection limit of qRT-PCR. Significant differences (*, $p<0.05$) between infections with and without 4G2 were calculated by Student's t-test.

than the WT vRNA in infections with and without 4G2, respectively (Figure 3.6C). These results suggested that binding of the virus-4G2 complex to FcγR on K562 cells was not sufficient to overcome the possible attachment and/or internalization deficiency of the mutant virus.

**B. Virus-mediated endosomal membrane fusion**

### *(1) G104S mutant in DII fusion peptide (cd loop)*

The cd loop (AA 98-111) of E DII is the fusion peptide that mediates virus-cell membrane fusion following virus entry through endocytosis. Substitution of G104 with S (G104S) resulted in fusion deficiency and minimal virus replication in Vero cells at 37$^{o}$C. However, its infectivity was recovered, although peak infectious titers were lower than WT, when the infected Vero cells was cultured at 28$^{o}$C (Huang et al., 2010).

In this study, we observed that the G104S mutant also exhibited the temperature sensitive characteristic in K562 cells. At 37$^{o}$C, percentages of G104S infected cells remained very low for at least 3 days pi in both ADE and non-ADE infections, while the percentage of WT DENV2-infected cells increased dramatically (Figure 3.7A). At 28°C, G104S replicated as well as WT DENV2, and the 4G2-opsonized G104S exhibited ADE infection similar to the opsonized WT virus (Figure 3.7B). Interestingly, ADE of G104S infection when K562 cells were cultured at 28$^{o}$C was not observed if the pre-incubation of G104S with 4G2 was conducted at 37$^{o}$C (Figure 3.7C), suggesting that G104S failed to form a virus-Ab complex with 4G2 at 37$^{o}$C.

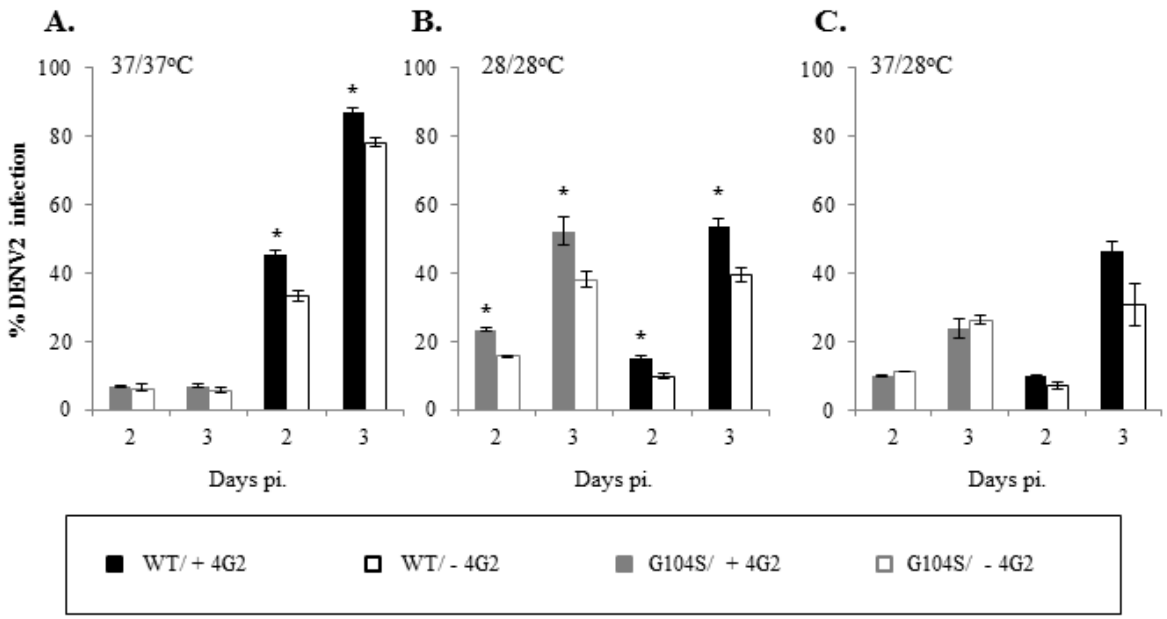


Figure 3.7 ADE of temperature-sensitive G104S (MOI=0.5) infection in K562 cells (A) to (C) Percentage of positive cells in G104S or WT infection with or without enhancing 4G2 (1:4,000) at different incubation temperatures: virus-Ab complex formation/virus-Ab complex infection. Significant differences (*, $p<0.05$) between infection with and without 4G2 complex were calculated by Student's t-test

### *(2) L135G mutant in molecular hinge region*

Four peptide strands, H1-H4 (Hurrelbrink and McMinn, 2001; McMinn et al., 1995) located between DI and DII of E protein function as a molecular hinge that allows E protein to undergo conformational rearrangement required for viral-endosomal membrane fusion and proper virion assembly in cells (Modis et al., 2004). The L135 located within the H2 strand is conserved among all DENV while isoleucine (I) at this position is conserved among other flaviviruses (Butrapet et al., 2011). The L135G mutant was previously demonstrated to be temperature sensitive at 37$^{o}$C, yet replication competent at 28$^{o}$C in Vero cells (Butrapet et al., 2011).

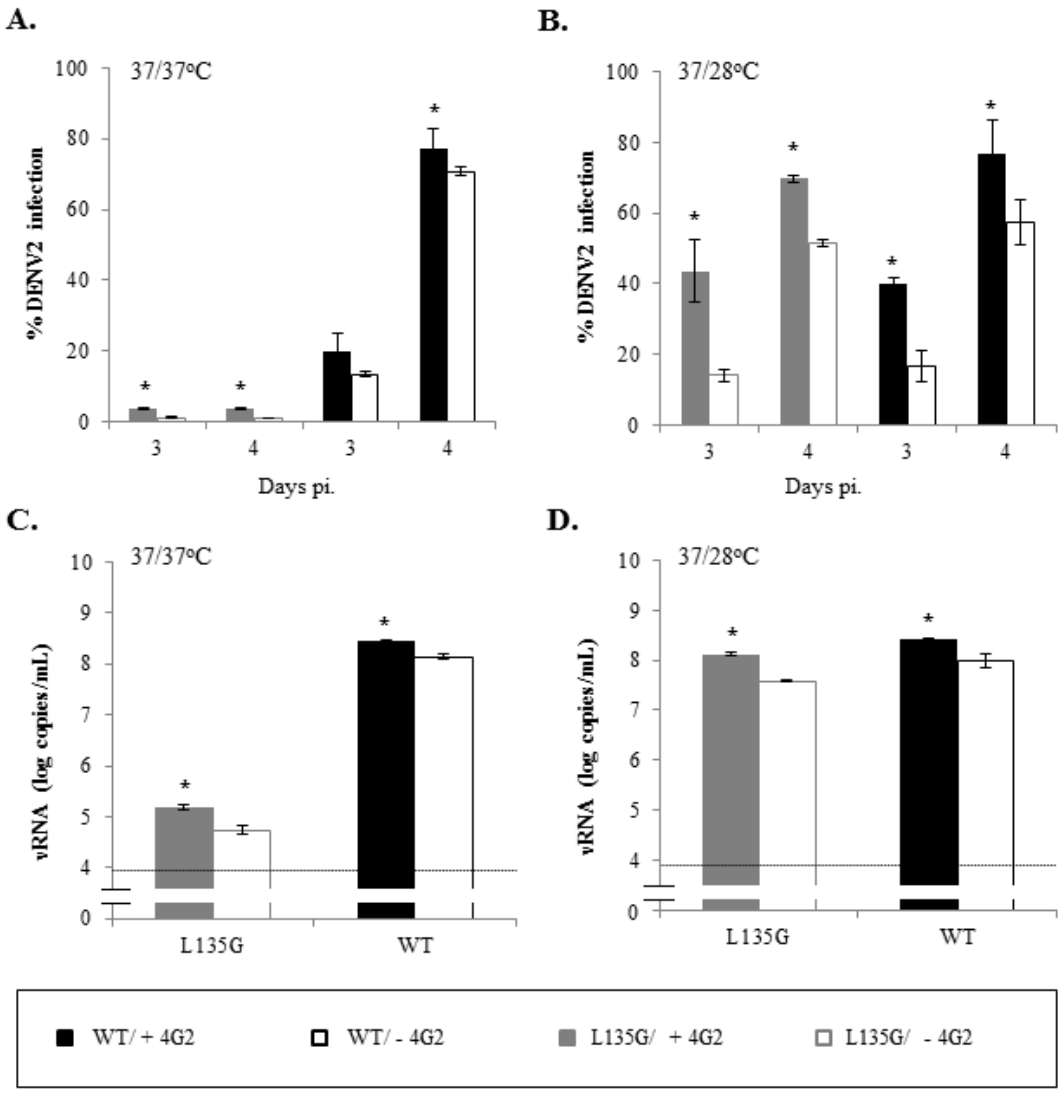


Figure 3.8 ADE of temperature-sensitive L135G infection (MOI=0.1) in K562 cells. (A) and (B) show percent of cells infected with L135G or WT at different temperatures: virus-Ab complex formation/virus-Ab infection. (C) and (D) show qRT-PCR determination of vRNA output in culture medium harvested on day 3 pi of cells infected with L135G or WT with and without 4G2. Dash lines show detection limit of qRT-PCR. Student's t-test was performed to calculate significant differences (*,$p<0.05$) between infection with and without 4G2.

As expected, we confirmed that L135G did not replicate in K562 cells at 37$^{o}$C, but infected K562 cells well at 28$^{o}$C (Figure 3.8 A and B). In addition, the 4G2-opsonized L135G demonstrated a clear ADE effect when cultured at this permissive temperature (Figure 3.8B).

Quantification by extracellular vRNA showed similar outcomes as the FACS analysis (Figure 3.8C and D). Although a minor ADE effect was also observed in 4G2-opsonized L135G at 37$^{o}$C based on statistical analysis between the non-ADE and ADE infections, we considered that as background artifact since the percentages of positive cells were all very low and without any progression from day 2 to day 3 pi in either type of infection (Figure 3.8A). According to these results, viral-mediated endosomal membrane fusion is required in ADE of infection.

## *ADE is not observed in CV-1 cells expressing FcγRIIA*

To further investigate whether FcγRIIA alone suffices for induction of ADE, we performed ADE infection of DENV2 WT in a stable FcγRIIA-transfected CV-1 cell line (CV-1-FcγRIIA) (Rodrigo et al., 2009). Despite multiple efforts and testing various MAbs, we were not able to detect any ADE of WT DENV2 infection in this cell line. Instead, virus neutralization was demonstrated at all dilutions of the MAbs (Figure 3.9). We observed that the percentage of CV-1- FcγRIIA cells infected with WT without Ab at 2 days pi was less than 2.5% (Figure 3.9), which was less than the proportion we typically observed with WT virus-infected K562. To further confirm this observation, we conducted 3 separate experiments, with 2-3 replicates in each experiment, to directly compare the percentage of K562 and CV-1-FcγRIIA cells infected with WT DENV2 at MOI of 0.5 without enhancing Ab on day 2 pi. The results revealed that the percentage of WT DENV2-infected K562 cells (7.76±0.97%) was significantly higher (Student t-test, $p<0.05$) than that of the infected CV-1- FcγRIIA cells (3.66±1.07%). This result indicates that CV-1-FcγRIIA are less susceptible to DENV2 infection than K562 cells

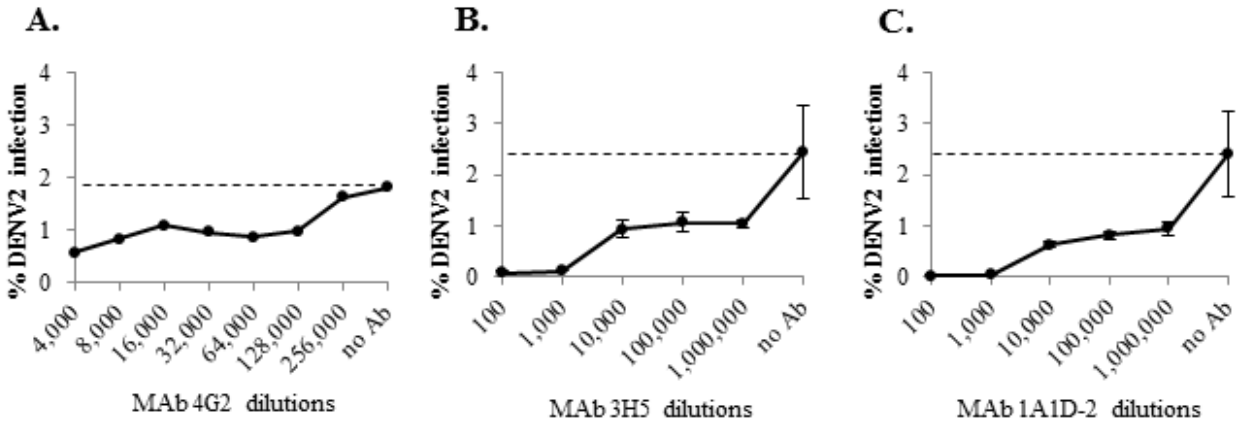


Figure 3.9 WT virus-Ab infection of CV-1-FcγRIIA cells with different MAb. (A) DENV2 WT at MOI of 1 was incubated with 4G2, (B) WT at MOI=0.5 was incubated with 3H5 and (C) WT at MOI=0.5 was incubated with 1A1D-2 at dilutions shown prior to infection. Cells were harvested on day 2 pi and the percentage of infected cells was measured by FACS.

To investigate whether variations in the level of FcγRIIA expression might contribute to the differences in ADE of DENV2 infection between K562 and CV-1-FcγRIIA cells, we measured by FACS the FcγRIIA on the cell surface of each cell type after anti-CD32-PE staining. The overlay histogram of negative control samples (stained with IgG1 isotype-PE) and FcγRIIA stained samples revealed a wide range of FcγRIIA staining intensity on K562 cell surfaces (Figure 3.10A). The histogram of CV-1-FcγRIIA staining exhibited a bimodal distribution, likely representing the FcγRIIA positive and negative populations (Figure 3.10B). The geometric mean fluorescence intensity (GMFI) of FcγRIIA and the percentage of cells expressing FcγRIIA were compared. The results from 3 separate experiments showed that both K562 cells and CV-1-FcγRIIA cells contained a similar percentage of cells expressing FcγRIIA,

60.26±7.22% and 66.32±8.06% respectively. The GMFI of FcγRIIA correlates to the amount of FcγRIIA expressed on the cell surface, and our results revealed that K562 cells expressed

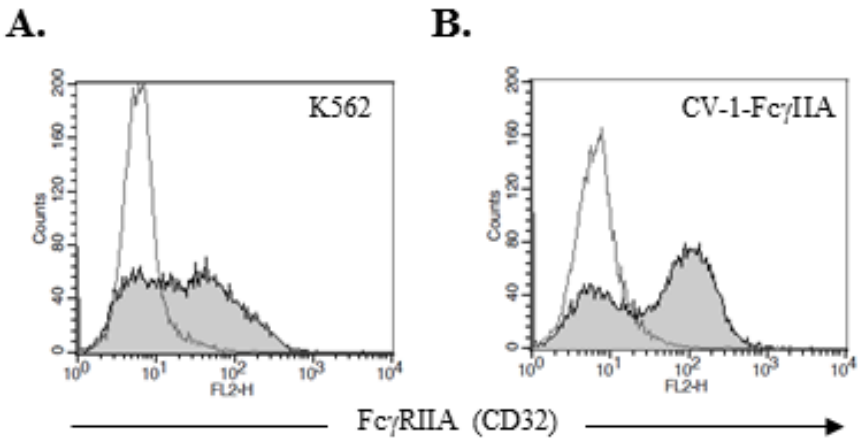


Figure 3.10 FcγRIIA (CD32) expression on the cell surface. FcγRIIA on (A) K562 and (B) CV-1-FcγRIIA cells were stained with anti-CD32-PE (shaded). Cells stained with anti-IgG1-PE (unshaded) were used as negative control. GMFI of FcγRIIA staining cells ± standard deviation and percent of cells expressing FcγRIIA were averaged from 3 separate experiments.

significantly ($p$<0.05) lower FcγRIIA (55.57±11.14) than that of CV-1-FcγRIIA cells (86.39±14.90). This observation suggested that the lack of ADE of DENV-2 infection in CV-1-FcγRIIA cells was not due to insufficient expression of FcγRIIA on the cell surface, and expressing of abundant FcγRIIA alone was not sufficient for ADE of infection.

# CHAPTER 4

# DISCUSSION

It has been shown that DENV enters cells mainly via receptor-mediated, clathrin-dependent endocytosis (Chu and Ng, 2004; Mosso et al., 2008; Suksanpaisan et al., 2009; van der Schaar et al., 2008). Viral attachment and virus-mediated endosomal membrane fusion are the two critical early events in the DENV entry pathway. Previous studies of mutations in the DENV E protein have identified several functional motifs involved in these early events of non-ADE infection (Butrapet et al., 2011; Erb et al., 2010; Huang et al., 2010; Roehrig et al., 2013). In this study, for the first time to our knowledge, we have identified motifs (molecular determinants) on the DENV2 E protein that are critical for virus entry during ADE infection with virus-Ab complexes. Through identification of such molecular determinants, we propose that ADE infection via FcγRIIA using similar viral entry pathway as that of the non-ADE infection.

Methods for inducing and detecting ADE of infection were optimized in a cell line well-known for studying ADE of infection, K562 (Balsitis et al., 2010; Boonnak et al., 2008; Huang et al., 2006; Nicholson et al., 2011; Rodenhuis-Zybert et al., 2010). This cell line constitutively expresses FcγRIIA (CD32), which plays a major role in ADE of DENV infection (Rodrigo et al., 2006). The flavivirus group cross-reactive MAb 4G2 has been previously shown to induce ADE of DENV2 infection (Balsitis et al., 2010; Boonnak et al., 2008) and was used successfully to establish the ADE assay in this lab. The MAb 4G2 used in the study was unpurified mouse ascitic fluid, and we determined that at a dilution of 1:4,000 it can induce significant ADE effect

(Figure 3.1). To validate that the enhancement of viral infection in our ADE assay was due to successful virus-Ab complex formation, we used the G106L mutant that cannot bind 4G2 (Huang et al., 2010) and observed no ADE of infection.

To minimize the chance of acquiring additional mutations, all viruses including WT were only passaged once in C6/36 cells after they were derived from transfection of the recombinant viral genome into C6/36 cells. Full genome sequencing, which verified the expected mutations, was performed previously for all the virus seeds used for the study. Due to engineered mutations, infectious titers of some mutant virus seeds were much lower than that of WT virus. Therefore, some experiments were conducted at a lower MOI for both mutant samples and WT controls. Even though the peak enhancement days point were established for WT DENV, we usually extended our analysis to 1 or 2 more days after the WT peak day to compensate for the delayed growth of the mutant viruses.

Because various MOI were used for different experiments in the study, it was necessary to determine the proper ratio between Ab and viruses for optimal ADE infection. The binding of Ab with flaviviruses can result in virus neutralization, virus enhancement, or no effect depending on the number of Ab molecules binding to the virus. Engagement of the Ab must exceed a particular threshold on the virion in order to neutralize the virus infectivity, and the minimum requirement for ADE has been shown to be approximately half the number required for neutralization (Pierson et al., 2007). Therefore, we expected that lower MOI of virus would require greater dilutions of Ab to maintain the proper ratio between virus and Ab for ADE. Moreover, it is well known that flaviviruses prepared from cell cultures contain large amounts of non-infectious viral particles, and the ratio between infectious and non-infectious particles can be widely different between viruses and even among different seed preparations of a given virus.

Consequently, available epitopes presented on the non-infectious particles can compete with infectious particles in Ab binding, which may result in inconsistent outcomes. Surprisingly, we found that the same amount of 4G2 can induce ADE of WT virus infection at various MOI inputs. These results suggested that the ratio between virion and 4G2 resulting in enhancement is widely flexible.

The ADE phenomenon was reported to resulting in higher numbers of target cells being infected, and possibly leads to higher virus production (reviewed in Flipse et al., 2013; Guzman and Vazquez, 2010). We used two methods to detect ADE of infection, FACS to determine numbers of infected cells and qRT-PCR to quantitate virus load. After infection, only early time points of these ADE assays can be used to measure the enhancement outcome, because at later times new rounds of nascent virus replication without enhancing 4G2 in the culture will mask the early enhanced cell infection rate (Figure 3.2).

Pierson and colleagues analyzed the enhancement capacity of more than 100 MAbs to WNV and DENV and showed that all Abs that were able to bind and neutralize viral infection had the potential to induce the enhancement of infection (Pierson et al., 2007). Anti-prM Ab was hypothesized to be a major Ab contributing to ADE of infection. Anti-prM Ab is usually weakly involved in virus neutralization, but has high potential to promote enhancement and is highly cross-reactive to multiple DENV serotypes (Dejnirattisai et al., 2010). Recent studies demonstrated that immune sera from patients with secondary DENV infection contained significantly more anti-prM Abs than the sera from the patients with primary infection (Lai et al., 2008), and secondary infection immune sera contained more anti-prM (60%) than anti-E Ab (40%) (Dejnirattisai et al., 2010). Rodenhuis-Zybert et al. showed that immature virions, which were non-infectious, became highly infectious when incubated with anti-prM 70-21 Ab prior to

infecting K562 cells (Rodenhuis-Zybert et al., 2010). Other studies also demonstrated successful ADE infection using anti-prM Ab opsonized to partially mature virus particles (da Silva Voorham et al., 2012; Dejnirattisai et al., 2010). It was reported that DENV produced from C6/36 cells, such as the WT DENV seed used in our study, may have high concentrations of partially mature virions containing prM (Junjhon et al., 2008; Zybert et al., 2008). When we pre-incubated WT DENV2 with the anti-prM MAb 2H2, we observed only subtle enhancement of infection and no neutralization at the levels tested. It is possible that the MAb 2H2 we obtained was already at subneutralizing titer, or the DENV2 used in this study did not contain sufficient prM for binding of MAb 2H2 to reach its neutralization threshold. As a result, we did not observe the typical Ab titration curve showing both neutralization and enhancing titers. DENV2 serotype-specific 3H5 MAb and DENV subcomplex-specific 1A1D-2 MAb (Crill and Roehrig, 2001; Sukupolvi-Petty et al., 2007) showed strong enhancement capacity. Our enhancement result with 3H5 agreed with those reported by Littaua et al (Littaua et al., 1990) but contradicted the observations of Boonnak et al. (Boonnak et al., 2008). The latter report did not detect enhancement using 3H5 in K562 cells, likely due to insufficient dilution of the 3H5 MAb used in that particular study.

To investigate whether the same molecular determinants in the DENV2 E protein are essential for both non-ADE and ADE infection, we investigated 4 previously characterized DENV2 E mutants with mutations targeting the putative receptor binding sites, VEPGΔ and KKK305/307/310EEE), or targeting important domains for membrane fusion (G106L and L135G). Due to its physical location on the accessible surface of the virion, its reacting to several strong neutralizing Abs, including the strongly neutralizing 3H5 MAb, and the fact that it is present only in mosquito-borne flaviviruses, the FG loop on D-III of the E protein has long been

proposed to be the major receptor binding domain for mosquito cells (Gromowski and Barrett, 2007; Hiramatsu et al., 1996; Hung et al., 2004). A previous study demonstrated that a peptide (AA380-389) containing some AA sequence as that of the FG loop can block the binding of soluble DENV2 E-DIII to C6/36 cells (Hung et al., 2004), suggesting the FG loop is critical for binding of the virus to C6/36 cells. However, using intact, infectious VEPGΔ mutant virus, we demonstrated that the FG loop is dispensable for infection of C6/36 cells (Erb et al., 2010). Nevertheless, we also found that deletion of the FG loop resulted in diminished midgut infection in orally infected *Ae. aegypti* mosquitoes, as well as low viral dissemination rates to head tissue following intrathoracic inoculation of these mosquitoes (Erb et al., 2010). In addition, we showed that deletion of the FG loop reduced viral replication efficiency and genetic stability in Vero cells, but it appeared that the mutant virus was capable of attaching to and entering these cells (Erb., 2010). It is still unclear whether the FG loop is directly involved in binding to cellular receptors in mosquito tissues, but our results suggested that this loop is not critical for attachment and internalization of DENV in Vero or C6/36 cells. In the current study, we further determined that the FG loop was also not required for either ADE or non-ADE infection in K562 cells.

The second DENV2 mutant targeting a putative receptor-binding domain on the E-DIII that was included in this study was the KKK305/307/310EEE mutant with triple AA changes from basic K to acidic E at AA 305, 307 and 310. Previous study of this mutant in our lab showed that the triple mutations are lethal in mammalian cells, including Vero, HepG2, and K562 cells (Roehrig et al., 2013; and CY-H Huang personal communication). In the current study, we confirmed that the mutant cannot grow in K562 cells. The mutant was found to be fusion competent by the fusion from within assay using C6/36 cells (Huang et al., 2010), but it did not show evidence of negative-strand vRNA replication in Vero cells (Roehrig et al., 2013).

In this study, we could not detect nascent prM in mutant-infected K562 cells by FACS at 3-5 days pi. These results suggested that blockage of the mutant virus infection in mammalian cells occurred before viral polyprotein translation and processing. Because this mutant also lost reactivity with several MAbs that have been shown to block attachment of DENV2 to Vero cells, it is likely that the triple K motif is critical for attachment of DENV2 to Vero cells.

Since FcγRIIA on K562 cells can bind the virus-Ab complex during ADE of virus infection, we reasoned that KKK305/307/301EEE opsonized with enhancing 4G2 could attach to K562 cells via FcγRIIA binding, thereby becoming infectious. However, our results showed that 4G2-opsonized mutant under ADE conditions still failed to overcome the non-susceptibility of K562 cells to infection by the mutant, indicating that binding of the virus-Ab complex to FcγRIIA on the cells was not sufficient to bypass the viral entry defect caused by the mutation in this virus (Figure 3.6B). Another DENV entry mechanism is via DC-SIGN on the cell surface, and the glycan at N67 of the E protein has been shown to be the moiety involved in DC-SIGN-mediated attachment. Interestingly, study of the KKK305/307/310EEE mutant in Raji cells expressing DC-SIGN on the cell surface showed that the glycan at N67 of the mutant E was intact, yet its attachment to DC-SIGN was also not sufficient to overcome the infectivity defect of the mutant in Raji-DC-SIGN cells (Roehrig et al., 2013). These results suggest that another, undefined cellular receptor or step engaging with this triple K motif is required for virus-Ab complex infection of FcγRIIA-bearing K562 cells, and for DENV2 infection of Raji-DC-SIGN.

The ability of DENV to escape the endosome and release its nucleocapsid into the cytoplasm for replication prior to lysosome degradation is a critical early event during virus entry via clathrin-mediated endosytosis. During ADE of DENV infection, virus-Ab complex binding to FcγR on the cell surface may permit the virus to enter cells directly through phagocytosis.

Because both clathrin-mediated endosomes (utilized by DENV non-ADE infection) and phagosomes (posible entry pathway through FcR in ADE infection) become acidified and fused with the lysosome (reviewed in Flannagan et al., 2012; Smit et al., 2011), we assumed that virus-mediated membrane fusion is still required to release the viral nucleocapsid into the cytoplasm after the virus-Ab complex is internalized in cells through either FcγR-triggered phagocytosis or other types of endocytotic pathways. Therefore, we expected that infectivity of mutant viruses with a fusion defect would not be rescued by the ADE. The mutants G104S and L135G are both fusion-defective at 37ºC and fusion-competent at 28ºC. This temperature-sensitive property allows us to directly compare the same mutant under both fusion defective and competent conditions in the ADE assay. Although both mutants were fusion defective, the mutation present in each virus was targeted to affect different steps in the fusion process. As expected, results from both mutants demonstrated that competent fusion is critical for infectivity of the mutants under both ADE and non-ADE conditions. We also determined that the epitope for MAb 4G2 on G104S was not stable at 37ºC, because ADE was only observed when opsonization of the virus with 4G2 was conducted at 28ºC. In addition, the temperature sensitivity of G104S was reversible, since pre-incubation of G104S at 37ºC did not block subsequent viral replication at 28ºC (Figure 3.7C). Replication of G104S was blocked only when infected cells were cultured at 37ºC (Figure 3.7A). On the other hand, we observed the enhancement of L135G infection when we conducted the temperature shifting involving pre-incubation of the virus with 4G2 at 37$^{o}$C and then shifting to 28$^{o}$C during cell infection (Figure 3.8B).

CV-1-FcγRIIA cells express FcγRIIA on the cell surface, but they may lack the FcγR-related signaling pathways that trigger the innate immune response and contribute to the intrinsic ADE of DENV. Certain signaling cascades following the ligation between DENV-Ab complex

and FcγRIIA in myeloid cells were demonstrated to be involved in the intrinsic ADE mechanism (Chareonsirisuthigul et al., 2007; Ubol et al., 2010). Using CV-1-FcγRIIA cells and the E mutants, we sought to further dissect the effect of FcγRIIA in the extrinsic ADE mechanism of DENV2 infection. However, enhancement of WT DENV2 was not observed in this cell line with any of the MAbs we tested. Further investigation of the FcγRIIA molecules expressed on CV-1-FcγRIIA and K562 cells indicated that the percentage of cells expressing FcγRIIA molecules was similar in both cell types, and yet CV-1- FcγRIIA cells expressed higher FcγRIIA level on the surface.We also found that CV-1-FcγRIIA cells were less susceptible than K562 cells to WT DENV2 infection under non-ADE conditions, which suggested that CV-1 cells may possess significantly fewer primary DENV2 receptors than the K562 cells. Based on ADE and non-ADE results of the DENV mutants in K562 cells, it was clear that molecular determinants of the DENV E protein critical in the non-ADE virus entry pathway were also critical in the virus entry pathway for ADE. Binding of FcγRIIA with virus-Ab complex alone was not sufficient for virus entry during ADE of infection. FcγR may only play an auxiliary role in concentrating DENV to the cell surface and other undefined primary DENV2 receptors may be required for effective virus internalization. This may explain the reason that CV-1-FcγRIIA cells with fewer primary DENV2 receptor than K526 cells did not exhibit detectable ADE of infection in our assay. However, lack of intrinsic ADE components in the FcγRIIA-transfected CV-1 cells could also be the reason that we could not detect ADE in this cell line.

The DENV entry pathway in non-ADE infection has been actively studied, and many functional domains on the E protein of DENV have been identified. However, knowledge regarding ADE of DENV infection is still very limited, and it is unclear whether vaccines or

anti-viral therapeutics against non-ADE of DENV infection can also be effective against ADE infection. Our results revealed that the viral components on the E protein essential for virus entry during non-ADE of DENV2 infection are also critical for ADE of infection. They also suggested that both types of infection might utilize the same or similar entry pathways regardless of the presence of Ab. Therefore, prevention measures against DENV entry and membrane fusion steps could be potentially effective for both types of infection. More studies are required for in-depth understanding of DENV entry through ADE of infection. Mutagenesis studies using intact recombinant DENV are critical to the understanding of the molecular mechanisms involved in virus replication. However, limitations occur when engineered mutations are lethal or result in crippled viral replication. We have successfully obtained mutants that replicate in mosquito C6/36 cells, yet exhibit significant replication defects in mammalian cells. As shown in this study, engineered temperature-sensitive mutants can be highly informative. Future studies using faster-growing DENV E mutants engineered in a more powerful chimeric WNV replicative backbone made in this lab (unpublished data) may overcome many experimental limitations resulting from the low-yield mutants such as KKK305/307/310EEE mutant.

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