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ABSTRACT

Our study of different human monoclonal antibodies showed that compared with group reactive anti-fusion loop monoclonal antibodies, type specific anti-domain III monoclonal antibodies have higher epitope accessibility and binding avidity to mature virus-like particles (VLPs), which presumably representing mature dengue virus (DENV) virions, the major infectious component in mixed DENV particles in culture. These findings not only suggest that type specific antibodies such as anti-domain III antibodies, though present as a small proportion of anti-envelope antibodies in polyclonal human serum, may contribute greatly to neutralizing activity, but also highlight the importance of inducing this type specific anti-domain III antibodies by using mature particles for vaccine strategy. Using DNA vaccines to test our hypothesis, we showed that DNA vaccine expressing mature VLPs could induce similar anti-DENV antibodies, neutralizing antibodies, less anti-fusion loop antibodies, no or little anti-precursor membrane antibodies compared with DNA vaccine expressing mixed VLPs. Future studies are needed to verify that mature DENV particles are better vaccine candidates than mixed DENV particles.
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ABBREVIATIONS

Abs  Antibodies
ADE  Antibody dependent enhancement
C    Capsid
CR   Complex-reactive
DALYs Disability-adjusted life years
DC   Dendritic cells
DENV Dengue virus
DF   Dengue Fever
DHF  Dengue hemorrhagic fever
DNA  Deoxyribonucleic acid
DSS  Dengue shock syndrome
E    Envelope protein
ER   Endoplasmic reticulum
FL   Fusion loop
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>GR</td>
<td>Group-reactive</td>
</tr>
<tr>
<td>JEV</td>
<td>Japanese encephalitis virus</td>
</tr>
<tr>
<td>Kb</td>
<td>Kilo-base</td>
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<tr>
<td>M</td>
<td>Membrane</td>
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<td>MVEV</td>
<td>Murray Valley encephalitis virus</td>
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<tr>
<td>NCR</td>
<td>Noncoding region</td>
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<tr>
<td>NS</td>
<td>Non-structural</td>
</tr>
<tr>
<td>NT</td>
<td>Neutralizing</td>
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<tr>
<td>ORF</td>
<td>Open reading frame</td>
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<tr>
<td>prM</td>
<td>precursor membrane</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>SLEV</td>
<td>St. Louis encephalitis virus</td>
</tr>
<tr>
<td>TGN</td>
<td>Trans-Golgi network</td>
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<tr>
<td>TS</td>
<td>Type-specific</td>
</tr>
<tr>
<td>VLPs</td>
<td>Virus-like particles</td>
</tr>
<tr>
<td>WB</td>
<td>Western blot</td>
</tr>
<tr>
<td>WNV</td>
<td>West Nile virus</td>
</tr>
<tr>
<td>YFV</td>
<td>Yellow fever virus</td>
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CHAPTER 1

INTRODUCTION
Flaviviruses

Flaviviruses, belonging to the genus Flavivirus in the family Flaviviridae can be transmitted by hematophagous arthropods (Gubler et al., 2007). Among the flaviviruses, there are several members that can cause extensive morbidity and mortality throughout the world and have become threats to public health (Guzman et al., 2010). Japanese Encephalitis Virus (JEV), West Nile Virus (WNV), Murray Valley Encephalitis Virus (MVEV), and St. Louis Encephalitis Virus (SLEV) are common encephalitic flaviviruses, whereas Yellow Fever Virus (YFV) and Dengue Virus (DENV) can cause hemorrhagic fever (Gubler et al., 2007).

Dengue virus

DENV is the most important mosquito-transmitted viral diseases in humans that can cause a wide spectrum of disease (Guzman et al., 2010; Murphy and Whitehead, 2011). Due to the geographic spread of its vector, global warming, and rapid globalization allowing people to travel half way around the world in less than 24 hours, dengue has been classified as an emerging infectious disease (Guzman et al., 2010; Murphy and Whitehead, 2011). DENV consists of four serotypes, DENV1, DENV2, DENV3, and DENV4; all four DENV serotypes can cause full spectrum of disease. The amino acid sequence homology among the four serotypes ranges from 63% to 77%. Homology among different viral strains of serotype DENV2 has been shown to exceed
90% (Lindenbach et al., 2007). Co-circulation of multiple DENV serotypes has been reported in endemic countries (Gubler et al., 2007).

**Genome**

DENV is a single-stranded, positive-sense RNA virus. The genome, approximately 10.6 kb in length, contains a single open reading frame (ORF) that is flanked by 5’ and 3’ noncoding regions (NCR) and encodes a polyprotein precursor (Lindenbach et al., 2007). The polyprotein is cleaved by viral and cellular proteases into three structural proteins, the capsid (C) protein, precursor membrane (prM) protein, and envelope (E) protein and seven non-structural (NS) proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (Fig. 1).

**Replication cycle**

Several cell surface receptors have been characterized to interact with DENV particles upon viral entry. DC-SIGN is a well-known C-type lectin that mediates DC infection by DENV and other flaviviruses. Following receptor mediated internalization, DENV is trafficked to the endosomal compartment where the low pH induces the conformational changes of E protein and allows the fusion between virus and the host cell membrane; the viral genome is released into the cell cytoplasm (Fig. 2A) (Lindenbach et al., 2007; Perera et al., 2008). A large polyprotein is produced as a result of viral translation, and it is cleaved into three structural proteins and seven NS proteins. The virus then
undergoes RNA replication at the endoplasmic reticulum (ER). Virion assembly occurs on the ER membranes, close to the viral RNA replication site. Immature virions, which contain prM and E proteins on the surface, bud into the lumen of the ER and are transported through the secretary pathway before they exit the cell. The maturation of virions takes place during the step in the trans-Golgi network (TGN), where immature virions undergo structural reorganization due to the low pH in the compartment. This reorganization allows the cellular serine protease furin to cleave prM into pr peptide and membrane (M) protein, resulting in the production of mature virions which contain M and E proteins on the surface (Perera et al., 2008). Studies have shown that this cleavage is not complete in DENV and several other flaviviruses, and the secreted virus particles in culture supernatants consist of a mixed population of mature virions, immature virions, and partially immature virions (Fig. 2B) (Junjhon et al., 2010; Plevka et al., 2011).

DENV virion

The DENV virion is spherical in shape and contains an envelope, which is a lipid bilayer with prM/M and E proteins present on the surface. Inside the envelope, there is a nucleocapsid consisting of C protein and RNA genome (Fig. 1B). Based on several cryo-electron microscopic (cryo-EM) studies (Kuhn et al., 2002; Yu et al., 2008; Zhang et al., 2003a; Zhang et al., 2003b), the mature virion has a smooth surface with a size of approximately 50nm in diameter and consists of 90 E protein dimers (Fig. 3A and 3B) whereas the immature virion has a spiky surface with an average size of 60nm in diameter.
and consists of 60 trimeric prM-E heterodimers (Figs. 3C and 3D) (Perera et al., 2008).

**E protein**

The E glycoprotein is a major protein organized on the surface of the virion. This protein mediates binding and fusion activities during virus infection, and it is also a major target for neutralizing (NT) antibodies (Abs). X-ray crystallographic studies have shown that the N-terminal ectodomain of E protein contains three domains (Modis et al., 2003, 2004). Domain I (DI) is located in the center, domain II (DII) is an elongated domain and contains a fusion loop (FL) at the tip, which is involved in membrane fusion and domain III (DIII) is an immunoglobulin-like domain and is involved in receptor binding. Following translation, RNA replication and assembly, the immature virions bud into the lumen of ER and go through the maturation process along the secretory pathway. This occurs as a result of a conformational change of the prM and E proteins on virion under the low pH environment in TGN, where the immature spiky particles become smooth and allows exposure of the prM cleavage site. This site is recognized by the cellular serine protease furin, and the pr peptides are cleaved from the prM (Yu et al., 2008; Perera et al., 2008). This disrupts the trimeric conformation; the E protein is rearranged to form a dimer and lays flat against the surface in a way that looks like a herringborn pattern, a characteristic of the mature smooth particles (Perera et al., 2008). Recently, an anti-DIII-monoclonal antibody (mAb) was reported to bind to its epitope on mature virions, which became completely accessible after
elevation of temperature. This was described as a phenomenon called “breathing”, suggesting the dynamic nature of epitope accessibility influenced by several factors such as temperature (Lok et al., 2008).

Epidemiology

DENV is the “most important mosquito-transmitted virus” (Guzman et al., 2010). The virus causes a significant burden to humans in terms of both public health and economy, as shown by a recent report in Nature that estimates a total of 390 million infections per year, with 500,000 cases of dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) per year (WHO, 2009; Bhatt et al., 2013). There are more than 100 countries endemic with dengue. The disease burden affects the economy as well with 264 disability-adjusted life years (DALYs) per million population being lost (Halstead et al., 2007; Suaya et al., 2009). By 1990, in areas like the Caribbean and South-East Asia, the disease burden could be comparable to meningitis, malaria or tuberculosis, and was more severe compared to hepatitis (Meltzer et al., 1998; Shepard DS et al., 2004). This number could be just the tip of the iceberg since many cases are underreported, misclassified, or misdiagnosed (Suaya et al., 2009; Beatty ME et al., 2011).

The mosquito, *Aedes aegypti* is the predominant vector for DENV, although another mosquito, *Aedes albopictus* can also sustain transmission (Gubler et al., 2007). *A. albopictus* was found as a secondary vector for DENV in Asia but has then spread to North America and Europe due to rapid transportation of people and goods (WHO, 2012).
Clinical features

Around 75 to 90% of the individuals infected with either of the four DENV serotypes have inapparent infection and remain asymptomatic. About 10 to 25% of individuals have symptomatic infections presenting as dengue fever (DF), which accompanies self-limiting febrile illness, rash, myalgia, arthralgia, headache, and retro-orbital pain. An even smaller percentage may develop more severe forms of clinical manifestations known as DHF or DSS, which are associated with capillary leakage, thrombocytopenia, and hemorrhage (Srikiatkhachorn et al., 2010; Simmons et al., 2012). The incubation period, which is the time from infection to onset of disease, is generally 4 to 7 days (Gubler et al., 2007). DF has been characterized as “break-bone fever”, due to constant aching all over the body. According to WHO, DF is defined as “febrile illness with 2 or more of the following: headache, retro-orbital pain, myalgia, arthralgia, rash, leukopenia, hemorrhagic manifestations, virus recovery, serological response and temporal occurrence with other cases” (WHO 1997, 2009).

DHF and DSS accompany hemorrhage, and more importantly, a severe intravascular fluid loss due to increased vascular permeability. Loss of plasma fluids mainly contributes to the severity of the disease, which may ultimately lead to a life-threatening shock. Secondary infection with a heterologous serotype is known to cause DHF and DSS more frequently rather than a primary infection (Halstead, 1988).
Immune response

Cellular immunity plays the main role in clearing the virus in an acute infection. DENV infects monocytes, DCs, and macrophages, and upregulates MHC class I on the surface of these cells. In vitro and in vivo experiments have shown DENV-specific CD8^+ cells associated with IFNγ production, cytotoxic activity, viral clearance, and its depletion resulted in increased viremia (Yauch et al., 2009; Gil et al., 2009). Humoral immunity also plays a role in clearing the virus and in protection against reinfection (Rothman, 2011).

DENV infection and Ab response

NT Abs developed after primary DENV infection confer life-long protection against the same serotype. Following primary DENV infection, patients develop an early IgM response, followed by an IgG response. On the other hand, patients with secondary DENV infection have a greater IgG response compared to IgM response. After recovering from primary infection by a DENV serotype, individuals will have life-long protection against that serotype, which correlated with the development NT Abs against that serotype (Guzman et al., 2007; Halstead 1974; Imrie et al., 2007; Innis 1997; Sabin 1952).

In the genus Flavivirus, there are several serocomplexes. Anti-E Abs that recognize members of different serocomplexes, members within the same serocomplex and a single member are called group (GR), complex-reactive (CR) and type-specific (TS), respectively (Calisher et al., 1989). Previous epitope studies of different mouse and human anti-E mAbs have shown that
GR mAbs primarily recognize the FL of DII, and TS and CR mAbs mainly recognize DIII residues (Brien et al., 2010; Costin et al., 2013; Crill et al., 2004; de Alwis et al., 2011; Gromowski et al., 2007; Shrestha et al., 2010; Sukupolvi-Petty et al., 2007, 2010; Tsai et al., 2013). TS human mAbs recognize DI/DII hinge or quaternary interdomain epitope have also been reported recently (de Alwis et al., 2012, Teoh et al., 2012). The NT potency of TS mAbs was generally greater than that of CR mAbs, which in turn was greater than that of GR mAbs (Gromowski et al., 2007; Sukupolvi-Petty et al., 2007). Thus, eliciting TS potent NT anti-E Abs is the main goal of current tetravalent dengue vaccines. Recent studies showed that anti-FL and anti-prM Abs cause ADE in vitro (Dejnirattisai et al., 2010; Huang et al., 2006; Rodenhuis-Zybert, 2010, 2011) and in vivo (Balsitis et al., 2010; Goncalvez et al., 2007; Williams et al., 2013; Zellweger et al., 2010).

Infection with one serotype may generate an immune response against both homotypic and heterotypic serotypes. Studies of polyclonal human sera after primary and secondary DENV infection have shown that a significant proportion of anti-E Abs was GR and recognized FL residues in DII and only a minor proportion was TS and recognized DIII (Crill et al., 2009; Lai et al., 2008; Lin et al., 2012; Wahala et al., 2009).

NT Abs generated from humoral immune response mainly target the E protein and its three domains. Neutralization activity of the Abs are described as a “multiple-hit” phenomenon, where the threshold for preventing infection is determined to be ~25% E protein on virion bound by Ab or ~30 Ab molecules per virion (Pierson et al., 2008; Pierson and Diamond, 2012). NT Abs play an important role in controlling disease outcome against DENV reinfection, but
weakly-NT Abs could play a converse role by contributing to enhancement of disease severity (Pierson and Diamond, 2012).

**Antibody-dependent enhancement (ADE)**

During secondary infection with a different serotype, the individuals have an increased risk of developing a severe clinical outcome such as DHF and DSS (Sangkawibha et al., 1984, Burke et al., 1988, Guzman et al., 1990). The increased risk of severe disease during secondary infection is believed to be due to ADE, a phenomenon mediated by cross-reactive weakly or non-NT Abs (Halstead, 1977, 1988). ADE is thought to occur in vivo when non-NT Ab from a previous DENV infection binds to a virus during a subsequent heterotypic infection, and the Ab is unable to neutralize the virus. The newly formed Ab-virus complex binds to Fc gamma receptors on monocytes (Fig. 5). The overall result is an increase in virus replication and the level of viremia (10-100 fold greater level in DHF/DSS compared to DF), which is associated with disease severity. This trend seems to fade after third or fourth infection with heterologous DENV serotypes.

**Diagnosis and treatment**

Clinical diagnosis of dengue is generally made from the patient's symptoms. Because of the variety of clinical manifestations caused by DENV, dengue could be misdiagnosed for many other viral or parasitic diseases.
(Simmons et al., 2012; Gubler et al., 2007). Co-infections are also a problem in many malaria endemic countries.

Well-trained and experienced clinicians may avoid misdiagnosis and mistreatment of the disease, thus reducing the number of severe dengue patients. Lab diagnosis depends on the capacity of the facility, and is mainly aimed to detect the virus or antibodies. Early diagnosis of DENV infection can be made by detection of NS1 protein in sera by rapid tests or ELISA. Detection of virus in serum, plasma, and tissues can be done after the onset of disease and up to 4 to 5 days by culture or detection of nucleic acid using RT-PCR. After this stage, diagnosis is based on serology using rapid tests or ELISA. The IgG and IgM responses found in serological results could lead to further determination of a primary or secondary DENV infection, which is important to assess the risk of ADE. Many serological tests are expensive for local clinics, and more research is necessary for improved and inexpensive diagnostic methods.

Since there are no antivirals available for dengue, the main method of treatment is through supportive treatment. In the case of plasma leakage, oral or intravenous rehydration of the patient in a timely manner is indispensible.

**Prevention and control**

Since there is no licensed vaccine currently available, prevention of DENV is focused on vector control of the mosquito (Simmons et al., 2012; Gubler et al., 2007). Current vector control methods include source reduction, insecticide space-spraying, and residual surface treatments of insecticides.
Source reduction can be carried out by elimination of containers and tires used for vector breeding. Insecticide use has been questioned, from aspects of sustainability and resistance of the vectors. Further research is also required on the effect of insecticide use in dengue endemic regions. WHO has set a goal to reduce dengue morality by at least 50% by 2010 (4). To control the dengue mortality and morbidity, a rapid and accurate clinical and laboratory diagnosis, appropriate rehydration, and training of physicians and medical staff is required. Proper reporting and monitoring of dengue cases is also necessary for an effective control strategy in terms of understanding the impact of the disease.

Vaccines

To induce protective NT Ab against all four serotypes, current DENV vaccine strategy focuses on a tetravalent approach (Murphy and Whitehead, 2011). To avoid the risk of ADE, simultaneous protection against all four serotypes is necessary. Development of DENV vaccines have been very challenging in terms of raising comparable levels of NT Abs against all four serotypes, conferring life-long protection, and the fact that there is no animal model available that completely mimic clinical manifestations seen in human patients.

Current DENV vaccine candidates range from live-attenuated vaccines to recombinant protein vaccines (Durbin and Whitehead, 2010). Live-attenuated vaccines take the lead, with the Sanofi group completing a phase IIb clinical trial in Thailand (Sabchareon et al., 2012). Although the study showed some
elevation of NTAbs, no protection was conferred against DENV2. This result revealed the difficulty of using the titer of NT Abs as correlate of protection in this study and highlighted the need of better NT tests and detailed understanding of immune correlates of protection in polyclonal sera. Several other vaccine candidates are in clinical trials, but it needs to be taken into consideration that the presence of cross-reactive weakly NT Abs increase the risk of ADE and DHF/DSS.

Research on supportive tools for vaccine development is ongoing. AG129 mice (interferon α/β and γ-receptor deficient) have been well established as a dengue model in mice to study immunopathogenesis, antivirals and vaccines, though mouse-adapted DENV strains are needed (Balsitis et al., 2010; Shresta et al., 2006; Williams et al., 2013; Zellweger et al., 2010). Many “humanized-mice” have been created to test DENV, as well as other virus and bacteria infections that have no ideal animal model for clinical evaluation (Frias-Staheli et al., 2014). These mice do not completely reflect the clinical aspects of human dengue patients, though they can be infected by human-infesting DENV strains, thus they still need great improvement.

Hypothesis and specific aims

Specific Aim 1. Investigate the binding of human mAbs to mature and immature DENV particles and their relationship to epitopes and neutralization potency

Hypothesis: Potent NT mAbs (such as mAbs recognizing DIII of E protein) bind mature and immature DENV particles similarly well. Weakly or non-NT
mAbs (such as anti-FL and anti-prM mAbs) preferentially bind immature to mature DENV particles.

**Rationale:** Most DIII epitopes are exposed similarly well on mature and immature DENV particles. FL is exposed on immature but poorly exposed on mature DENV particles; pr, the epitope of most anti-prM mAbs, is present on immature but not on mature DENV particles (Kuhn et al., 2002; Zhang et al., 2003a, 2003b; Li et al., 2008).

**Experimental Plan:** A panel of human and mouse mAbs targeting different epitopes will be tested for binding to immature and mature virus-like particles (VLPs). VLPs will be produced by transfection of 293T cells. Binding will be assessed by VLP-capture ELISA (Crill et al., 2009; Lin et al., 2012).

**Specific Aim 2. Test if DNA vaccines expressing mature DENV particles induce better Ab response than DNA vaccines expressing mixed DENV particles**

**Hypothesis:** DNA vaccines expressing mature DENV particles induce less cross-reactive anti-FL Abs and no anti-prM Abs, compared with DNA vaccines expressing mixed DENV particles (Fig. 6).

**Rationale:** FL is exposed on immature but poorly exposed on mature DENV particles; pr, the epitope of most anti-prM mAbs, is present on immature but not on mature DENV particles (Kuhn et al., 2002; Zhang et al., 2003a, 2003b; Li et al., 2008). Anti-FL Abs and anti-prM Abs are weakly or non-NT and cause ADE (Huang et al., 2006; Goncalvez et al., 2007; Rodenhuis-Zybert, 2010, 2011; Dejnirattisai et al., 2010).
**Experimental Plan:** 6-week-old female SW mice will be immunized intramuscularly (IM) with 2 doses of DNA vaccines expressing mature or mixed DENV VLPs of each serotype. Sera will be assayed for total anti-DENV Abs by ELISA, proportion (%) of anti-FL Abs by capture ELISA using wild type (WT) and mutant VLPs, anti-prM Abs by Western blot (WB) analysis, NT Abs by focus-reduction neutralization test (FRNT), and ADE activity (Crill et al., 2012; Hughes et al., 2012).

**Significance and Innovation.** Compared with mixed DENV particles, vaccination with mature DENV particles can induce NT Abs, less cross-reactive weakly or non-NT Abs and no anti-prM Abs, thus reducing the risk of ADE and severe diseases. This represents a novel strategy for safe and effective DENV vaccines.
**Fig. 1.** Genome and virion of DENV. (A) The RNA genome of DENV contains a single ORF encoding a polyprotein precursor, which is cleaved into 3 structural proteins and 7 non-structural proteins. (B) Pseudoatomic structure of mature DENV virions based on cryo-EM study of virion and X-ray crystallographic structure of E protein. E protein is present on surface with DI, DII and DIII shown in red, yellow and blue, respectively. FL of DII is shown in green (Murphy and Whitehead, 2011).
**Fig. 2.** Replication cycle of DENV and DENV particles produced in culture supernatants. (A) Schematic drawing of the replication cycle of DENV (Perera et al., 2008). (B) Cryo-EM study of DENV particles derived from culture supernatants revealed a mixture of immature (3.2%), partially immature (41.7%) and mature (55.1%) virions (Junjhon et al., 2010; Plevka et al., 2011).
**Fig. 3.** Structure of immature and mature DENV virions. (A) Cryo-EM picture showed spiky immature particles, formed by 60 trimeric prM-E heterodimers (mean diameter=60 nm). Pseudoatomic structure showed pr peptide (cyanine) and FL (green). (B) Cross section. (C) Cryo-EM picture showed smooth mature particles with 90 E dimers in herringbone pattern (mean diameter=50 nm). (F) Pseudoatomic structure showed poorly exposed FL (green). (D) Cross section (Lindenbach et al., 2007; Perera et al., 2008).
**Fig. 4.** Structure of dimers of E protein ectodomain, including DI, DII and DIII shown in red, yellow and blue, respectively. FL of DII is shown in green (Modis et al., 2003, 2004). Based on epitope mapping of mouse and human mAbs, GR mAbs mainly recognize FL. CR mAbs mainly recognize DIII. TS mAbs mainly recognize DIII, DI/DII hinge or quaternary interdomain epitope (Brien et al., 2010; Costin et al., 2013; Crill et al., 2004; de Alwis et al., 2011; Gromowski et al., 2007; Shrestha et al., 2010; Sukupolvi-Petty et al., 2007, 2010; Tsai et al., 2013). The NT potency of TS mAbs is generally higher than that of CR mAbs, which in turn is higher than that of GR mAbs (Gromowski et al., 2007; Sukupolvi-Petty et al., 2007).
Fig. 5. Model of ADE (Murphy and Whitehead, 2011).
**Fig. 6.** Central hypothesis of this thesis. Pictures of immature, partially immature and mature particles were adapted from Plevka et al., 2011.
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CHAPTER 2

MATERIALS AND METHODS
Preparation of VLPs

293T cells, prepared in a 10-cm culture dish at 5 x 10^5 cells per dish 1 day earlier, were transfected with 10 µg of plasmid DNA using the Lipofectamine method (Life Sciences). At 48 h post-transfection, culture supernatants were collected, and cells were washed with phosphate-buffered saline (PBS) and treated with 1% NP40 lysis buffer (100 mM Tris [pH 7.5], 150 mM NaCl, 20 mM EDTA, 1% NP40, 0.5% sodium deoxycholate, and protease inhibitors [Roche Diagnostics]), followed by centrifugation at 20,000 x g and 4°C for 30 min to obtain cell lysates (Hsieh et al., 2011).

Culture supernatants were clarified by centrifugation at 1,250 x g and 4°C for 20 min, filtered through a 0.22-µm-pore-sized membrane (Millipore), layered over a 20% sucrose buffer, and ultracentrifuged at 65,000 x g and 4°C for 5 h to obtain pellets containing VLPs, which were re-suspended in 30 µL 1 x PBS buffer (Hsieh et al., 2011).

Western blot analysis

Western blot analysis was performed by first adding cell lysates or pellets to 4 x non-reducing buffer (2% SDS, 0.5 M Tris [pH 6.8], 20% glycerol, 0.001% bromophenol blue [final concentrations]) and subjected to 12% polyacrylamide gel electrophoresis (PAGE) using an apparatus (Bio Rad) at 80 – 120 V. The gel was then transferred at 250 mA for 1 h to a nitrocellulose membrane (Hybond-C; Amersham Biosciences). After blocking with blocking buffer (4% milk in wash buffer; 10 mM Tris [pH 7.4], 150mM NaCl, 0.2%
Tween-20 [final concentrations]), the membrane was incubated at 4°C overnight with primary antibody (mouse or human mAb). After washing, the membrane was incubated at RT for 1 h with secondary Ab (donkey anti-human IgG at 1:10000, Jackson; anti-rabbit IgG at 1:2500, Cell Signaling; anti-mouse IgG at 1:5000, Pierce). After final wash, the signals were detected by enhanced chemiluminescence reagents and exposed to a film (Perkin Elmer Life Sciences) (Lai et al., 2008; Hsieh et al., 2011). The intensities of mature and immature VLPs were compared.

**Dot blot analysis**

Pellets containing mature or immature VLPs were mixed with 4 x non-reducing buffer, serially diluted and added on to the nitrocellulose membrane (Hybond-C; Amersham Biosciences) using a 96-dot formatted dot blotter (Labrepco). After blocking with 4% milk for 1 h, the membrane was hybridized with primary antibody (mouse mAb FL0232, 1:100000) at 4°C overnight. After washing the membrane with wash buffer (10 mM Tris [pH7.4], 150mM NaCl, 0.2% Tween-20 [final concentrations]) 3 times, secondary antibody (Pierce goat anti-mouse, 1:10000) was added and incubated for 1 h. After washing the membrane 3 times, signals on the membrane were detected by enhanced chemi-luminescence reagents and exposed to a film (Lin et al., 2012).
Four-layer VLP-capture ELISA

96-well plates were coated with mouse mAb (FL0251, 1:5000) or human serum (Palau #17, 1:12000) at 4°C overnight. After adding blocking buffer (Starting Block™, Thermo) at room temperature for 1 h, immature, mature or mixed VLPs or virions were added and incubated at 37°C for 2 h. After washing with 1 x wash buffer (0.5% Tween-20 in 1 x PBS) 4 times, primary Ab (panel of human or mouse mAbs) was added and incubated at 37°C for 2 h. After washing 4 times, secondary Ab (goat anti-mouse Ab, 1:5000, donkey anti-human Ab, 1:10000, Jackson Laboratory) was added and incubated at 37°C for 1 h. After washing 6 times and adding TMB substrate (KPL, Gaithersburg, MD) at room temperature for 10 to 15 min, stop solution (2N H₂SO₄) was added promptly. Optical density (OD) was measured at a wavelength of 450 nm with reference wavelength of 650 nm using an ELISA plate reader (BioTek ELx50). Binding curves, maximum binding (Bmax) and relative binding (% of Bmax), and dissociation constant (Kd) were determined by a nonlinear regression analysis using GraphPad Prism 5.0 (GraphPad Inc., CA) (Lin et al., 2012; Tsai et al., 2013).

Human and mouse monoclonal antibodies

A panel of 16 human mAbs targeting different epitopes/domains were tested for binding to immature and mature VLPs. These include 5 mAbs derived from a patient with primary DENV1 infection (Tsai et al., 2013) (Table 1) (kindly provided by Drs. S. Halstead and S. Kliks from the Pediatric Dengue
Vaccine Initiative at Seoul, South Korea), 7 mAbs derived from a patient with secondary DENV1 infection (Betramello et al., 2010) (Table 2) (kindly provided by Dr. F. Sallusto at the Institute for Research in Biomedicine, Switzerland), and 4 mAbs derived from three patients with secondary DENV1 infection (Tsai et al., 2013) (Table 3) (kindly provided by Dr. J. Mongkolsapaya at the Imperial College of London, UK). Four previously reported mouse mAbs (Shrestha et al., 2010) (Table 4) were kindly provided by Dr. M. Diamond at the Washington U. St. Louis. In addition, three human anti-prM mAbs (65.5, 58.3 and 38.1) were kindly provided by Drs. S. Halstead and S. Kilks from the Pediatric Dengue Vaccine Initiative at Seoul, South Korea (Tsai et al., 2012).

**Constructs of DNA vaccine**

Previously described prM/E expression plasmids of all four serotypes (pCB-D1, pCB-D2, pCB-D3 and pCB-D4), designated as pCB-WT constructs, were used as DNA vaccines (Hu et al., 2007). Four prM/E expression plasmids containing furin, designated as pCB-furin constructs, were generated by inserting IRES-furin-flag into the pCB-WT constructs. Insertions were verified by the patterns of restriction enzyme digestion and sequencing of the entire insert. DNA plasmids were amplified using DH5α competent cells and purified using an endotoxin-free plasmid DNA Purification Kit (OMEGA BIO-TEK, QIAGEN).
Vertebrate Animals

This study was conducted according to the principles in the NIH Guide for the Care and Use of Laboratory Animals. All animal experiment protocols were approved by the Institutional Animal Care and Use Committees (IACUC) at the University of Hawaii at Manoa. Procedures comply with the USDA Animal Welfare Act Regulations, American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals: 2013 Edition, and the Guide for the Care and Use of Laboratory Animals 8th Edition (2011).

Mice immunization schedule

Mice were obtained from Charles River Laboratories and were maintained in the animal facility of John A. Burns School of Medicine, University of Hawaii at Manoa. Seven week-old female Swiss Webster mice (n = 4-5/group) were immunized with PBS, pCB-WT or pCB-furin DNA vaccines at 0 week and 4 weeks. Immunization was conducted by intramuscular (IM) injection with 20 µL PBS or DNA vaccine injected into the thigh of each mouse. Mice were bled submandibularly (SM) at 0 week, 4 weeks, 8 weeks, and bled via cardiopuncture (CP) at 10-12 weeks.
Serum collection

Whole blood collected from SW mice were centrifuged at 2,500 rpm for 5 min); the supernatant were collected, centrifuged again to get serum, and then stored in -80°C freezer. Pooled sera of either 4 or 5 mice from each group as well as individual mice sera were tested for ELISA, FRNT, and ADE assay.

Preparation of inactivated DENV for ELISA

Vero cells, prepared in a 75 mL flask at 7 x 10^6 cells per flask were infected with DENV1 (Hawaii strain), DENV2 (NGC strain), DENV3 (H87 strain), DENV4 (H241 strain) at a multiplicity of infection (MOI) of 0.05. Culture supernatant was harvested when 50% cytopathic effect (CPE) was observed and subjected to ultracentrifugation (25,000 rpm, 5 h at 4°C) to collect virions in the pellets. Virions were inactivated by UV (Strategen, 600 V, twice, 5 min).

Three-layer virion ELISA

Three-layer ELISA was performed on collected sera of PBS and DNA vaccinated groups at various time points. 96-well plates were coated with inactivated DENV or VLPs of WNV and incubated at 4°C overnight. Blocking buffer (Starting Block™, Thermo) was added the following day and the plate was incubated at room temperature for 1 h, followed by incubation of primary
Ab (pooled mice sera or individual mice sera with serial dilutions) at 37°C for 2 h and secondary Ab (goat anti-mouse Ab, 1:5000, donkey anti-human Ab, 1:10000, Jackson Laboratory) at 37°C for 1 h. After washing 6 times, TMB was added and incubated at room temperature for 10 to 15 min. Stop solution (2N H2SO4) was added promptly. OD was measured at a wavelength of 450 nm with reference wavelength of 650 nm using an ELISA plate reader (BioTek ELx50) (Tsai et al., 2013). O.D. results were analyzed by Graph Pad Prism 5.0 and endpoint titers were calculated.

Focus Reduction Neutralization Test (FRNT)

FRNT assay against 3 serotypes (DENV2, 3, and 4) were performed on collected pooled and individual sera to test the neutralization potency. Vero cells were seeded on a 96-well ELISPOT plate one day prior to virus infection and incubated at 37°C. Two-fold serial dilutions of each serum were prepared and incubated with 50 focus-forming unites (FFU) of each virus serotype at 37°C for 1 h. Virus-serum mixture was inoculated into the Vero cell monolayers and incubated at 37°C for 90 min. Plates were gently shaken every 15 min during the incubation. After 150 µL of overlay with methylcellulose, plates were incubated at 37°C for 65 to 70 h (65-68 h for DENV2, 4, 70 h for DENV3). The plates were then washed with 200 µL of 1 x PBS for 5 times, and fixed with 200 µL fixation buffer (50% methanol/50% acetone) for 30 min. After removing the fixation buffer and drying at room temperature for 1 h, 380 µL of blocking solution (5% milk in 1 x PBS) was added and incubated on a shaker at room temperature for 30 min. After
removing the blocking buffer, primary Ab (mouse mAb; 4G2, 1:6400 dilution) was added and incubated at 37°C for 1 h. After washing 3 times, secondary Ab (goat anti-mouse Ab, 1:6000 dilution, Pierce) was added and incubated at 37°C for 1 h. After washing 3 times, 45 µL of True Blue substrate (KPL) was added and incubated at room temperature for 10 to 20 min. The plate was then washed with ddH₂O and dried over night. Foci were counted by the CTL Biospot Analyzer and the titers of FRNT₅₀ were determined by a nonlinear regression analysis using Graph Pad Prism 5.0 (Tsai et al., 2013).

Preparation of DENV virions for ADE assay

Vero cells were subjected to infection with each DENV serotype at a MOI of 0.05. Supernatants were collected when 50% CPE was observed and were mixed with 20% FBS (Hyclone). Virions were kept in -80°C for further use of Vero cell infection and ADE assay.

In vitro ADE assay

K562 cells were grown in 10% RPMI to a density of 2-5 x 10⁵ cells/mL two to three days prior to infection. Pooled and individual mice sera were prepared in five-fold serial dilutions. A mouse monoclonal Ab (4G2) was simultaneously prepared at two dilutions, which showed strong ADE in vitro. In a 96-well plate, diluted sera were incubated with 1.25 x 10⁵ plaque-forming units (PFU) of each virus serotype at 37°C for 1 h. 2 x 10⁵ cells/50 µL of K562 cells were added to the virus-serum mixture and were incubated at 37°C for 2 h. After
centrifugation at 2,000 rpm for 5 min, supernatants containing virus were discarded, and cells were re-suspended in 100 µL culture media. Cells were then transferred to a 24-well plate with 500 µL culture media added to each well and were incubated for 37°C for 48 h. Approximately 500 µL of supernatants were collected into 1.5 mL collection tubes followed by centrifugation at 2,000 rpm for 5 min. 450 µL of supernatants were collected in new collection tubes and stored in -80°C for future use in focus assay (Crill et al., 2012; Hughes et al., 2012).

In order to observe ADE in vitro, focus assay was conducted following virus infection. Focus assay was performed as FRNT, except that the virus was not mixed with any Abs.

**Analysis of epitope accessibility by structure-based modeling**

To determine the epitope accessibility of different mAbs on mature and immature virions, the program “UCSF chimera (http://www.cgl.ucsf.edu/chimera)” was used.
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Tsai et al., 2013
<table>
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Betramello et al., 2013
Table 3. Characteristics of human mAbs derived from patients secondary DENV infection

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Tsai et al., 2013
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</table>

Shrestha et al., 2010


Lai CY, Tsai WY, Lin SR, Kao CL, Hu SP, King CC, Wu HC, Wang WK. 2008. Antibodies to envelope glycoprotein of dengue virus during the natural course of infection are predominantly cross-reactive and recognize epitopes containing highly conserved residues at the fusion loop of domain II. J. Virol. 82: 6631-8843.


CHAPTER 3

RESULTS
RESULTS

Specific Aim 1: Investigate the binding of human monoclonal Abs (mAbs) to mature and immature DENV particles and their relationship to epitopes and neutralization potency

Preparation of immature, mature and mixed VLPs

A panel of 16 human and 4 mouse mAbs targeting different epitopes/domains was tested for binding to immature and mature VLPs (Tables 1 to 4 in Chapter 2). Mature and mixed VLPs were successfully produced by transfection of 293T cells with pVD1-furin and pVD1-WT, respectively (Fig. 1A). Expression of each vector was confirmed previously by Western blot analysis (data not shown). For production of immature VLPs, 293T cells were transfected with pVD1-WT and treated with NH₄Cl post-transfection. Production of each VLPs was confirmed by Western blot analysis using anti-M rabbit serum, and anti-prM and anti-E Ab from human serum (Fig. 1B). M protein was observed only in mixed and mature VLPs. PrM was only observed in mixed and immature VLPs for both experiments. E protein was highly expressed in all three VLPs, suggesting a stable expression of the particles.

A capture-ELISA using immature, mature and mixed VLPs

As shown in Fig. 2C, we have established a capture (four-layer) ELISA using mixed, mature, and immature VLPs. In order to determine comparable
amounts of VLPs were added in the four-layer capture ELISA, immature, mature and mixed VLPs were treated with 4X non-reducing (4NR) buffer, serially diluted and subjected to a dot blot assay using an anti-E mAb FL0232 (Fig.2A) (Lin et al., 2012). 4NR contains detergent SDS and can disrupt the membrane of particles and conformation of E protein, allowing similar accessibility of epitopes in immature, mature and mixed VLPs. Expression of immature and mixed VLPs were generally higher than that of mature VLPs, showing at least a four-fold difference. Based on the ratio of expression, comparable amounts of immature, mature and mixed VLPs were added. We then used a mouse anti-DIII Ab, FL0251, which has been shown to bind mature and immature VLPs similarly well (under both conditions with or without 4NR treatment) (Fig. 2B). This was further confirmed by capture-ELISA using a dengue-immune human serum (#17), which also recognized immature and mature VLPs similarly well (Fig. 2B).

In contrast, another mouse mAb FL0232, which recognized FL and bound immature VLPs much better than mature VLPs under condition without 4NR treatment (data not shown). Therefore, we used FL0251 and #17 serum as capturing Abs in capture-ELISA to study binding of human and mouse mAbs, respectively (Fig. 2C).

**Binding of human mAbs to immature, mature and mixed VLPs**

A panel of human mAbs including 6 anti-FL mAbs and 2 anti-DIII mAbs were first tested for binding to mature and immature VLPs. The binding curves based on the OD readout and % of Bmax are shown in Fig.3. Generally, anti-
FL mAbs bound immature VLPs better than mature VLPs, as shown by the binding curve with much higher Bmax value to immature VLPs than mature VLPs in all five anti-FL mAbs tested (Figs. 3A-3D). On the other hand, anti-DIII mAbs bound mature VLPs slightly better than immature VLPs in the two mAbs tested (Fig. 3E-3H). Since previous studies reported that DENV particles derived from cell culture consist of a mixture of immature, partially immature and mature particles (Junjhon et al., 2010; Plevka et al., 2011), we also tested the binding to mixed VLPs derived from culture supernatants in the four-layer ELISA (Fig. 4). As expected, the binding curves of mixed VLPs based on OD readouts and % of Bmax were between those of mature and immature VLPs for both anti-FL mAbs (Fig. 4A, 4B) and anti-DIII mAbs (Fig. 4C, 4D). As a control for the capture ELISA involving immature, mature and mixed VLPs, human serum #17, which recognizes immature and mature VLPs similarly well (Fig.2B), bound the three VLPs comparably (Fig. 4E). In addition, three human anti-prM mAbs (65.5, 58.3 and 38.1) bound immature and mixed VLPs but not mature VLPs (Fig. 4F); this is in agreement with our Western blot analysis that prM is present only in immature and mixed VLPs (Fig. 1B). Since only two human anti-DIII mAbs were tested initially, we also tested four mouse anti-DIII mAbs using the four-layer ELISA (Fig.5). Although these four anti-DIII mAbs have different binding specificity (TS or CR) and recognize different epitopes on DIII of DENV protein (Table 4, Chapter 2), they seem to have very similar binding pattern, namely, binding to immature VLPs slightly better than mixed and mature VLPs (Fig. 5).
Bmax and Kd of different mAbs to immature and mature VLPs

Previous reports have shown that DENV particles derived from culture supernatants contain immature, partially immature and mature particles in a proportion of 3.2%, 41.7% and 55.1%, respectively (Junjhon et al., 2010; Plevka et al., 2011). Mature DENV particles were reported to have 10000-fold higher infectivity compared to immature DENV particles (Zybert et al., 2008). Taking these into consideration, we then analyzed the Bmax and Kd of all 13 human anti-FL mAbs and 3 human anti-DIII mAbs to mature and immature VLPs (Fig. 6). Bmax, based on the binding curve, is indicative of epitope accessibility on particles and Kd, the dissociation constant based on half maximum binding, is indicative of the binding avidity of Abs; both epitope accessibility and binding avidity are important determinants of the NT potency of Abs (Diamond et al., 2008; Pierson et al., 2008, 2012). A shown in Figs. 6A and 6D, anti-FL mAbs had significantly higher Bmax to immature VLPs than to mature VLPs but comparable Kd to immature and mature VLPs (p<0.0001 and p=0.33 respectively, two-tailed Mann-Whitney test), suggesting that anti-FL mAbs have more accessible epitopes on immature than mature VLPs and similar avidity to both VLPs. As for anti-DIII mAbs, they had similar Bmax to immature VLPs and mature VLPs, and slightly low Kd to mature VLPs compared with immature VLPs (Fig. 6A, 6D) (p=0.7 and p=0.1 respectively, two-tailed Mann-Whitney test), suggesting that anti-DIII mAbs have similar epitope accessibility and avidity to both VLPs. Comparing the binding of anti-FL mAbs and anti-DIII mAbs to immature VLPs, they have no difference in Bmax and Kd (p=0.35 and p=0.79 respectively, two-tailed Mann-Whitney test).
Comparing the binding of anti-FL mAbs and anti-DIII mAbs to mature VLPs, anti-DIII mAbs had higher Bmax and lower Kd than anti-FL mAbs (p=0.01 and p=0.022 respectively, two-tailed Mann-Whitney test), suggesting that anti-DIII mAbs have higher epitope accessibility and avidity to mature VLPs, which presumably represent the major infectious component in mixed DENV particles derived from culture supernatants. These findings suggest that TS Abs such as anti-DIII Abs, though present as a small proportion of anti-E Abs in polyclonal human serum compared with GR anti-FL Abs, may contribute greatly to NT potency due to their higher epitope accessibility and avidity to mature particles, the major infectious component in mixed DENV particles in culture.

**Repertoire of human mAbs derived from single individual**

We further examined the repertoire of human mAbs derived from a single individual after primary and secondary DENV1 infection; of the five mAbs derived from a patient with primary DENV1 infection, four were GR recognizing FL of DII and one was TS recognizing DIII (Table 1 in Chapter 2); of the seven mAbs derived from a patient with secondary DENV1 infection, five were GR, one was CR, and one was TS recognizing DIII (Table 1 in Chapter 2). This is in agreement with previous studies of polyclonal human sera after DENV infection that a significant proportion of anti-E Abs was GR and recognized FL of DII and only a minor proportion was TS and recognized DIII (Lai et al., 2008; Crill et al., 2009; Wahala et al., 2009; Lin et al., 2012). Consistent with the findings in Fig.3, GR anti-FL mAbs derived from the
patient with primary DENV1 infection showed better binding to immature VLPs than mature VLPs whereas TS anti-DIII mAbs bound slightly better to mature VLPs (Fig. 7A). Similar trends were seen in 7 mAbs derived from the patient with secondary DENV1 infection in that GR mAbs, most likely recognizing FL (Tsai et al., 2013), bound immature VLPs better than mature VLPs and anti-DIII mAbs bound mature VLPs slightly better or equally compared with immature VLPs (Fig. 7B).

**Comparing the binding of mAbs to VLPs and virions**

We used VLPs to study the binding of different mAbs based on previous observation that the biophysical antigenic properties of flaviviral VLPs are similar to infectious virions (Chang et al., 2003; Davis et al., 2001; Ferlenhi et al., 2001; Purdy et al., 2004; Schalich et al., 1996; Stiasny et al., 1996). To further verify this, we examined the Kd of different human mAbs based on DENV1 VLPs and DENV1 virions in our capture ELISA. As shown in Fig.8A, a linear relationship was found between the Kd of 9 mAbs based on VLPs and Kd based on virions derived from Vero cells (correlation coefficient \( r=0.72 \), \( p=0.019 \) respectively, two-tailed Spearman correlation test), suggesting that the antigenic properties of DENV1 VLPs derived from transfection of 293 T cells are similar to those of DENV1 virions derived from Vero cells. Similarly, a linear relationship was found between the Kd of 5 mAbs based on DENV1 virions derived from Vero cells and Kd based on DENV1 virions derived from BHK cells, though not significant probably due to small sample size (\( r=0.70 \), \( p=0.23 \) respectively, two-tailed Spearman correlation test). Taken together,
these findings suggest that the antigenic properties of DENV1 VLPs are similar to those of DENV1 virions derived from two cell lines and support our approach of using VLPs to study the binding characteristics of different mAbs.

**Specific Aim 2. Test if DNA vaccines expressing mature DENV particles induce better Ab response than DNA vaccines expressing mixed DENV particles**

**Experimental plan**

Two groups of 7 to 9 week-old female SW mice were immunized IM with 2 doses of DNA vaccines expressing either mature (pCBD3-furin) or mixed (pCBD3-WT) DENV3 VLPs. Same volume of PBS was used for the control group. Pooled and individual sera from each group were assayed for total anti-DENV Abs by ELISA, proportion (%) of anti-FL Abs by capture ELISA, anti-prM Abs by Western blot analysis, NT Abs by FRNT, and ADE activity (Crill et al., 2012; Hughes et al., 2012) (Fig. 9).

**Anti-DENV Abs and NT activity**

Mice injected with either pCBD3-WT or pCBD3-furin produced anti-DENV Abs that bind DENV3 virions as early as 4 wk post-immunization, as shown in Fig. 10A. Individual sera collected at 10 to 12 wk post-immunization showed some variation in anti-DENV Ab titers, but there was no significant difference in the Ab titers between mice immunized with pCBD3-WT and those
immunized with pCBD3-furin (p=0.28, two-tailed Mann-Whitney test), (Fig.10A). This suggests that the immunization with DNA vaccine is successful and mice produce detectable amounts of Abs against DENV.

FRNT_{50} titers were then measured at 4 wk and 8 wk post-immunization. At 4 wk, both pCBD3-WT and pCBD3-furin immunized groups showed FRNT_{50} titers below the detection cutoff in our assay (1:20), but at 8 wk post-immunization both groups showed increased FRNT_{50} titers (mean=25 and 100 for pCBD3-WT and pCBD3-furin groups, respectively (Fig. 10B). NT_{50} titer higher than 1:20 is generally regarded as protective level based on study in JEV vaccine (Roehrig and Barrett, 2008).

**Anti-prM Abs and anti-FL Abs**

Western blot analysis was then conducted on pooled sera of 4 to 5 mice from each group to determine the anti-DENV Abs profile and the existence of anti-prM Abs. As expected, pCBD3-WT immunized mice recognized a strong prM band at 4 wk or 8 wk, indicating high levels of anti-prM Abs being produced (Fig. 11A). Mice immunized with pCBD3-furin recognized none or a very faint prM band (Fig. 11A). Consistent with the notion that anti-E Abs were the predominant Ab response after DENV infection, the intensity of E protein band was the strongest in all groups.

Previous work from our lab has established a method of measuring the proportion of anti-FL Abs in total anti-E Abs in polyclonal sera by capture-ELISA using WT and FL-mutant VLPs (Lai et al., 2013). Pooled sera from pCBD3-WT- immunized mice had higher % anti-FL Abs compared with those
from pCBD3-furin-immunized mice (Fig. 11B). Similar trend was observed for individual sera from 3 mice of each group at 8 wk (Fig. 11C), though not statistically significant probably due to the small sample size.

**ADE activity**

We further examined the ADE activities of sera in these mice by performing ADE assay with K562 cells, a FCγ receptor-bearing cells, in vitro. We first established the ADE assay using serial dilution of a mouse anti-E mAb, 4G2, which has been shown to cause a range of infection enhancement at different sub-NT concentrations. After pre-incubating with DENV2 and then inoculating to K562 cells, 4G2 showed 8- to 22-fold increase in virus titers at dilutions of 10,000, 40,000 and 160,000 but not 640,000, compared with DENV2 only control (Fig. 12A); we thus chose 4G2 at dilutions of 40,000 and 160,000 as our positive control for ADE assay.

Using DENV2 in the ADE assay, pooled sera from mice immunized with pCBD3-WT showed some enhancement (at dilutions of 300 and 7,500), though generally low enhancement compared to the 4G2 positive control (Fig. 12B). In contrast, pooled sera from mice immunized with pCBD3-furin showed no ADE. Using DENV4 in the ADE assay, while there was no enhancement compared to the 4G2 positive control, pooled sera from mice immunized with pCBD3-WT showed low enhancement at dilution of 40 (Fig. 12C).

Taken together, these results suggest that immunization of mice with DNA vaccine producing mature VLPs, compared to that with DNA vaccine
producing mixed VLPs can induce similar titers of anti-DENV Abs, higher NT Abs, no or little anti-prM Abs, lower % anti-FL Abs and Abs with less ADE activity in vitro, of which the statistical significance remains unknown due to small sample size.
Fig. 1. Preparation of mature, immature and mixed VLPs. (A) Schematic drawing of the constructs, pVD1-WT and pVD1-furin. Culture supernatants derived from 293T cells transfected with pVD1-furin, pVD1-WT in the presence of NH₄Cl and pVD1-WT were ultracentrifuged to get pellets containing mature, immature and mixed VLPs, respectively. (B) Mature, immature and mixed VLPs were subjected to Western blot analysis probed with rabbit anti-M serum and human dengue-immune serum.
Fig. 2. Establishment of four-layer capture-ELISA using mature, immature and mixed VLPs. (A) Serial 2-fold dilutions of mature, immature and mixed VLPs were treated with SDS-containing sampling buffer (4NR) and subjected to dot blot assay probed with mouse anti-E mAb FL0232. Boxes indicate comparable amounts of different VLPs. (B) Mouse anti-DIII mAb FL0251 recognized mature and immature VLPs (in PBS) similarly well in dot blot assay and capture-ELISA. Human #17 dengue serum recognized mature and immature VLPs (in PBS) comparably in capture-ELISA. (C) The format of four-layer capture-ELISA using FL0251 as the capturing Ab.
Fig. 3. Binding curves of human anti-FL and anti-DIII mAbs to mature and immature VLPs. Serial 4-fold dilutions of different mAbs were subjected to capture-ELISA using mature and immature VLPs. (A,C) The binding curves of one (A) and four (C) anti-FL mAbs. (B,D) The % Bmax curves of one (B) and four (D) anti-FL mAbs. (E,G) The binding curves of one (E) and two (G) anti-DIII mAbs. (F,H) The % Bmax curves of one (F) and two (H) anti-DIII mAbs.
Fig. 4. Binding curves of human anti-FL and anti-DIII mAbs to mature, immature and mixed VLPs. Serial 4-fold dilutions of different mAbs were subjected to capture-ELISA using mature, immature and mixed VLPs. (A,B) The binding curves of anti-FL mAbs. (C,D) The binding curves of anti-DIII mAbs. (E) Human #17 dengue serum recognized mature, immature and mixed VLPs comparably. (F) Three human anti-prM mAbs recognized immature and mixed VLPs but not mature VLPs.
Fig. 5. Binding curves of mouse anti-DIII mAbs to mature, immature and mixed VLPs. Serial 4-fold dilutions of mouse mAbs were subjected to capture-ELISA using mature, immature and mixed VLPs. The binding curves of one (A) and four (B) mouse anti-DIII mAbs.
Fig. 6. Epitope accessibility and binding avidity of human anti-FL and anti-DIII mAbs to mature and immature VLPs. (A,B,C) Epitope accessibility, presented by Bmax, of mature and immature VLPs recognized by 13 anti-FL and 3 anti-DIII mAbs. (D,E,F) Binding avidity, presented by Kd, of 13 anti-FL and 3 anti-DIII mAbs to mature and immature VLPs.
A. Primary DENV Infection

TS, anti-DIII mAbs
GR, anti-FL mAbs

B. Secondary DENV Infection

TS, anti-DIII mAbs
CR, anti-DIII mAbs

Fig. 7. Binding curves on mature and immature VLPs of repertoire of anti-E mAbs derived from patients with primary and secondary DENV infections. (A) Four GR anti-FL and one TS anti-DIII mAbs derived from a patient with primary DENV1 infection (Tsai et al., 2013). (B) Seven GR anti-FL, one CR anti-DIII, and one TS anti-DIII mAbs derived from a patient with secondary DENV1 infection (Betramello et al., 2013).
**Fig. 8.** Relationship between Kd based on DENV1 VLPs and Kd based on DENV1 virions derived from two cell lines (A) Relationship between Kd based on DENV1 VLPs and Kd based on DENV1 virions derived from Vero cells. (B) Relationship between Kd based on DENV1 virions derived from Vero cells and that from BHK cells.
Fig. 9. Experimental plan. Groups of 7 to 9 week-old female SW mice were immunized IM with 2 doses of DNA vaccines pCBD3-WT (n=9), pCBD3-furin (n-10) or PBS (n-5) Pooled and individual sera from each group were assayed for anti-DENV Abs, % of anti-FL Abs, anti-prM Abs, NT Abs, and ADE activity.
**Fig. 10.** The titers of anti-DENV Abs and NT Abs in pCBD3-WT and pCBD3-furin immunized mice. (A) Pooled sera of pCBD3-WT and pCBD3-furin immunized mice at 4 and 8 wk (n=5, each group) were subjected to DENV3 virion-ELISA. Individual sera of mice immunized with pCBD3-WT (n=9) and pCBD3-furin (n=10) at 10-12 wk were subjected to DENV3 virion-ELISA. The endpoint titers were determined. (B) Pooled sera of mice immunized with pCBD3-WT (n=4) and pCBD3 (n=5) at 4 and 8 wk were subjected to FRNT and the FRNT$_{50}$ titers were determined.
Fig. 11. Anti-prM Abs and % anti-FL Abs in pCBD3-WT and pCBD3-furin immunized mice. (A) Pooled sera of pCBD3-WT immunized mice (group 1, n=5; group 2, n=4) and pCBD3-furin immunized mice (group 1, n=5; group 2, n=5) at 4, 10 and 10-12 wk were subjected to Western blot analysis using mock- (Mo) or DENV3-infected Vero cell lysates as antigen. (B) Pooled sera of mice immunized with pCBD3-WT and pCBD3-furin (n=5, each group) at 4 and 8 wk were subjected to capture-ELISA using WT and FL-mutant VLPs to determine the % anti-FL Abs (Lai et al., 2013). The % anti-FL Abs = [1 - endpoint titer to mutant VLPs/endpoint titer to WT VLPs] X 100%. (C) The % anti-FL Abs in individual mice sera (n=3, each group) at 8 wk were determined as above.
Fig. 12. ADE activity in sera of pCBD3-WT and pCBD3-furin immunized mice. (A) Serial 4-fold dilutions of mAb 4G2 (10K to 640K) were pre-incubated with DENV4, and the immune complex was inoculated to K562 cells. The virus titers in supernatants at 48 h were determined by focus assay. Relative infection = virus titers with pre-incubated Ab / virus titers without Ab. (B) DENV2 ADE assay. Serial 5-fold dilutions (60 to 7500) of pooled sera from pCBD3-WT and pCBD3-furin immunized mice (n=5, each group) at 10-12 wk were pre-incubated with DENV2, and subjected to ADE assay. (B) DENV4 ADE assay. Serial 5-fold dilutions (40 to 5000) of pooled sera from pCBD3-WT and pCBD3-furin immunized mice (n=5, each group) were pre-incubated with DENV4, and subjected to ADE assay.
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CHAPTER 4

SUMMARY AND DISCUSSION
Overview

In this study, we first investigated the binding of various human mAbs to DENV particles of different maturation statuses in order to understand their relationship to epitope accessibility and neutralization potency. Secondly, we tested DNA vaccines expressing mature and mixed DENV particles to examine characteristics of the Abs produced by immunized mice. Analysis of the human and mouse mAbs revealed that potent TS anti-DIII mAbs bind mature VLPs comparably to immature VLPs, whereas weakly NT anti-FL mAbs preferentially bound immature VLPs. For binding to mixed VLPs, both anti-DIII and anti-FL mAb showed the binding curves and Bmax between those of the mature and immature VLPs.

Previous studies have revealed that both epitope accessibility and binding avidity, assessed by Bmax and Kd in our study, are important determinants of the NT potency of Abs (Diamond et al., 2008; Pierson et al., 2008, 2012). Interpretation of binding assays using our human mAb panel suggests that anti-DIII mAbs have similar accessibility to epitopes on mature and immature VLPs, and similar avidity to mature VLPs compared to immature VLPs (Figs. 3, 4, 6). On the other hand, anti-FL mAbs have significantly high accessibility to epitopes on immature VLPs than that on mature VLPs, but comparable avidity to both mature and immature VLPs. Comparison of the binding of anti-DIII and anti-FL mAbs to immature VLPs showed no difference in epitope accessibility and avidity (Fig. 6). In contrast, comparison of the binding of anti-DIII and anti-FL mAbs to mature VLPs showed significant difference with anti-
DIII mAbs having higher epitope accessibility and avidity than anti-FL mAbs (Fig. 6). Repertoire of anti-E mAbs produced by patients with primary and secondary DENV infections also showed the same trend (Fig. 7). However, the results from our mouse mAb panel revealed that anti-DIII mAbs bind immature VLPs slightly better than mature VLPs. This may be due to different binding specificity (TS or CR), different epitopes or small sample size of the mouse and human anti-DIII mAbs studied.

These findings suggest that at least for humans, TS Abs such as anti-DIII Abs, though present as a small proportion of anti-E Abs in polyclonal human serum compared with GR anti-FL Abs, may contribute greatly to NT potency due to their higher epitope accessibility and avidity to mature particles, the major infectious component in mixed DENV particles in culture (Junjhon et al., 2010; Plevka et al., 2011; Zybert et al., 2008).

Considering that the molecular conformation of E protein is highly dependent on the pH of the environment, four-layer capture ELISA rather than three-layer ELISA was used (Fig. 2). By using four-layer capture ELISA, the influence of a basic enviroment (pH 9, due to the coating buffer) to the E protein could be avoided.

To further explore the immunogenicity of mature virions in vivo, we immunized two groups of 7 to 9 week-old female SW mice with pCBD3-WT or pCBD3-furin, which express mature or mixed VLPs, respectively. Sera collected from the mice groups were subjected to ELISA, WB, FRNT and ADE
assay. Other mice strains and bleeding methods have been tested initially, such as BALB/c mice and tail-bleed, but due to low volume of blood collected from these tests, SB bleeding on SW mice was chosen in this study. DNA vaccines in other backbone constructs, such as pVAX, have also been tested, but due to low response, pCB-based construct was chosen in this study. Results of total anti-DENV Abs based on 3-layer ELISA, % anti-FL Abs based on VLP-capture ELISA, anti-prM Abs detected by WB, and FRNT_{50} titers generally followed the trend as we expected (Figs. 10A-B, Figs. 11A-C). Mice were able to produce anti-DENV Abs with similar titers induced by the two vaccines, suggesting that the DNA vaccine producing mature VLPs (pCBD3-furin) is immunogenic compared with pCBD3-WT (Fig. 10A). Variation in titers among individual mice was observed (Fig. 10A right panel), which could be due to the nature of outbred mice or the technique of handling/injection. DNA vaccine, pCBD3-furin, which expresses mature VLPs, induced a stronger FRNT_{50} titer than pCBD3-WT, which expresses mixed VLPs (Fig. 10B). Both vaccines had the potential to induce a “protective level”, higher than 1:10, of FRNT_{50} titer at 4 to 8 wk post-immunization (Roehrig et al., 2008). Our WB analysis revealed that pCBD3-furin can induce none or a small amount of anti-prM Abs (Fig.11A). The faint prM band shown in group 1 pCBD3-furin immunized-mice could be due to anti-prM Abs raised against pr peptide that was cleaved from prM protein and released at the point of maturation in vivo, however, nothing was detected group 2 pCBD3-furin immunized-mice (Fig. 11A). Consistent with our prediction, pCBD3-furin immunized mice produced much less anti-FL Abs compared to pCB-D3 WT immunized mice (Figs. 11B-C). However, only a small number of mice were included in this experiment,
further study with a larger number of mice is necessary to confirm this observation (Fig. 11C).

In order to examine the ADE activity in sera of our immunized mice, we have established an assay to evaluate ADE in vitro (Figs. 12A-C). Among the three serotypes (DENV1, 2 and 4) heterologous to the immunized serotype DENV3, DENV2 and DENV4 rather than DENV1 were tested in the ADE assay, considering the ease to determine virus titers based on the more clear foci of DENV2 and DENV4 in our system. Compared to our positive control 4G2 mAb, mice immunized with pCBD3-WT or pCBD3-furin had very low enhancement in general (Figs. 12B-C). This may be due to the relative low titers of anti-DENV Abs induced by our DNA immunization. As a comparison, we have also tested the ADE assay on sera from ICR mice immunized with pVAXD1-WT or pVAXD1-furin, kindly provided by our collaborator Dr. J.Chang at CDC Fort Collins, and found higher titers of anti-DENV Abs and higher folds of enhancement in our ADE assay (data not shown).

Limitations and future plans

This study faced several limitations. In Aim 1, mature, immature and mixed DENV VLPs rather than DENV virions were used to investigate the binding properties of human and mouse mAbs. This is based upon previous studies that VLPs and virions have similar antigenic properties (Chang et al., 2003; Davis et al., 2001; Ferlenhi et al., 2001; Purdy et al., 2004; Schalich et al., 1996; Stiasny et al., 1996). Although our study of 5 to 9 mAbs using the
Spearman correlation test suggested that the antigenic properties of DENV1 VLPs derived from 293 T cells were similar to those of DENV 1 virions derived from Vero cells (Fig.8), further investigation with more mAbs and DENV virions derived from different cell lines is needed to verify this observation.

The correlation of antigenic properties between DENV 1 VLPs derived from 293 T cells and DENV 1 virions derived from Vero cells were determined by the Spearman correlation coefficient (Fig. 8). The Spearman correlation test is a nonparametric method that can determine the correlation of non-linear variables. The Pearson r test cannot measure the relation between two variables if the correlation is not linear even though there is a very close relationship between the two. It needs to be taken into consideration when interpreting results that the Spearman test cannot completely reflect the relationship between two variables if the sample size is too small.

In Aim 2, one issue is to obtain comparable Ab titers from the same groups, since we were using outbred mice. We have planned to overcome this by using liposomes to stabilize DNA constructs, and to utilize liposome as an adjuvant (kindly suggested by Dr. P. Hoffmann). Another technical issue is to increase and stabilize the amount of sera collected from each mice. We have improved the quality of bleeding and immunization by using anesthetic measures, which also reduces the overall stress of mice. This study was conducted as a pilot study to determine the appropriate mouse strain, vector, immunizing periods and to establish the ADE assay. Further study on a larger number of mice, with different serotypes is required.
Concluding summary

In summary, our study of different human mAbs showed that compared with GR anti-FL mAbs, TS anti-DIII mAbs have higher epitope accessibility and binding avidity to mature VLPs, which presumably representing mature DENV virions, the major infectious component in mixed DENV particles in culture (Junjhon et al., 2010; Plevka et al., 2011; Zybert et al., 2008). These findings not only suggest that TS Abs such as anti-DIII Abs, though present as a small proportion of anti-E Abs in polyclonal human serum, may contribute greatly to NT activity, but also highlight the importance of inducing this type TS anti-DIII Abs by using mature particles for vaccine strategy. Using DNA vaccines to test our hypothesis, we showed that DNA vaccine expressing mature VLPs can induce similar anti-DENV Abs, NT Abs, less anti-FL Abs, no or little anti-prM Abs compared with DNA vaccine expressing mixed VLPs. Future studies are needed to verify mature DENV particles are better vaccine candidates than mixed DENV particles.
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