Generation and Characterization of Human Monoclonal Antibodies with Neutralizing Activity for Dengue Virus

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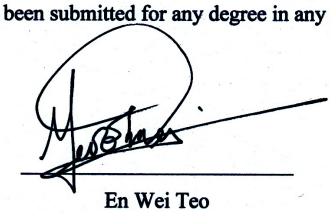
I hereby declare that this thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information that have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.



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List of Publications

Ee Ping Teoh*, Petra Kukkaro*, En Wei Teo*, Angeline P. C. Lim, Tze Tong Tan, Andy Yip, Wouter Schul, Myint Aung, Victor A. Kostyuchenko, Yee Sin Leo, Soh Ha Chan, Kenneth G. C. Smith, Annie Hoi Yi Chan, Gang Zou, Eng Eong Ooi, D. Michael Kemeny, Grace K. Tan, Jowin K. W. Ng, Mah Lee Ng, Sylvie Alonso, Dale Fisher, Pei-Yong Shi, Brendon J. Hanson, Shee-Mei Lok,† Paul A. MacAry†. The Structural Basis for Serotype-Specific Neutralization of Dengue Virus by a Human Antibody. *Science Translational Medicine*. 2012 June 20;4(139):139ra83

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Laura Rivino, Emmanuelle A. P. Kumaran, Vojislav Jovanovic, Karen Nadua, En Wei Teo, Shyue Wei Pang, Guo Hui Teo, Victor Chih Hao Gan, David C. Lye,d,e Yee Sin Leo, Brendon J. Hanson, Kenneth G. C. Smith, Antonio Bertoletti, David M. Kemeny, and Paul A. MacAry. Differential targeting of viral components by $CD4^+$ versus $CD8^+$ T lymphocytes in dengue virus infection. Journal of Virology. March 2013; 87(5): 2693–2706.

List of Patents

Human Monoclonal Antibody with Specificity for Dengue Virus Serotype 1 E Protein and Uses Thereof. Paul Anthony MacAry, Ee Ping Evelyn Teoh, Brendon John Hanson, En Wei Teo, Angeline Pei Chiew Lim, Mah Lee Mary Ng, Shee Mei Lok, Petra Eveliina Kukkaro. Publication Number: US 2013/0259871 A1. Publication Date: October 3 2013.

A Fully Human Anti-Dengue Serotype 2 Antibody and Uses Thereof. Paul Anthony MacAry, En Wei Teo, Shee Mei Lok, Wang Jiaqi, Brendon John Hanson, Conrad En Zuo Chan. Invention Disclosure submitted October 2014.

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List of Abbreviations

Å	Angstrom
Ab	Antibody
ADE	Antibody dependent enhancement
AF	Alexa Fluor
BHK-21	Baby hamster kidney 21
CD	Cluster of differentiation
cryoEM	Cryo-electron microscopy
DC	Dendritic cell
DC-SIGN	Dendritic Cell-Specific Intercellular adhesion molecule-3-Grabbing Non-integrin
DENV	Dengue virus
DF	Dengue fever
DHF	Dengue Hemorrhagic fever
DSS	Dengue Shock Syndrome
EBV	Epstein-Barr virus
E	Envelope protein
EDI	Envelope protein domain I
EDII	Envelope protein domain II
EDIII	Envelope protein domain III
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
Fab	Fragment, antigen binding
Fc	Fragment, crystallizable
FcγR	Fc gamma receptor
H chain	heavy chain
HRP	Horse radish peroxidase
hu	humanized
IFNα	Interferon alpha
IFNβ	Interferon beta
IFNγ	Interferon gamma
Ig	Immunoglobulin

IL	Interleukin
i.p.	Intraperitoneal
JEV	Japanese encephalitis virus
kDa	kilo Daltons
L chain	light chain
log_{10}	logarithm with base 10
M	membrane
mAb	monoclonal antibody
mg	milligrams
ml	milliliters
mu	Murine
NS	Non-structural protein
PFU	Plaque forming units
prM	pre-membrane
p.i.	post-infection
PRNT	Plaque reduction neutralization test
s.c.	sub-cutaneous
SD	Standard deviation
SHM	Somatic hypermutation
TBEV	Tick-borne encephalitis virus
TNFα	Tumor necrosis factor alpha
V_H	Variable heavy
V_L	Variable light
WHO	World Health Organization
WNV	West Nile virus
YFV	Yellow fever virus
µg	microgram
µl	microliter

Summary

Dengue virus (DENV) is a member of the family *Flaviviridae* and the genus Flavivirus. DENV is the etiological agent of dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), the most common arthropod-borne viral diseases of global importance. DENV includes four related although antigenically-distinct serotypes (DENV1, 2, 3 and 4). All four DENV serotypes can be found throughout the tropical and sub-tropical regions of the world and transmission of DENV takes place in more than 100 countries in the Americas, Middle East, Africa and Asia-Pacific region. Latest estimate puts the number of people in 2012 living in dengue endemic areas at 3.6 billion, which constitutes more than half the world's population. A recent study using new modeling techniques estimated 96 million apparent and 294 million inapparent dengue infections worldwide in 2010. Infection with one DENV serotype confers lifetime immunity to that serotype although not the remaining serotypes. There are presently no licensed vaccines nor specific treatments for dengue and therapy is mainly supportive in nature. Natural long term immunity to DENV is mediated by serotype-specific antibodies. Specifically, antibodies generated as part of a natural human immune response against DENV have been postulated to decrease viremia and disease severity. In this regard, they represent a possible therapeutic modality that has not been exploited. In this study, we have generated and characterized two fully human monoclonal antibodies, one specific for DENV1 and the other DENV2 from convalescent patients. We demonstrate that they have good neutralizing activity both *in vitro* and *in vivo*, making them potential therapeutic candidates for the future treatment of DENV infections.

1 Introduction

1.1 Dengue Virus

Dengue viruses (DENV) belong to the family *Flaviviridae* and the genus Flavivirus. DENV is the etiological agent of dengue fever (DF), dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), one of the most prevalent arthropod-borne viral diseases. Mosquito vectors transmit DENV between humans in urban areas (epidemic cycle) or in non-human primates in the jungle (enzootic cycle) (Yang et al., 2013).

The name flaviviruses originated from the Latin word *"flavus"* which means yellow that signifies jaundice, which is a common trait of infection with the prototypic Yellow fever virus. Flaviviruses comprise around eighty viruses with widespread geographical distributions. The most important human pathogenic flaviviruses are yellow fever virus (YFV), DENV, West Nile virus (WNV), tick-borne encephalitis virus (TBEV) and Japanese encephalitis virus (JEV). The RNA of flavivirus virion is single stranded and positive sensed with a size of approximately 10.5kb (Yu et al., 2005). Flaviviruses can infect a number of vertebrate and arthropods species. Most flaviviruses are arthropod-borne and are sustained in nature between hematophagous arthropod vectors and their vertebrate hosts.

1.1.1 Classification of Dengue Viruses

All flaviviruses are related serologically, demonstrated by hemagglutination-inhibition assays with polyclonal sera. Thus, they were originally classified into eight serocomplexes which consist of closely related flaviviruses that exhibit cross neutralization (Calisher et al., 1989). More recently, phylogenetic analysis of the Flavivirus genus based on partial sequences of the 3' terminus of the non-structural 5 (NS5) gene or structural envelope (E) gene have further classified Flaviviruses into clusters, clades and species (Kuno et al., 1998), defined by their epidemiology and disease manifestations. Approximately 50% of identified flaviviruses are mosquito-borne, 28% tick-borne while the rest are transmitted between rodents or bats with no known arthropod vectors. These three major clusters are summarized in Figure 1 (Gaunt et al., 2001).

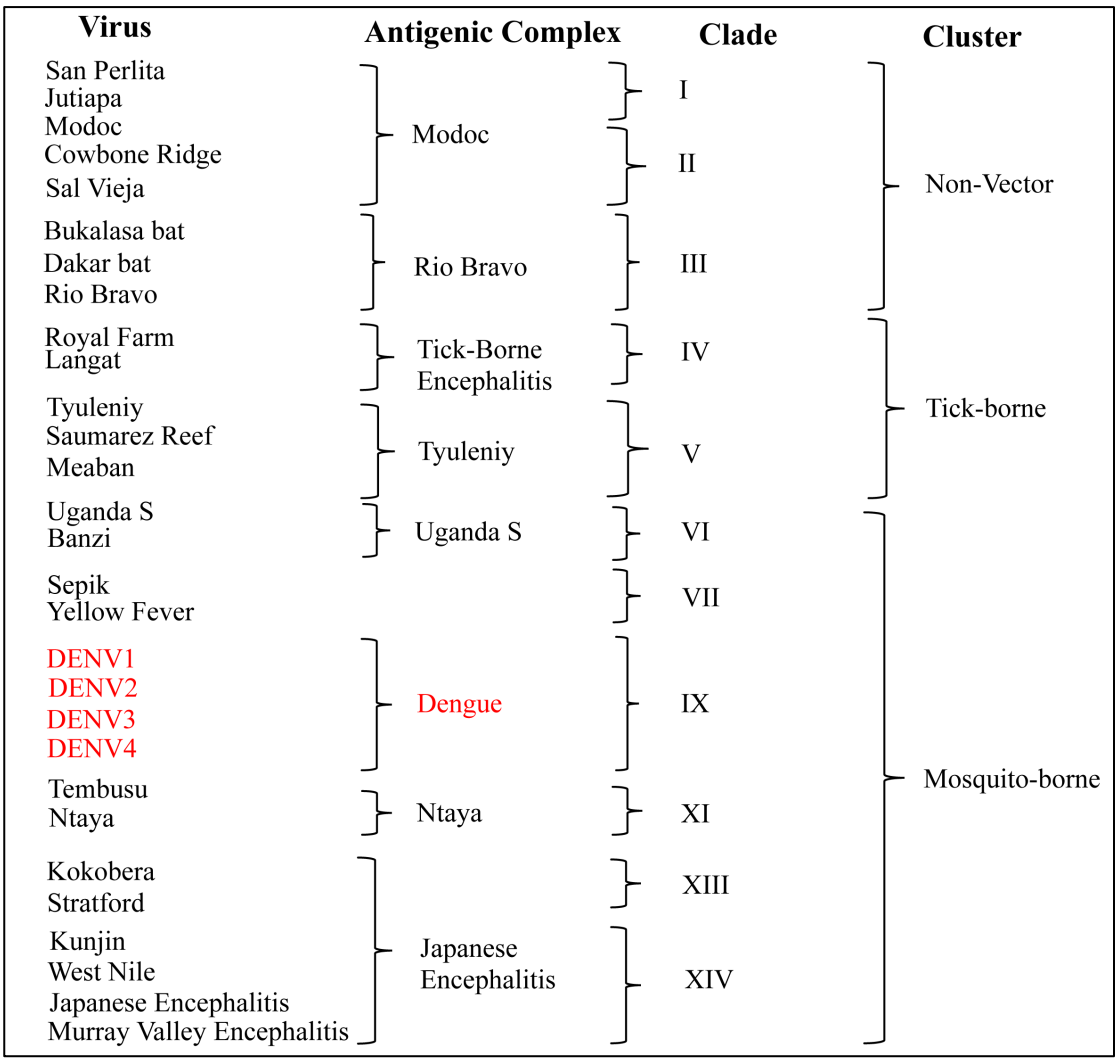


Figure 1 **Phylogenetic relationships of flaviviruses.** Adapted from Gaunt et al, Journal of Virology 2001.

DENV includes four distinct but related serotypes (DENV1, 2, 3 and 4) in the dengue antigenic complex (Calisher, et al., 1989). A fifth DENV serotype (DENV5) was recently identified in Sarawak, Malaysia in 2007, although there is still skepticism over its identity as a new serotype or merely a variant of an existing serotype (da Silva Voorham, 2014). DENV of all serotypes were originally classified genetically into topotypes using T1 RNase fingerprinting (Repik et al., 1983). The genetic relationship between the four DENV serotypes has been studied by cDNA-RNA hybridization using serotype specific cDNA probes (Blok, 1985). DENV1 and DENV4 are found to be genetically very similar (sharing approximately 70% of their genomes), as are DENV3 and DENV4 (sharing approximately 50% of their genomes) However, DENV2 is not very closely related to the other serotypes. The four DENV serotypes are defined by the amino acid sequence of the E protein – which is well conserved,

ranging from 90% to 96% similarity within each serotype and 60% to 70% similarity between serotypes (M. C. Chu et al., 1989; Lanciotti et al., 1997; Lanciotti et al., 1994; J. A. Lewis et al., 1993).

Rico-Hesse later classified DENV into genetically distinct groups or 'genotypes' within each serotype using nucleic acid sequencing. DENV within each genotype have nucleotide sequence divergences of less than 6% within the E/NS1 junction of their genomes (Rico-Hesse, 1990). The various genotypes within each of the DENV serotypes derived from various phylogenetic analyses are summarized in Table 1.

Serotype	Genotype	Name	Description	Basis	Reference
1	I	Genotype I	Southeast Asia, China, East Africa	Partial E/NS1 or complete E nucleotide sequences	(Rico-Hesse, 1990), (Goncalvez et al., 2002)
	II	Genotype II	Thailand in 1950s to 1960s		
	III	Genotype III	Sylvatic strains from Malaysia		
	IV	Genotype IV	West Pacific islands and Australia		
	V	Genotype V	All strains from the Americas, West Africa and limited number from Asia		
2	I	Asian	Asian Genotype 1 from Malaysia and Thailand, Asian Genotype 2 from Vietnam, China, Taiwan, Sri Lanka and the Philippines	E nucleotide sequences	(Twiddy et al., 2002), (Rico-Hesse et al., 1997), (Vasilakis et al., 2008)
	II	Cosmopolitan	Australia, East and West Africa, the Pacific and Indian ocean islands, Indian subcontinent and the Middle East		
	III	American	Latin America, the Caribbean, Indian subcontinent and Pacific Islands		
	IV	Southeast Asian / American	Thailand and Vietnam strains collected in the Americas		
	V	Sylvatic	Collected from humans, forest mosquitoes or sentinel monkeys in West Africa and Southeast Asia		
3	I	Genotype I	Indonesia, Malaysia and the Philippines and recent isolates from South Pacific islands	prM/E nucleotide or complete genome sequences	(Lanciotti, et al., 1994), (Chao et al., 2005)
	II	Genotype II	Thailand, Vietnam and Bangladesh		
	III	Genotype III	Sri Lanka, India, Africa, Samoa and 1962 strain from Thailand		
	IV	Genotype IV	Puerto Rico, Latin and central America and 1965 strain from Tahiti		
4	I	Genotype I	Thailand, the Philippines, Sri Lanka, Japan	E gene or complete genome sequences	(AbuBakar, Wong, et al., 2002), (Foster et al., 2003), (Klungthong et al., 2004)
	II	Genotype II	Indonesia, Malaysia, Tahiti, the Caribbean and the Americas		
	III	Genotype III	Thailand (recent samples distinct from other Thai strains)		
	IV	Genotype IV	Sylvatic strains		

Table 1 **Summary of the various genotypes of DENV within each serotype.**

1.1.2 History of Dengue Virus

The geographical origin of DENV is still the subject of debate. It was suggested that DENV originated in Africa based on the circulation of several mosquito-borne flaviviruses and the origin of Aedes aegypti, the most important vector for inter-human transmission (Gaunt, et al., 2001). However, there is also indication from phylogenetic analyses of an Asian origin (Wang et al., 2000). DENV1-4 evolved in non-human primates from a common ancestor, with each virus serotype entering the urban cycle independently approximately 500 to 1000 years ago (Wang, et al., 2000). It has been suggested that DENV evolved as an arboreal mosquito virus before it adapted to lower primates in forest environments and eventually into urban environments with the increase of deforestation and growth of human settlements. Benjamin Rush reported the first definitive case of dengue disease in 1789 and he coined the term 'breakbone fever'. Major outbreaks have since been recognized worldwide every 20-40 years (A. Guzman et al., 2010).

In the 18th and early 19th century, the African Aedes aegypti mosquito vector spread to the tropics via the movement of migrants and their water storage tanks by commercial sailing ships. Additionally, World War II brought about vast ecologic, demographic and epidemiologic changes, as well as rapid urbanization at the end of the war (Weaver et al., 2009). Sub-optimal housing and sewage management systems led to a sharp increase in vector densities that in turn facilitated dissemination of all four DENV serotypes throughout diverse geographic regions. Such conditions were optimal for the emergence of DHF in Southeast Asia (Hammon et al., 1960).

1.1.3 Current Status of the Spread of Dengue

Although the natural amplification and reservoir host range for DENV is restricted to primates, DENV is one of the most widely disseminated flaviviruses. All 4 DENV serotypes can be found throughout the tropical and sub-tropical regions of the world and dengue fever transmission occurs in more than 100 countries in the Asia-Pacific region, the Americas, the Middle East and Africa. Local spatial variations in risk have

been found to be closely dependent on rainfall, temperature and the degree of urbanization (Bhatt et al., 2013).

The World Health Organization (WHO) estimates 50-100 million DENV infections to occur annually, of which 500,000 are DHF requiring hospitalization and 22,000 deaths, mainly in children. The latest estimate puts the number of people in 2012 living in dengue endemic areas at 3.6 billion, more than half the world's population (Wilder-Smith et al., 2012). A recent study on the global distribution and burden of dengue using a formal modeling framework accounting for an exhaustive collection of known dengue occurrence worldwide has estimated 96 million apparent and 294 million inapparent dengue infections worldwide in 2010 (Bhatt, et al., 2013). Annual economic burden of dengue disease in Southeast Asia over the decade of 2001 to 2010 has been estimated to be US$950 and annual number of disability-adjusted life years (DALYs) at 372 per million inhabitants (Shepard et al., 2013).

1.1.4 Transmission and course of infection

The main vectors of dengue virus are the Aedes aegypti and Aedes albopictus. Infection with DENV begins with the bite of an infected mosquito during their blood meal. The virus is deposited subcutaneously, where it is thought to infect and skin-resident macrophages and dendritic cells (DCs) (St John et al., 2013). These infected cells eventually migrate to lymph nodes where recruited macrophages and monocytes recruited are infected, leading to the amplification of infection and subsequent dissemination through the lymphatic system and blood to other tissues resulting in viremia (Marchette et al., 1973). The main sites of DENV replication in humans have been reported to be in DCs, monocytes and macrophages (Jessie et al., 2004) (Limon-Flores et al., 2005), though DENV could also be detected in the spleen, kidney, lungs and liver (Jessie, et al., 2004). Viremia in infected individuals is detectable 24 to 48 hours prior to the onset of clinical symptoms and can persist for up to 10 days. Mosquitoes that take a blood meal from viremic individuals take up the virus, which subsequently infects epithelial cells of the midgut. The virus is then disseminated into the hemocoel and eventually the salivary glands (Salazar et al., 2007). These

mosquitoes become infectious 4 to 12 days post-feeding and are then able to transmit DENV and are then able to transmit DENV (Salazar, et al., 2007).

Incubation period for DENV is typically 4 to 7 days, before the presentation of a range of clinical symptoms – from asymptomatic or self-limiting sub-clinical febrile illness (50% to 90% of DENV infections) to severe and fatal hemorrhage. The clinical manifestations in children can vary from those in adults, with cough, vomiting and abdominal pain more common in children (Hanafusa et al., 2008). In a study conducted in a Vietnamese cohort, mortality rates observed in young children (aged 1 to 5 years) infected with DENV were significantly higher than that in older children (aged 6 to 10) and adults (Anders et al., 2011). Furthermore, in another comparative study in Nicaragua, the disease burden and severity was most predominant in infants (aged 4 to 9 months) (Hammond et al., 2005). The most common symptomatic manifestation of DENV is dengue fever (DF), characterized by fever and a range of generic symptoms including rash, headache, retro-orbital pain, myalgia, arthralgia and some degree of hemorrhagic manifestations such as petechiae, and ecchymoses (Tantawichien, 2012; Whitehorn et al., 2011). The disease is usually self-limiting with the acute febrile phase lasting for up to a week, followed by a convalescent phase that can last for several weeks. Up to 2% of dengue cases, the majority of which are children under the age of 15, progress to the more severe and potentially fatal DHF characterized by increased vascular permeability (plasma leakage), thrombocytopenia, and hemorrhagic manifestations of the skin, nose, gum and gut (Halstead, 2007; Kyle & Harris, 2008). DSS occurs when the leakage of fluid into interstitial spaces results in a sudden drop in blood pressure which may be fatal without appropriate interventions (St John, et al., 2013). The case fatality rates range from <1% to 5% (Gubler, 1998).

The case definitions for DHF and DSS were revised in 2009 by the WHO to distinguish between dengue and severe dengue using warning signs for disease progression as summarized in Figure 2. Patients without warning signs can be safely managed as outpatient cases, reducing hospital resource burden (Leo et al., 2013)

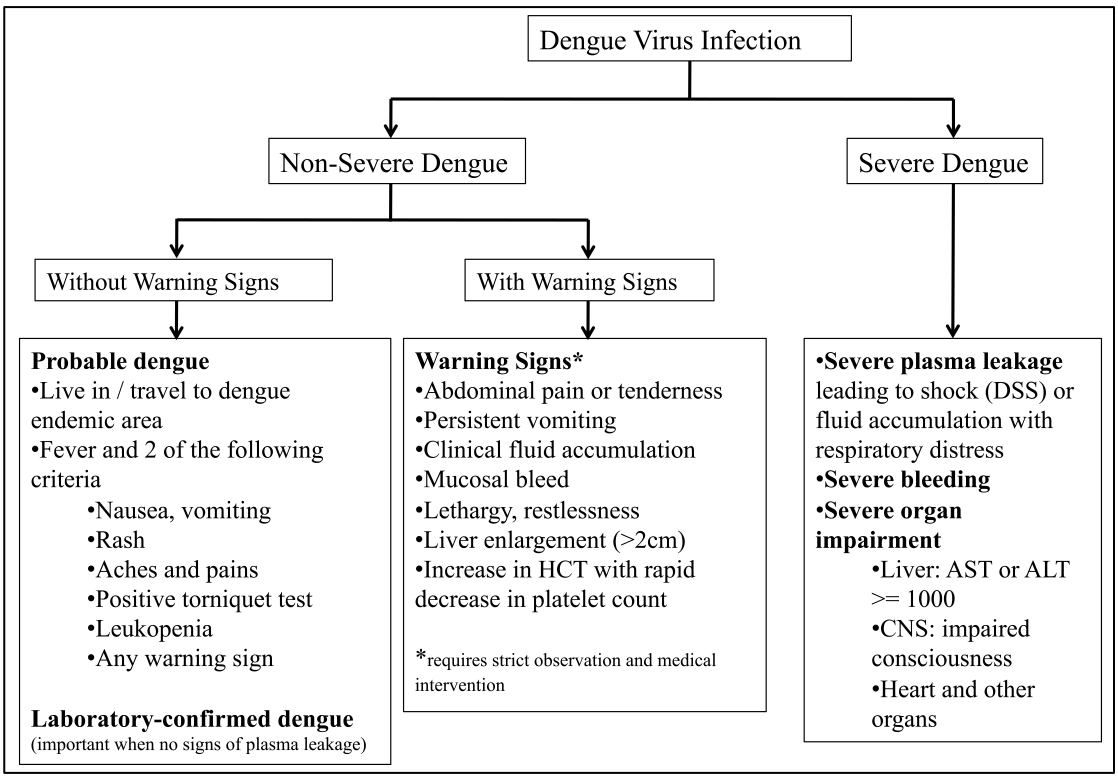


Figure 2 **WHO classification for dengue severity.** The new classification for dengue severity is divided into Dengue without Warning Signs, Dengue with Warning Signs, and Severe Dengue.

1.2 Molecular Biology of DENV

1.2.1 Dengue Virus Proteins

DENV belongs to the genus Flavivirus of the family Flaviviridae. Other members of the Flavivirus genus include yellow fever virus (YFV), West Nile virus (WNV), Japanese encephalitis virus (JEV) and tick-borne encephalitis virus (TBEV). The Flavivirus genome comprises of a single-stranded, positive-sense RNA about 10.7kB in length and contains a 5' methyl guanosine cap, a 5' untranslated region (UTR) followed by a single open reading frame (ORF) and a 3' UTR (Clyde et al., 2006). The ORF codes for a polyprotein that is co- and post-translationally modified by proteases of both cellular and viral origin into three structural proteins and seven non-structural proteins. The structural proteins include the capsid (C), premembrane (prM) that is cleaved to form the membrane (M) in the mature virus and the envelope (E). The non-structural proteins include NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. Structural proteins form the virus particle and are essential for viral entry, fusion and assembly while non-structural proteins function in viral RNA replication, evading the host innate immune system and assembly of the virus (Guo et al., 2005; Kummerer et al., 2002; Xie et al., 2013).

1.2.1.1 Capsid (C) Protein

The DENV C protein has 100 amino acid residues with a molecular weight of 12-15 kDa. Containing 25% lysine and arginine residues, the protein's highly basic character enables it to neutralize the negatively charged viral RNA. The primary function of the C protein is to encapsulate the viral RNA to make up the nucleocapsid. The nucleocapsid is approximately 30nm in diameter and appears as a dense particle when viewed with an electron microscope. A hydrophobic segment of the C protein that is 21 amino acids in length is essential for the maturation process and assembly of viral particles (Markoff et al., 1997). Flaviviruses have poorly conserved C protein sequence homology but are structurally and functionally similar. The mature C protein is generated when the hydrophobic signal sequence at its C terminal is cleaved by NS2B-NS3 proteins (Amberg et al., 1999).

1.2.1.2 Pre-Membrane (prM) and Membrane (M) Protein

The dengue membrane (M) protein is made up of 75 amino acids, weighing approximately 9kDa. prM is the uncleaved precursor of M and is found as prM–E heterodimers on the immature virion. Antibodies targeting prM are found to be a key component of the human immune response against DENV infection (Dejnirattisai et al., 2010). However, these antibodies are cross-reactive and poorly neutralizing even at high concentrations. Instead, these prM specific antibodies are potent enhancers of infection.

As the immature virion transits through the acidic milieu of the trans-Golgi network (TGN), prM-E heterodimers dissociate to become E homodimers (Rodenhuis-Zybert et al.). These conformational arrangements facilitate the cleaving of prM protein into M protein and a pr peptide by cellular endo-protease furin (I. M. Yu et al., 2008). The prM protein and pr peptide functions to prevent the premature fusion of E protein with the acidic compartments within the secretory pathway (Keelapang et al., 2004). Hence, this cleavage of prM to M is essential for viral infectivity (Zybert et al., 2008). However, this cleavage is not always complete and partially cleaved prM from the viral surface decreases antigen density accessible for viral neutralization, rendering DENV susceptible to ADE by anti-prM antibodies (Dejnirattisai, et al., 2010).

1.2.1.3 Envelope (E) Protein

The envelope (E) protein is the main protein found on the virion surface. The E protein is approximately 500 amino acids in length and the amino acid sequence homology between E proteins of different DENV serotypes is approximately 60-70% (Hiramatsu et al., 1996). 180 copies of the E protein are arranged on the surface of the virus. It forms a head-to-tail homodimer and consists of three distinct domains designated domains I, II and III (EDI-III), which lie over a helical stem region, tethered onto the viral membrane by a transmembrane anchor (Modis et al., 2004). Mature DENV virions consist of 90 anti-parallel E protein homodimers in a quasi-icosahedral symmetry arrangement flat along the surface of the virion. In contrast, the immature virion in which prM is not cleaved from E protein, consists of 60 spikes each composed of 3 prM-E heterodimers (Cherrier et al., 2009; Y. Zhang et al., 2003). As the virus matures when exposed to the mildly acidic conditions in the trans-Golgi

network, the E proteins undergo structural rearrangement and prM is cleaved to M (Li et al., 2008; I. M. Yu, et al., 2008).

N-terminal EDI makes up the central region of the protein and forms an eight-stranded β-barrel. Dimerization domain EDII is a finger-like protrusion from EDI containing the highly conserved fusion loop at its distal end that mediates type II fusion of the virus with acidic endosomal membrane (Allison et al., 2001; Modis, et al., 2004). It also contains a second N-linked glycan that recognizes dendritic cell-specific ICAM3 grabbing non-integrin (DC-SIGN) (Pokidysheva et al., 2006), which is thought to be an ancillary receptor for DENV infection of human DCs (Tassaneetrithep et al., 2003). EDI and EDII are discontinuous and are joined by four peptide linkers that constitute the EDI/EDII hinge. EDIII makes up the C terminal region (residues 292 to 395) of soluble E and is a continuous peptide that extends from EDI to form an immunoglobulin-like structure consisting of seven anti-parallel β-sheets joined by flexible hoops and is most likely involved in receptor binding (Rey et al., 1995). EDIII is linked to EDI via amino acid residues 291-301, a flexible region that mediates the large inter-domain rearrangement of the E protein, which mediates fusion process (Modis, et al., 2004). The lateral ridge epitope is the target of most strongly neutralizing murine antibodies. This epitope consist of the BC loop, N linker region and FG loop of EDIII (Wahala et al., 2012).

In its native conformation, E protein exists as a homodimer that lies flushed against the surface of the virion. E protein is a "class II" viral fusion protein, found in flaviviruses and alphaviruses, as it mediates receptor binding (Crill et al., 2001) and induces fusion of the cell membranes of the virus and the host to release viral RNA into the cytoplasm (Stiasny et al., 2005). The four DENV serotypes is differentiated by the amino acid sequence of their E proteins, since amino acid sequence is 90% to 96% similar within each serotype but 60% to 70% similar between serotypes (M. C. Chu, et al., 1989; Lanciotti, et al., 1997; Lanciotti, et al., 1994; J. A. Lewis, et al., 1993). The crystal structures of E protein of several flaviviruses including DENV (Modis et al., 2003, 2005), WNV (Nybakken et al., 2006) and tick-borne encephalitis (Rey, et al., 1995) have been solved.

Since the E protein is the key protein exposed on the surface of the virion, neutralizing antibodies that confer immunity to the flavivirus primarily target the E protein, though antibodies that target the prM (Vazquez et al., 2002) and NS1 (Schlesinger et al., 1987) proteins have also been shown to be protective. Amino acids that differ between serotypes are usually located at the outermost surface of the virion and are spread between domains EDI, II and III (Modis, et al., 2005), suggesting that the evolution of DENV serotypes may be brought about by selecting pressure of neutralizing antibodies. This also implies that the target of neutralizing antibodies is widely distributed throughout the surface areas of EDI, II and III that antibodies can access. Studies with murine monoclonal antibodies (mAbs) against WNV that is structurally similar to DENV suggest that serotype-specific neutralizing mAbs against WNV usually target epitopes on the surface of EDIII (Nybakken et al., 2005). Antibodies against EDI are usually non-neutralizing. Conversely, antibodies against EDII where the highly conserved fusion loop is located, are usually cross-reactive with other flaviviruses but are still neutralizing, possibly by preventing membrane fusion (Nybakken, et al., 2005; Thompson et al., 2009). However, when the B cell repertoire of humans infected with WNV were analyzed, most B cell clones were found to produce antibodies that target epitopes in EDII, particularly the fusion loop but not EDIII as seen in mAbs generated in mice (Throsby et al., 2006). Similarly, studies on convalescent DENV-infected human patients found that only a small proportion of neutralizing antibodies in immune sera were EDIII-specific (Wahala et al., 2009). Several groups have identified human antibodies with potent neutralizing activities that bind to epitopes around the EDI/EDII hinge region (Messer et al., 2014; Smith et al., 2013; Teoh et al., 2012). de Alwis and colleagues found that the majority of neutralizing antibodies in human immune sera bound to whole intact virions but failed to bind to the ectodomain of purified soluble E protein, leading them to conclude that neutralizing antibodies produced by humans as part of a natural infection bound to a quaternary epitope that is present only when E proteins are assembled on the virion. They identified this epitope to span adjacent E protein dimers and include the EDI/EDII hinge region (de Alwis et al., 2012). EDI/EDII hinge region of DENV3 and DENV4 was recently found to be the primary target of long-lasting serotype-specific neutralizing antibody response in humans (Messer, et al., 2014).

1.2.1.4 Non-Structural Protein 1 (NS1)

NS1 protein has a molecular weight of 46-55kDa dependent on its degree of glycosylation. It exists as several forms – associated with the cell membrane (mNS1), within vesicular compartments in cells or secreted as a soluble hexamer (sNS1) (Muller et al.). Cell membrane-associated and secreted forms of NS1 are both highly immunogenic, with the protein itself and the antibodies elicited associated with disease pathogenesis (Avirutnan et al., 2006a; D. S. Sun et al., 2007). NS1 can be detected in the bloodstream of infected individuals from the onset of fever to early convalescence, ranging from several nanograms to micrograms per milliliter of serum (Alcon et al., 2002). Patients with an elevated sNS1 level (≥ 600ng/ml) within 72 hours of fever onset are found to be at risk of developing DHF (Libraty, Young, et al., 2002). Intracellular NS1 is a cofactor in virus replication and co-localize with viral dsRNA replicative form (Mackenzie et al., 1996) although its precise function in viral replication has yet to be elucidated. NS1 has been linked to the dysfunction of endothelial membranes, a hallmark of severe dengue, by mimicking or hijacking lipid metabolic pathways (Gutsche et al.). sNS1 has also been implicated in complement activation and its subsequent pathogenesis of the vascular leakage seen in patients with severe dengue (Avirutnan, et al., 2006a).

1.2.1.5 Non-Structural Protein 2A, 2B (NS2A, NS2B), 4A and 4B (NS4A and NS4B)

There is limited information on the functions of the small hydrophobic proteins NS2A, NS4A and NS4B. These proteins have been shown to be integral membrane-associated, spanning the endoplasmic reticulum (ER) membrane with numerous transmembrane regions (S. Miller et al., 2007a; S. Miller et al., 2006; Xie, et al., 2013). They may also function to anchor viral replicase proteins to cellular membranes (Chambers et al., 1989), contribute to virion assembly as shown in yellow fever virus (Kummerer, et al., 2002) and inhibit IFNα/β response (Munoz-Jordan et al., 2003).

NS2A was shown to function in viral replication and assembly (Leung et al., 2008; Mackenzie et al., 1998; Xie, et al., 2013). The topology model of DENV NS2A on the ER membrane has recently been reported and mutagenesis studies conducted have suggested that DENV NS2A is associated with RNA synthesis and virion

assembly/maturation (Xie et al.). NS2B mainly functions as a cofactor of NS3 (Section 1.1.5.6).

NS4A has been found to reside primarily in cytoplasmic dot-like structures derived from the ER, which also contain dsRNA and other DENV proteins, indicating that NS4A is a component of the membrane-bound viral replication complex (S. Miller et al., 2007b). NS4A induces membrane arrangement to form the replication complex. NS4B is an approximately 27kDa hydrophobic integral protein. Flavivirus NS4B is required for viral replication and suppression of IFN-induced JAK/STAT signaling (Munoz-Jordan et al., 2005). The membrane topology of DENV NS4B has recently been identified using anti-NS4B monoclonal antibodies – the N-terminus is located in the ER lumen while amino acids 130-148 of NS4B are in the cytosol (Xie et al., 2014).

1.2.1.6 Non-Structural Protein 3 (NS3)

NS3 is a 618 amino acid protein with multiple functions. It consists of the N terminal region that is a serine protease domain (requiring association with NS2B), and the C terminal region containing RNA helicases/NTPases (Luo et al., 2008; Warrener et al., 1993; Wengler et al., 1991). NS3 has been found to be essential to flavivirus replication and polyprotein processing.

1.2.1.7 Non-Structural Protein 5 (NS5)

NS5 is a large protein with a molecular weight of 105kDa. It is also the most highly conserved dengue viral protein with at least 67% homology between DENV1-4. It comprises of a methyl-transferase (MTase) domain at its N-terminus (L. J. Yap et al.) and a RNA-dependent RNA polymerase (RdRp) domain at its C-terminal end (T. L. Yap et al., 2007). The MTase domain carries out sequential guanine N7 and ribose 2'-O methylation to form the cap structure on the 5' end of the viral genome and internal RNA methylation (Dong et al., 2012; Egloff et al., 2002). The RdRp domain has *de novo* RNA synthesis activity (Ackermann et al., 2001). The F1 motif in dengue NS5 is also involved in promoter-dependent RNA synthesis (Iglesias et al., 2011). Additionally, NS5 blocks type 1 interferon (IFN) response by binding to STAT-2 (signal transducer and activator of transcription 2) and induces its degradation in the proteasome (Ashour et al., 2009).

1.2.2 Structure of DENV

The DENV particle consists of a nucleocapsid containing a single copy of genomic RNA associated with 180 copies of the C protein (Y. Zhang et al., 2007). The nucleocapsid is surrounded by a lipid bilayer consisting of 180 copies of E protein and prM. Cryo-EM reconstruction of immature virions have determined its size to be 60nm and consists of 60 icosahedrally arranged trimeric spikes each made up of three prM-E heterodimers (Y. Zhang, et al., 2003), with the pr peptide of the uncleaved prM covering the fusion peptide of the E protein. The virions then undergo a reversible conformational change brought about by the low pH in the trans golgi network (TGN). The E proteins rearrange, forming head-to-tail dimers lying parallel on the surface of the virion in a herringbone-like fashion in 30 groups of 3 dimers each. Hence the virion appears smooth instead of spiky and has a diameter of 53nm (I. M. Yu, et al., 2008). This rearrangement makes the virion accessible to furin cleavage. The pr peptide does not dissociate from the fusion loop of the E protein after furin cleavage, suggesting that pr is retained on the virion to prevent membrane fusion in the TGN (I. M. Yu, et al., 2008). This interaction of the pr peptide with E protein is pH dependent – the pr peptide dissociates from the virion when pH increases to 7 in the extracellular space, rendering the virion competent for infection. Mature virions have smooth surfaces and a diameter of 50nm (Kuhn et al., 2002). Cryo-EM reconstruction determined the virion to consist of a series of spherical shells, with the outermost shell made up of E protein and an inner shell made up of the ectodomains of M protein and stem region of E protein. Beneath these 2 shells lies the lipid bilayer surrounding the nucleocapsid with little structural organization. The orientation of the nucleocapsid is unrestrained due to the lack of interactions of E and M proteins with the capsid (W. Zhang et al., 2003).

The structure of DENV changes at elevated temperature of 37°C without affecting infectivity. This change in structure has been termed as “breathing” (Pierson et al., 2012)– a phenomenon discovered from the realization that neutralizing epitopes on viruses are better exposed at elevated temperatures rendering the effectiveness of neutralizing antibodies. This occurs because E protein interactions on the virus surface loosen at elevated temperatures to expose previously cryptic epitopes for antibody binding (Cockburn, Navarro Sanchez, Fretes, et al., 2012; Lok et al., 2008).

This structural change brought about at elevated temperatures was not observed in immature DENV (Fibriansah et al., 2013).

1.2.3 Replication cycle of DENV

The life cycle of flaviviruses are very similar. It begins with the binding of the infectious virus particle to host cell surface receptors and internalization via endocytosis. Subsequent fusion of viral and endosomal membranes in acidic conditions lead to the disassembly of the virion and the release of viral RNA into the cytoplasm. Viral RNA is then translated into a polyprotein subjected to processing by viral and host proteases. Genomic RNA is also replicated before virus assembly takes place to form immature virions. Immature virions mature as they pass through the secretory pathway – notably via the cleavage of prM by furin in the trans golgi network (TGN). Mature virus particles are then released from the host cell.

1.2.3.1 Receptor interaction and entry

It is generally believed that in a natural infection, the initial targets of infection are the macrophages and DCs – through the mannose receptors on macrophages and DC-SIGN (DC-specific ICAM-3-grabbing non-integrin 1) on DCs. Additionally, Langerhans cells have also been shown to be permissive to DENV infection in human skin explants (S. J. Wu et al., 2000). DENV has been found to be permissive in a variety of cell lines including human (K562, U937, THP-1, HepG2, HUVEC, ECV304, Raji, HSB-2, Jurkat, LoVo, KU812), mosquito (C6/36), monkey (Vero, BS-C-1, CV-1, LLC-MK2), hamster (baby hamster kidney, BHK) and murine macrophage (Raw, P388D1, J774) (Cabrera-Hernandez et al., 2007; Chareonsirisuthigul et al., 2007; King et al., 2000; Moreno-Altamirano et al., 2007). Several candidate receptors / attachment factors have been identified, suggesting that DENV might be able to enter cells using multiple molecules. DENV has been shown to interact with heat-shock protein 70 (Hsp70) (Reyes-Del Valle et al., 2005), R80, R67 (Mercado-Curiel et al., 2006) and a 45-kDa glycoprotein (Yazi Mendoza et al., 2002) in mosquito cells. Mammalian cell receptors include heparan sulfate (Y. Chen et al., 1997; Germi et al., 2002), Hsp90 (Reyes-Del Valle, et al., 2005), CD14 (Y. C. Chen et al., 1999), glucose regulating protein 78 (GRP78/BiP) (Jindadamrongwech et al., 2004) and 37/67-kDa high affinity laminin receptor (Thepparit et al., 2004). DENV interact with human myeloid cells via C-type lectin receptors which include

DC-specific intracellular adhesion molecule 3 (ICAM-3)-grabbing non-integrin (DC-SIGN, CD209) (Lozach et al., 2005; Navarro-Sanchez et al., 2003; Tassaneetrithep, et al., 2003), mannose receptor (J. L. Miller et al., 2008) and C-type lectin domain family 5, member A (CLEC5A) (S. T. Chen et al., 2008). One of the newest receptors to be identified, CLEC5A has been proposed to be a crucial macrophage receptor for DENV and a proinflammatory receptor which orchestrates lethal disease in mice (S. T. Chen, et al., 2008; Watson et al., 2011).

DENV has been shown to enter target cells exclusively via clathrin-mediated endocytosis using live-cell imaging to track single virus particles (van der Schaar et al., 2008). In this model, DENV particles diffuse along target cell surfaces before being trapped in a pre-existing clathrin-coated pit which buds into the cytoplasm of the cell to deliver DENV particles to Rab5-positive early endosomes which matures into Rab7-positive, Rab5-negative late endosomes. Fusion of the viral and endosomal membrane occurs primarily in the late endosomal compartment. An alternative clathrin, caveolae and lipid rafts - independent endocytic pathway has also been proposed for the infectious entry of DENV2 into Vero cells (Acosta et al., 2009).

The DENV E protein is generally believed to be the main player in binding to host cell receptor and virus entry. It contains the fusion peptide at the distal end of DII and DIII which is responsible for receptor binding. It has been proposed that the low pH in endosomal compartment triggers dissociation of E homodimers, leading to EDII projecting outwards to expose the fusion peptide to its target membrane. Hydrophobic residues in the fusion loop subsequently inserts into the target membrane, triggering E trimers to assemble. EDIII then shifts and folds back towards the fusion peptide forming a hairpin-like structure. This mechanism of folding back forces the target membrane and viral membrane to bend towards each other for its subsequent fusion. The nucleocapsid of the virus particle is then released into the cytoplasm of the target cell (Harrison, 2008; Heinz et al., 2003; Modis, et al., 2004).

1.2.3.2 Replication and assembly

After the nucleocapsid is released into the cytoplasm of the target cell, the C protein and viral RNA dissociate, releasing the RNA genome and initiating its replication and assembly of viral particles. The RNA genome is translated as a single polypeptide,

which is co- and post-translationally modified by proteases from both the host and virus into 3 structural (E, C and prM) and 7 NS proteins (NS1 NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5).

NS proteins then initiate the replication of viral RNA (Clyde, et al., 2006). The positive strand viral RNA from the infecting virus is used for the synthesis of a negative-strand intermediate, which becomes the template for a new positive-strand viral RNA. As the polypeptide is translated, the prM of the C protein is translocated into the ER lumen from the ER surface where it is synthesized. Similarly, E protein is also translocated into the lumen of the ER, where it forms a stable heterodimer with prM protein. The prM/E heterodimers then associate into trimers, which project from the virus surface as spikes. In contrast, the C protein, which is still attached to the ER and viral RNA, is localized in the cytoplasm. After several rounds of translation and viral RNA replication, the viral RNA is packaged by C protein to generate the nucleocapsid. The outer shell formed by prM/E proteins then encapsulates the nucleocapsid by a mechanism that has yet to be identified, forming new progeny viruses (Clyde, et al., 2006). As previously detailed these newly assembled immature viruses undergo maturation as they travel through the secretory pathway. Mature viruses exit the host cell via exocytosis. However, this cleavage can be incomplete, especially in mosquito cells, resulting in many prM-containing immature virions to be released (van der Schaar et al., 2007).

1.2.4 Immunopathogenesis of DENV

It is not yet fully understood why the majority of individuals infected with DENV resolve dengue infection without complications, while some individuals progress to severe and potentially fatal vascular leakage and hemorrhagic manifestations. Epidemiological observations point to secondary infections with a heterologous dengue serotype as the single greatest risk factor for severe disease. The pathogenesis of dengue infection is complicated and still remains unclear to date. It involves the interplay of multiple factors of both the host and the virus including the individual's immune status, the role of T cells, complement activation, host genetics and virus virulence which will be looked into in the following sections.

1.2.4.1 Humoral Immune Response and Antibody Dependent Enhancement (ADE)

The humoral immune response has been hypothesized to play a key role in controlling DENV infection. Pioneering work carried out by Sabin in the 1950s demonstrated that long-lasting immunity to DENV is linked to the development of neutralizing antibodies that target the original infecting serotype (Sabin, 1952). Sabin found that when human volunteers were completely immune for the length of time the study was carried out, which was at eighteen months, after which they were re-inoculated with the same strain of virus they were initially inoculated with(Sabin, 1952). The hypothesis that dengue immunity is antibody-mediated could also be inferred from other related flaviviruses, for example Japanese encephalitis and Yellow Fever. The vaccines available for both viruses elicit neutralizing antibodies against the virus and the level of neutralizing antibody induced is used as a correlation of immunity (Belmusto-Worn et al., 2005; Hoke et al., 1988; Monath et al., 2002). Similarly in the recent ChimeriVax tetravalent dengue vaccine phase 2b trial in Thailand, protective efficacy of the vaccine was measured by the protective antibody response elicited against each serotype (Sabchareon et al., 2012).

The observation that infection with one dengue serotype conferred life-long protection to that specific serotype (homotypic immunity) but a transient protection against the other serotypes (heterotypic immunity) was first made by Sabin in the 1950s (Sabin, 1950). Immunity to DENV is primarily mediated by neutralizing antibodies targeting the E protein, which is the major protein expressed on the surface of DENV. As discussed in Section 1.2.1.3, there is a 60% to 70% sequence homology of the E protein between serotypes, which could account for the transient nature of heterotypic immunity brought about by cross-reactive antibodies that target E protein, which are protective above a certain threshold. As the concentration of these cross reactive antibodies decrease over time, the individual becomes susceptible to infection with other DENV serotypes.

Numerous epidemiological studies conducted in Asia and Latin America have pointed to secondary infections with heterotypic DENV as a significant risk factor for progression to severe forms of dengue (Endy et al., 2010; Halstead et al., 1967; Sabin,

1952). One of the earliest retrospective studies of the two dengue epidemics in Cuba (caused by DENV1 in 1977 and DENV2 in 1982) demonstrated that children first infected with DENV1 then DENV2 had a high risk of acquiring severe dengue (M. G. Guzman et al., 1990).

One of the hypotheses put forth to account for this phenomenon is known as antibody-dependent enhancement (ADE). This is the process whereby non-neutralizing cross-reactive antibodies elicited by a primary infection bind DENV of a heterologous serotype during a secondary infection, rendering it more likely to infect Fc-receptor bearing cells. The risk of developing severe disease increases since a greater number of cells become infected with DENV and consequently results in a higher viral load *in vivo* (Halstead, 2003; Halstead, et al., 1967). There has also been suggestions that complexes of DENV with non-neutralizing IgG antibodies can ligate Fcγ receptors on monocytes or macrophages to suppress innate immunity, increase IL-10 production and bias Th1 responses to Th2 responses thus increasing infectious output by infected cells (Halstead et al., 2010).

The strongest evidence of the enhancing role of antibodies can be inferred from the occurrence of severe dengue in infants less than 1 year of age born to dengue-immune mothers, of severe dengue during their primary dengue infection (Halstead et al., 1970). Passively acquired maternal anti-DENV antibodies have been found to play a dual role – initially protective, they subsequently increase the risk for developing severe dengue (Halstead, et al., 1970; Kliks et al., 1988). This was first observed in a group of Thai infants where the primary infection with DENV2 resulted in DHF/DSS. It was proposed that these infants received protective maternal anti-DENV antibodies since most women in Thailand of childbearing age are usually immune to more than one DENV serotype. As these antibodies become catabolically degraded, it decreases to a level that is no longer protective but potentially enhancing to cause severe disease (Kliks, et al., 1988).

More recently, it was found that anti-prM antibodies that are highly cross-reactive between DENV serotypes and poorly neutralizing, which could potently promote ADE (Dejnirattisai, et al., 2010). This occurs when the cleavage of prM to M is incomplete and a proportion of viruses express prM on the viral surface. prM-

expressing virus particles are "immature" and by definition non-infectious. However in the presence of anti-prM antibodies, these immature viruses become infectious through the process of ADE when they enter Fc receptor bearing cells as a virus-antibody complex (Rodenhuis-Zybert et al., 2010).

1.2.4.2 The cellular immune response

DENV-specific $CD4^+$ and $CD8^+$ T cell responses are developed when humans are infected with DENV. Such T cell responses have been found to play both a protective role in resolving human DENV infections as well as a pathogenic role that increases disease severity. In a primary DENV infection, $CD4^+$ and $CD8^+$ T cell responses have been observed. These responses are serotype cross reactive but strongest for the infecting serotype (Kurane et al., 2011). $CD4^+$ T cells specific for DENV NS3 have been shown to proliferate, produce gamma interferon (IFNγ) and can lyse target cells (Gagnon et al., 1999). It has been recently showed that there is a preferential targeting of epitopes by $CD8^+$ T cells to NS3 and NS5, while $CD4^+$ epitopes are skewed towards E, C and NS1 (Rivino et al., 2013).

The T cell response to DENV in humans has been implicated in dengue pathogenesis during secondary infection. This phenomenon, known as the "original antigenic sin" involves the dominant expansion of lower avidity cross-reactive T cell responses to the "original" DENV serotype (of the primary infection) over that of naïve T cells with higher avidity for the current (secondary) infecting serotype. Consequently, the elimination of DENV-infected cells is less effective with these low-avidity T cells. It has also been suggested that some of these memory T cells specific for the primary infecting serotype can undergo altered signaling resulting in faulty cytotoxicity for DENV infected cells but instead shift to produce more inflammatory cytokines including gamma interferon (IFNγ) and tumor necrosis factor alpha (TNFα), which can directly or indirectly lead to increased vascular leakage and severe disease (Rothman, 2009). Mongkolsapaya and colleagues showed that in a group of Thai children that T cells specific for dengue had low affinity for the current infecting serotype but a higher affinity for previous infecting serotypes, supporting the "original antigenic sin" theory (Mongkolsapaya et al., 2003). This has been experimentally proven by a study that concluded that the ratio of cells producing IFNγ and TNFα was higher when DENV-specific $CD4^+$ T cells were stimulated ex vivo with antigens from

a heterologous rather than homologous serotype (Mangada et al., 2005). Heightened levels of TNFα have also been detected more often in patients with DF (Green et al., 1999). Additionally, T cell responses in patients suffering from severe dengue produce IFNγ and/or TNFα only but rarely CD107a, a marker of cytotoxic degranulation – unlike patients with mild infections, whereby more CD8+ T cells express CD107a and a minority produce IFNγ and/or TNFα (Duangchinda et al., 2010). This suggests that the delay of viral clearance coupled with cytokine mediated effects increase the risk of severe dengue.

1.2.4.3 Cytokines and Chemokines

As discussed in Section 1.2.4.2, the expression of cytokines and other pro-inflammatory molecules could be triggered in a portion of individuals with secondary DENV infections from innate and activated cross-reactive T cells. This "cytokine storm" following massive T cell activation has been hypothesized to be responsible for targeting vascular endothelial cells, leading to fluid and protein leakage to result in the critical pathological event of plasma leakage and eventually shock which can be fatal, as seen in numerous severe dengue patients (Basu et al., 2008; Rothman, 2011).

In addition to IFNγ and TNFα, IL-6, IL-1, IL-8, CCL2, CCL3 and CXCL10 have also been found to be elevated in the serum of patients with DHF/DSS (Chaturvedi et al., 2000; J. P. Chen et al., 2006; Medin et al., 2005; Navarro-Sanchez et al., 2005; Raghupathy et al., 1998; Rothman, 2011; Tolfvenstam et al., 2011). A lowered level of nitric oxide (NO) associated with elevated IL-10 levels has also been observed in patients with severe dengue. Lowered NO levels are correlated with higher levels of pro-inflammatory cytokines (Khare et al., 1997) while elevated IL-10 indicates reduced platelet levels and function (Libraty, Endy, et al., 2002) which could account for bleeding tendencies in severe disease. It has also recently been shown that DENV activates platelets that produce IL-1β contained within microvesicles that increases vascular permeability (Hottz et al., 2013). Levels of TNFα produced by T cells isolated from patients with DHF and stimulated ex vivo with dengue antigens were found to be higher, suggesting the positive correlation of TNFα levels with disease severity in humans (Chaturvedi, et al., 2000). Macrophage migration inhibitory factor (MIF) is another proinflammatory cytokine, when produced with TNFα during the

host response to DENV infection, favor the manifestation of more severe disease (Assuncao-Miranda et al., 2010; Chuang et al., 2011).

The chemokine system appears to have both a protective and pathological role in DENV infection. CXCL10 competes with DENV for cellular receptors resulting in lowered viral replication (J. P. Chen, et al., 2006). Mice deficient for CXCL10 and CXCR3 also exhibited lowered resistance to primary DENV infection as a result of faulty activation of $CD8^+$ T cells and NK cells (Hsieh et al., 2006). On the other hand, CCR2 and CCR4 are associated with pathological roles – $CCR2^{-/-}$ mice have reduced lethality rates after primary DENV infection as in $CCR4^{-/-}$ mice, although in both models there is no change in viral load (Guabiraba et al., 2010).

1.2.4.4 Complement

The potential pathogenic role of complement activation in the initiation of DSS was first proposed in the 1970s, from a study conducted in hospitalized Thai patients with serologically confirmed dengue (Bokisch et al., 1973). Elevated levels of DENV NS1, complement component 5a (C5a) and terminal complement complex SC5b-9 were found in pleural fluids of patients with DSS (Avirutnan et al., 2006b). Notably, DENV NS1 has been found to be the major protein responsible for the activation of human complement (Avirutnan, et al., 2006b). Anti-NS1 antibodies also direct complement attack to infected cells, generating terminal SC5b-9 complement complexes that can damage plasma membrane and increase vascular permeability (Bossi et al., 2004). Cross-reactive non-neutralizing anti-DENV antibodies have been found to activate complement on surfaces of endothelial cells infected with DENV leading to the release of complement peptides (Avirutnan et al., 1998).

1.2.4.5 Virus virulence

The occurrence of severe dengue may not always be during secondary infections - there have been reports of severe dengue in primary infections. This would challenge the hypotheses of cross-reactive antibodies and/or T cells in the pathogenesis of severe dengue. The various genotypes within each serotype of DENV are distributed across various geographical locations. Although several DENV serotypes have been co-circulating in the Americas, it was not until the 1981 Cuban epidemic that the first cases of DHF occurred in the region, coinciding with the introduction of a Southeast

Asian genotype of DENV2 to the area (Rico-Hesse, et al., 1997). Conversely, it was observed that there were no DHF cases during an epidemic caused by DENV2 of the American genotype, 5 years following a DENV1 epidemic in Peru. This further supports the notion of the lower virulence of the American genotype DENV2 (Watts et al., 1999). Conversely, the Southeast Asian genotype of DENV2 seems to have higher virulence, replicating to higher titers in human DCs than that of the American genotype and possessing higher potential for transmission in Aedes aegypti mosquitoes which can account for its potential for causing severe epidemics (Anderson et al., 2006). Increased disease severity was observed in clinical studies conducted in Managua, Nicaragua, when a new DENV2 virus clade NI-2B replaced Asian/American NI-1 which was originally circulating in the area (OhAinle et al., 2011). Subsequent analysis of the virus in patient blood samples revealed that the new clade is a fitter virus. Avirulent strains have been shown to replicated less well *in vitro* compared to virulent strains (Morens et al., 1991). Antigenic variations between DENV strains have been proposed as a likely factor for severe dengue. Sera from DENV1 immune individuals could neutralize the American but not Asian genotypes of DENV2 (Kochel et al., 2002), a phenomenon confirmed in vivo using primate model of infection (Bernardo et al., 2008). One likely explanation for the increased virulence of some DENV strains is that highly pathogenic strains have structural differences in several viral proteins conferring them higher replicative ability in major human target cells, producing more progeny viruses per target cell and hence higher viremia compared to a strain with lower pathogenic potential (Leitmeyer et al., 1999).

1.2.4.6 Host genetic factors

Host genetic factors have also been implicated as a contributing factor to severe dengue. It has been observed that people of the Negroid race have a lower risk of DHF/DSS compared to people of Caucasoid race (de la et al., 2007). Variants of the vitamin D receptor (VDR) gene containing the t allele at position 352 as well as the individuals homozygous for arginine at position 131 of the FcγRIIA gene have been associated with resistance to severe disease (Loke et al., 2002). A strong association between the protection against DF and G allele of variant DCSIGN1-336 (a promoter variant of CD209 which encodes for DC-SIGN1) has been found in three independent cohorts in Thailand (Sakuntabhai et al., 2005). Several studies have investigated the association between the variation in human leucocyte antigen (HLA) genes and the

severity of DENV infections. Polymorphisms in HLA class I have been found to be associated with susceptibility to DHF, including HLA-A2 and HLA-B in a cohort of Thai children (Chiewsilp et al., 1981), HLA-A*0207 in patients with secondary DENV1 and DENV2 infections and HLA-B*51 in patients with secondary DENV infections (Stephens et al., 2002). HLA-DR4 (a HLA class II product) homozygous individuals have been found to be more likely to develop severe dengue compared to DR4-negative individuals in a group of Mexican patients, suggesting that HLA-DR4 could be a protective genetic factor against severe dengue in Mexicans (LaFleur et al., 2002).

1.2.5 Prevention of Dengue

With the wide geographical spread of multiple serotypes of DENV and the potentially fatal risk of DENV infections, there is an urgent need to control the spread of DENV and design specific treatments for DENV infection, which is currently non-existent. The lack of an approved vaccine for human use, DENV-specific treatment and even markers to predict disease severity has resulted in massive economic and healthcare burden on endemic countries. Strategies for the prevention and/or treatment of dengue include vector control, development of a vaccine for DENV and specific therapeutics for DENV infections.

1.2.5.1 Vector Control

One of the most straightforward strategies for the prevention of DENV infections would be the elimination of vector populations that transmit the virus. The use of chemical pesticides for this purpose has been met with varying degrees of success in various endemic regions. Larvicides could be used to control Aedes aegypti vector in the field, and new formulas with higher efficacies have been developed and reported (Thavara et al., 2013). However there are concerns with using chemical control for the mosquito vectors. Firstly there are environmental concerns that the release of insecticides will upset the ecological balance in the affected areas and toxic compounds could be magnified through the food chain that can eventually adversely affect human health. Secondly is the development of pyrethoid resistance in Aedes aegypti which can confer the mosquitoes knockdown resistance (Linss et al., 2014). Alternatively, amore environmentally friendly, non-toxic and biodegradable strategy for vector control could be achieved with synthesized silver nanoparticles containing leaf extracts of Leucas aspera, which has been shown to have good larvicidal activity (Suganya et al., 2013). Similarly, extracts of the medicinal plant Erythrina indica has also been shown to be a potential eco-friendly insecticide for Aedes aegypti (Govindarajan et al., 2014). Another strategy for vector control is the use of Wolbachia, an insect symbiont that can reduce the ability of Aedes aegypti to transmit DENV (Bourtzis et al., 2013).

1.2.5.2 Vaccines undergoing clinical evaluation

A recent study using mathematical modeling of the transmission of dengue in Thailand, accounting for seasonality, population age, co-infection with all DENV serotypes, cross protection, ADE effect and vector-host transmission dynamics, has found vaccination to be the single most effective method of combatting DENV infection (Knerer et al., 2013). Indeed, the development of an effective vaccine against all four serotypes of DENV is instrumental in the control and eventual eradication of dengue. The main focus of dengue vaccine development is to elicit a neutralizing antibody response since T cells are thought to play a lesser role where dengue vaccine-mediated protection is concerned. The E protein expressed on the surface of DENV is responsible for host-cell binding and entry. Hence, neutralizing antibodies that target E protein is a probable correlate of protection. Most vaccines under development strive to present the E protein to the host in the hope of inducing protective anti-E antibody. Since DENV exists as 4 antigenically distinct serotypes, a DENV vaccine should effectively present all 4 E proteins in a tetravalent formulation for the induction of homotypic neutralizing antibody against each serotype to confer long-lasting protection against all 4 serotypes of DENV. This is particularly challenging since sub-neutralizing levels of anti-E antibodies could lead to immune enhancement via the ADE effect resulting in potentially fatal severe disease. The ideal vaccine should possess a range of characteristics including the ability to confer life-long immunity, have a good safety profile, an effective immunization regime, the ability to stimulate both neutralizing antibodies and cell mediated immunity to all four serotypes, be affordable as well as be easily stored and transported (Webster et al., 2009). A variety of vaccine strategies are currently being pursued, with several in clinical development stage.

1.2.5.2.1 Chimeric Vaccines

The chimeric vaccine candidate that has progressed the furthest along clinical evaluation is Sanofi Pasteur's CYD-TDV (ChimeriVax). It consists of DENV prM and E structural genes inserted into yellow fever (YF) 17D vaccine backbone (Guy, Saville, et al., 2010). The YF 17D vaccine backbone was selected as this YF vaccine has proved to be safe and efficacious in its 60 years of use (Guy, Guirakhoo, et al.,

2010). Phase 1 trials of this vaccine found it to be safe with relatively low viremia detected in vaccinated individuals (Capeding et al., 2011; Morrison et al., 2010). The results of a phase 2b clinical study conducted in Thailand on children aged 4 to 11 years were recently reported in 2012 (Sabchareon, et al., 2012). The vaccine was found to be safe but overall protective efficacy was a mere 30.2%. This was mainly due to the fact that DENV2, which was endemic in the region at the time of the trial, had an efficacy of only 9.2%, skewing the overall efficacy. Although the protective efficacy observed from this trial was somewhat disappointing, the vaccine was found to be safe with no disease enhancement or vaccine-related severe adverse events (SAE) observed. Interestingly, it was observed that although there was satisfactory immunogenicity engendered towards all 4 serotypes, the vaccine was not protective against DENV2 – against the general consensus that the protective efficacy of a vaccine is correlated with the induction of protective neutralizing antibodies. Large scale phase 3 studies involving 30,000 subjects in Latin America and Asia begun in 2011 and will soon offer additional data on the safety and efficacy of this vaccine candidate.

1.2.5.2.2 Live Attenuated Vaccines

Live attenuated vaccines involve the usage of weakened viruses that has sufficiently lessened replicative ability to reduce pathological effects but still retain the ability to elicit adaptive immune responses. The existence of effective live attenuated vaccines against other members of the *Flavivirus* family like yellow fever (17D vaccine) and Japanese encephalitis virus (Ixiaro) suggests that such a vaccine approach for DENV is probable, but is unfortunately more elusive. Attenuated DENV vaccine candidates were developed by passaging each DENV serotype multiple times in Vero cells and combined into a tetravalent formula, by researchers from Mahidol University in Bangkok (Bhamarapravati et al., 2000). This formulation was used for phase I and II clinical trials, however some adverse reactions to the vaccine were observed and not all subjects seroconverted to all four DENV serotypes (Sabchareon et al., 2004; Sabchareon et al., 2002; V. Sanchez et al., 2006). Hence, this vaccine candidate was not considered for further clinical evaluation. Another vaccine formulation made using similar methods by the Walter Reed Army Research Institute (WRAIR) also exhibited uneven immunogenicity and reactogenicity (W. Sun et al., 2003). Its

protective efficacy has yet to be evaluated although improved formulations were found to be safe and immunogenic in a phase II study (Thomas et al., 2013).

Site directed mutagenesis of the viral genome could also be used to attenuate the viral genome. The National Institute of Allergy and Infectious Diseases (NIAID) utilized a 30-nucleotide deletion (Δ30) in the 3'-UTR and found it to be effective in attenuating DENV1 and DENV4 yet retaining their immunogenicity (designated DEN1Δ30 and DEN4Δ30) (Men et al., 1996; Whitehead et al., 2003). However there was less success with DENV2 and DENV3, so DEN4Δ30 was used as a genetic backbone (designated DEN2/4Δ30 and DEN3/4Δ30)(Blaney, Hanson, Firestone, et al., 2004; Blaney, Hanson, Hanley, et al., 2004). Each monovalent form was tested for attenuation and immunogenicity both in animal models and human trials, and various tetravalent mixtures are currently undergoing Phase I clinical evaluation (Durbin et al., 2011).

DENVax is developed by Division of Vector-Borne Infectious Diseases of the Centers for Disease Control and Prevention (CDC) and licensed by Inviragen. Using PDK-53 vaccine strain DENV2 as a backbone, DENV2 structural proteins were replaced with those from DENV1,3 or 4 to derive the tetravalent formulation (Butrapet et al., 2000). After initial *in vivo* testing in cynomolgus macaques (Osorio, Brewoo, et al., 2011), the optimal dosage combination of each serotype is currently being tested in phase I and II clinical trials (Osorio, Huang, et al., 2011).

1.2.5.2.3 Subunit Vaccines

Recombinant subunit vaccines offer several advantages over live attenuated vaccines – they are safe, able to induce a balanced immune response to the four DENV serotypes and it is possible administer them with an accelerated immunization schedule that could elicit complete immunity to lower the risk of ADE. However, these vaccines require the use of adjuvants and multiple doses for optimal immunogenicity hence they may not confer the long-lasting immunity that could be achieved with live attenuated vaccines. Traditionally, the target for subunit vaccines has been the E protein that contains most of the neutralizing epitopes. Recombinant E

protein can be produced in a variety bacterial and mammalian cells. Hawaii Biotech (later acquired by Merck & Co) engineered constructs containing prM and 80% of E protein (80E) from each of the four DENV serotypes, to be stably transformed into Drosophila melanogaster Schneider 2 (S2) cell expression system for the expression of E protein in its native structure (Clements et al., 2010; Coller et al., 2011). ISCOMATRIX adjuvant was found to induce long lived neutralizing antibody titers across all DENV serotypes, when administered as a tetravalent formulation *in vivo* (Clements, et al., 2010) and phase I clinical trials began in 2012.

1.2.5.3 Antibodies as Therapeutics

The development of a safe and efficacious tetravalent dengue vaccine is no doubt the best strategy for reducing global dengue burden. Its development has been thought to be feasible since there are licensed vaccines against other flaviviruses such as YFV (Pulendran, 2009) and JEV (Ohrr et al., 2005) . However, there is currently no commercially available dengue vaccine despite more than 70 years of research (Thomas, 2014). As such, there is justification to explore other prophylactic and/or therapeutic options in the form of an antiviral or anti-inflammatory dengue drug.

Antibodies are an important component of the adaptive immune response, critical for recognition of target antigens as well as signaling of effector functions. Human monoclonal antibodies (mAbs) have become an increasingly important class of therapeutic drugs, making up the single largest class of molecules entering clinical evaluation in recent years (A. L. Nelson et al., 2010). Additionally, more than half of the FDA-approved biologics in the past 3 years are formulations based on mAbs (Kling, 2012, 2014; Osborne, 2013).

1.2.5.3.1 Antibodies

Human antibodies, collectively known as immunoglobulins (Ig), have the same general structure. Differentiated B cells produce antibodies in response to infection or immunization. Antibodies bind to and neutralize pathogens or prepare them for uptake and destruction by phagocytes. Antibodies are highly specific – each recognizes binds one particular antigen. They are roughly Y-shaped proteins consisting of 2 identical pairs of heavy (H) and light (L) polypeptide chains forming the two arms of the Y.

The tips of the two arms of the Y make up the variable (V) region of the antibody unique to the antibody and are responsible for antigen recognition. The stem of the Y is the constant (C) region made up of the unpaired regions of the H chains that determines the isotype of the antibody and is responsible for interaction with effector molecules. The stem region is termed the fragment crystallizable (Fc) region while the antigen-binding arms are termed fragment variable (Fv) regions and a flexible "hinge" links these two regions.

There are five subclasses or isotypes of Ig in humans defined by the structure of the H chain that determine their functional activity. They comprise of IgM, IgD, IgG, IgA and IgE. Antibodies of different subclasses are adapted to work in various compartments within the body to provide protective functions best suited for that compartment. Since each V region can class switch to associate with any C region, progeny of a single B cell with a pre-defined specificity can be adapted to best function in each body compartment. B cells expressing IgG have undergone selection in germinal centers for their increased affinity for its antigen and constitute the principal class of antibody found in serum and non-mucosal tissues.

Almost all approved therapeutic antibodies are of the IgG format (Reichert et al., 2005). IgG molecules are large molecules of approximately 150kDa in molecular weight. They consist 2 H chains of approximately 50kDa and 2 L chains of approximately 25kDa. Disulphide bonds link each pair of H and L chain. The H chains consist of a variable domain (V_H domain) and three constant domains (C_H1, C_H2 and C_H3 domains). L chains contain a variable domain (V_L domain) and a single constant domain (C_L domain). The complementarity-determining regions (CDRs) of the antibody are made up of six loops, three of which are in each of the V_H and V_L domains. The sequence of amino acids in the CDRs is unique to the antibody and determines its specificity. Diversity generated in the CDRs through genetic recombination can be immense, making up an antibody repertoire large enough to potentially bind a variety of antigens on pathogens. Human IgG bound to its antigen on a target cell can interact with IgG receptors expressed by effector cells or with complement 1q (C1q) through its Fc region to support destruction of the target cell via antibody dependent cell mediated cytotoxicity (ADCC) or complement dependent cytotoxicity (CDC) respectively. The long half life of human IgG (approximately 21

days) is mediated by the ability of the Fc region of IgG to bind the neonatal Fc receptor (FcRn) - FcRn interaction with IgG protects it from degradation as well as recycles it back into the circulation from the acidic endosomal compartments of endothelial cells and circulating monocytes that internalize serum IgG (Roopenian et al., 2007).

1.2.5.3.2 Strategies to Generate Human Antibodies

The natural human antibody response is a rich and diverse source of therapeutic reagent with exquisite specificity and neutralizing activity. With the advancement in technology, there are various tools that allow researchers to harness the therapeutic potential of the human antibody response by isolating monoclonal antibodies (mAbs) generated as part of a natural infection or vaccination. mAbs are defined as monospecific antibodies derived from a single clone of B cells. The earliest work involving the generation of mAbs was done by Milstein's group in 1975, who developed the hybridoma technology to produce mouse mAbs (Kohler et al., 1975). It involved the fusion of primary murine B cells with a suitable fusion partner, usually non-secretory myeloma cell lines deficient for the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT). Cell fusion is made possible by polyethylene glycol that enhances cell adherence and nuclei exchange. Successful fusion cells are selected by growing cells in hypoxanthine, aminopterin and thymidine medium (HAT medium) where only hybrid cells can proliferate. Fusion cells or hybridomas as they are known as, are then screened for specificity and cloned by limiting dilution to obtain homogenous long-lived cell clones secreting mAbs (C. Zhang, 2012).

With the advent of the hybridoma technology, large quantities of consistent and predictable antibody preparations with exquisite specificity against the desired antigen could be produced. However, murine mAbs have limitations in terms of being successful therapeutic agents for humans, as they had short serum half-life in humans, are not sufficiently able to activate human effector functions and could possibly elicit immune reactions against proteins of murine origin (Winter et al., 1993). These problems were addressed to a large extent with advances in genetic engineering to produce chimeric mouse-human or "humanized" antibodies that have been licensed

for human therapeutic use (Carter, 2006). More recently, methodologies for generating mAbs directly from human B cells with a pre-defined specificity have led to the isolation of fully human mAbs naturally generated in response to pathogens. The advantages of these human mAbs include heightened safety and efficacy profiles and greater relevance to human disease when compared to mAbs generated in other species like rodents.

B cells that have encountered pathogen antigen become activated, subsequently differentiating into memory B cells and antibody-secreting cells. These B cells that secrete antibodies can be further separated into short-lived plasmablasts and long-lived plasma cells. Both memory and antibody-secreting B cells have been utilized in the generation of human mAbs. A major difficulty in generating such human mAbs is to pinpoint B cells that express immunoglobulins with the chosen specificity and functional characteristic, which is usually its neutralising capacity. To achieve this, there are currently several methods including (1) the usage of Epstein barr virus (EBV) to "immortalize" antigen-specific B cells into cell lines that constantly secrete a pre-selected immunoglobulin (Kozbor et al., 1981; Roome et al., 1984; Steinitz et al., 1977) (2) phage display technology to obtain antigen-specific immunoglobulins and (3) directly cloning and expressing immunoglobulin genes from human B cells with a pre-selected antigen specificity using single B cell cloning techniques.

1.2.5.3.2.1 Immortalization of Human B Cells

The immortalization of human memory B cells using Epstein-Barr virus (EBV) transformation was first described in 1977 (Steinitz, et al., 1977). EBV is a virus of the family *herpesviridae*, best known as the causative agent for infectious mononucleosis. They demonstrated that human B cells could be converted into established cell lines upon infection with EBV, a process termed 'immortalisation', that preserves the antibody-secreting capabilities of the B cells. This paved the way for establishing human cell lines that produced antibodies of choice. This methodology was used to generate human mAbs against various pathogens like hepatitis C virus, Mycobacterium Leprae and human immunodeficiency virus (HIV) but achieved limited success since this method was found to be inefficient in producing large quantities of specific B cells (Atlaw et al., 1985; Habersetzer et al., 1998). It was later found that activating B cells using toll-like receptor (TLR9) agonist

CpG DNA or other polyclonal mitogens before and during EBV infection enhanced transformation efficiencies considerably. Traggiai and colleagues first described this improved methodology in 2006, to generate human mAbs against severe acute respiratory syndrome (SARS) virus (Traggiai et al., 2004). Since then there have been mAbs produced against a variety of pathogenic viruses using this methodology or adaptations of it, including respiratory syncytial virus (RSV) (Collarini et al., 2009), chikungunya virus (CHIKV) (Warter et al., 2011), human cytomegalovirus (Macagno et al., 2010), influenza (Corti, Suguitan, et al., 2010; Krause et al., 2012; Krause et al., 2011; X. Yu, T. Tsibane, et al., 2008), HIV (Corti, Langedijk, et al., 2010; Walker et al., 2009), and DENV.

Immortalisation of human B cells with EBV generally involves isolating IgG^+ memory B cells or total peripheral blood mononuclear cells (PBMCs) in the presence of the supernatant of an EBV-producing cell line with a TLR9 ligand and allogenic PBMCs to provide additional co-stimulatory signals. Primary B cells cultured under such conditions proliferate and secrete antibodies, which are screened for their antigen binding and/or neutralizing capacity. At this stage, the antibodies produced by the B cell pool are usually polyclonal. B cells producing antibodies of the desired specificity are cloned by limiting dilution to obtain single clones that secrete monoclonal antibodies. After further screening for the desired specificity at the single B cell clone level, individual Ig H and L chains are cloned and sequenced (Traggiai, et al., 2004).

Besides using EBV to immortalize human B cells, an alternative approach to obtain human B cell lines as a source of human mAbs involve the expression of B cell lymphoma-6 (Bcl-6) and BCL-xL proteins in peripheral blood memory B cells and subsequently culturing them in the presence of follicular helper T cells, CD40 ligand (CD40L) and interleukin-21 (IL-21) (Kwakkenbos et al., 2010). These transduced B cells express high levels of signaling-competent membrane-bound B cell receptor (BCR) that facilitates isolation of B cells based on antigen binding on a flow cytometry assay, appreciably increasing the frequency of antigen-specific cells and eliminates the need for extensive subcloning. Another group combined EBV immortalization of human B cells with hybridoma-based technology to obtain human hybridomas secreting the desired mAb using a novel electrofusion protocol that fuses EBV-transformed human B cells with myeloma partners (X. Yu, P. A. McGraw, et

al., 2008). They demonstrated that functional human mAbs specific for RSV and influenza H3N2 virus strain could be obtained.

The advantages of using the strategy of EBV-immortalized B cells is the potential for combining it with high-throughput screening, which can enable the isolation of B cells from the memory B cell pool many years after exposure to the pathogen. Antibodies specific for the influenza virus strain responsible for the 1918 H1N1 pandemic that is no longer in circulation have been isolated using this method (X. Yu, et al., 2008). A major disadvantage of this methodology is the involvement of screening massive numbers of B cells, typically in tens of thousands, to obtain approximately ten B cells with the desired specificity (Wilson et al., 2012).

1.2.5.3.2.2 Phage Display Technology

Phage display technology is a high throughput methodology that allows the isolation of B cells with rare specificities. It is a component of human antibody library display technique, which can include bacteria, yeast, mammalian cell and ribosome besides phage. Phage libraries consist of pools of bacteriophage virions that express a unique Ig fragment on the exterior of the virion. These libraries are constructed from variable Ig genes of immunized or non-immunized individuals, to obtain an immune library and a naïve library respectively. The library is constructed by randomly amplifying the V_H and V_L genes of human B cells obtained from immune or non-immune sources before they are combined as random V_H and V_L pairs. This library is cloned for expression as single chain fragment variable (scFv) or antigen-binding fragments (Fab) onto the phage surface. The phage library is then selected against the target antigen using a process known as panning. This involves several rounds of binding the Ig fragment displayed on the phage to the target antigen, washing away unbound phages, eluting the binders. This process is repeated for higher enrichment of specific binders. Antigen-specific phages are amplified by infection of Escherichia coli (E. coli). To screen for the desired specificity, several *in vitro* assays including enzyme linked immunosorbent assay (ELISA) and fluorescent-activated cell sorting (FACS) can be utilized. As soon as antibodies with the desired specificity has been identified, the Ig genes of the variable regions can be cloned into human IgG expression vectors and transfected into suitable cell lines for expression as fully human mAbs (Marasco

et al., 2007). This methodology has made possible to generation of neutralizing mAbs against various pathogens including WNV, rabies virus, human and avian influenza strains using B cells isolated from the bone marrow, bloods and spleen of immunized or convalescent subjects (Marasco, et al., 2007).

Although phage display technology has enabled researchers to isolate antibodies to a variety of pathogens, a major drawback of this methodology is that the antibodies that are isolated may not be an accurate representation of the antibody repertoire originally expressed by the B cell pool. This is due to the fact that the V_H and V_L regions of human Ig are cloned separately and randomly paired. Hence some of these pairings are artificial and a given H and L chain pair may not have gone through germinal center selection and/or may not be self-tolerant. However, this artificial pairing of H and L chain could result in a greater diversity of antibody that can be desirable. The affinity of a human phage antibody has been improved by chain shuffling of V_H and V_L genes with the variable gene repertoire of non-immune donors (Marks et al., 1992).

1.2.5.3.2.3 Single-Cell Expression Cloning

The latest advancements in human mAb isolation involves the use of single-cell reverse transcription polymerase chain reaction (RT-PCR) to obtain V_H and V_L Ig gens from single B cells that have been pre-selected for their antigen specificity. These B cells are selected using antigen baiting, which involves sorting B cells bound to fluorescently labeled antigens using FACS. These genes can be subsequently transfected and expressed in eukaryotic expression systems from which mAbs can be expressed and purified. The mAbs produced are then tested for antigen specificity and neutralizing activity. By using FACS to detect B cells specific for the antigen of interest, only B cell subsets that express Ig on their surface can be detected. Moreover, the antigen bait used has to be highly specific and stable. Despite these limitations, this methodology is highly efficient and selective – 80% to 90% of sorted B cells were found to produce Ig with the desired specificity (Di Niro et al., 2012). It should be noted that this methodology screens for binding specificities rather than functional characteristics, although the design of the antigen bait can potentially isolate B cells expressing neutralizing mAbs (Wilson, et al., 2012).

1.3 Animal Models of DENV Infection

The lack of licensed vaccines and antivirals for DENV has been hampered largely by the lack of a suitable animal model. The natural hosts for DENV are humans and mosquitoes, although a sylvatic cycle has been observed in non-human primates (NHP) in Africa (Diallo et al., 2003) and Southeast Asia (Wolfe et al., 2001). Infection of certain NHPs can engender detectable viremia but minimal clinical manifestations. Immunocompetent mice do not fully support viral replication (Yauch et al., 2008) hence various immunodeficient mouse models have been utilized.

1.3.1 Non-Human Primates (NHP)

Several NHPs have been used in dengue research, including rhesus monkeys (Macaca mulatta), cynomolgus monkeys (Macaca fascicularis), and owl monkeys (Aotus nancymaae). NHPs have been observed to recapitulate certain hallmarks of DENV infection in humans like viremia and antibody response but with limited clinical signs of disease that can be observed in humans (Zompi et al., 2012). Studies conducted with WNV have found that mosquito inoculate 10^4 to 10^6 PFU of virus with each feeding so similar levels have been assumed for DENV, with this dosage commonly used to mimic a mosquito bite (Styer et al., 2007). Rhesus monkeys infected subcutaneously with 10^5 PFU of DENV resulted in sustained viral replication, albeit at diminished levels compared to that in humans and limited to lymphoid rich tissues and skin, accompanied by lymphadenopathy, lymphocytosis and leukopenia (Marchette, et al., 1973). Intravenous infection of rhesus monkeys with a high dose ($1x10^7$ PFU/animal) of 16681 DENV2 resulted in hemorrhage 3 – 5 days post-infection, a major clinical sign of DHF, including petechiae and hematomas, coagulopathy and increased levels of D-dimers but no other clinical signs commonly seen in humans such as fever, anorexia or lethargy (Onlamoon et al., 2010). Rhesus monkeys immunosuppressed with cyclophosphamide, which lowers total white cell count, then infected with DENV2 displayed prolonged infection (Marchette et al., 1980). Rhesus macaques sometimes displayed thrombocytopenia although no other apparent clinical signs were observed after s.c. DENV infection (Halstead et al., 1973a, 1973b). ADE has also been demonstrated in NHP models. Higher levels of viremia were observed upon secondary infection with a heterologous DENV serotype,

suggesting ADE might have caused this increase in viral load via the action of cross-reactive antibodies (Halstead, et al., 1973b). Rhesus monkeys passively infused with human dengue-immune serum also developed 50-fold higher viremia compared to control animals (Halstead, 1979). In a more recent study, passive transfer of sub-neutralizing levels of a humanized monoclonal antibody IgG 1A5 resulted in 100-fold higher viremia in rhesus monkeys compared to control animals (Goncalvez et al., 2007). Sequential infections with heterologous serotypes of DENV induced cross-reactive antibody responses in NHPs, with the highest antibody titers against the primary infecting serotype, similar to the "original antigenic sin" phenomenon observed in humans (Koraka et al., 2007).

NHPs have been used in the testing of vaccines since the viremia and anti-DENV antibody profile they develop after infection mimics what is observed in humans (Marchette et al., 1974). A typical readout consists of any reduction in the duration of viremia, highest viral titer and magnitude of antibody response. Several live-attenuated vaccines that have reached clinical trials have been tested for their infectivity, replication kinetics and immunogenicity for optimal tetravalent formulations in NHP models (Durbin, et al., 2011; Guy, et al., 2010; Osorio, et al., 2011).

1.3.2 Mice

Wild-type mice are generally resistant to infection with wild-type DENV clinical isolates since rodents are not a natural host of DENV. However there is an urgent need for a small animal model to understand the immunopathogenesis of DENV infections as well as to test potential antivirals and vaccines since the use of NHPs can be prohibitive due to its technicality and high cost. To overcome the problem of the lack of virus replication in mice, a number of strategies have been utilized to develop mouse models of DENV infection. Some of these include intracerebral (i.c.) inoculation with mouse-brain adapted virus, the use of immunocompromised mice (which lack one or more components of the interferon response pathway) and humanized mice.

1.3.2.1 Wild-Type (WT) Mice

The initial mouse models of DENV infection involved intracerebral route of infection using DENV passaged in the brain of suckling mice and possessed increased neurovirulence (Cole et al., 1969). This mouse adapted virus led to paralysis in some mice after a period of incubation.

Certain strains of wild-type immunocompetent mice can support some level of virus replication. When immunocompetent A/J mice are infected intravenously with $1x10^8$ PFU per mouse of PL046 DENV2, a non-mouse adapted local isolate from Taiwan, transient viremia could be detected by RT-PCR 2 days post infection (p.i.) while transient thrombocytopenia developed 10 to 13 days after primary or secondary infection (Huang et al., 2000). However, paraplegia was observed in most of these mice, a clinical manifestation that is not commonly observed in humans. In the same study, the susceptibility of BALB/c and C57BL/6 to PL046 DENV2 was found to vary – less than 10% of these mice displayed signs of hind limb paralysis and manifestation of thrombocytopenia varied in the mice. In another study with A/J mice infected with $1x10^8$ PFU per mouse of PL046 DENV2, a subset of mice developed paralysis and infectious DENV particles could be recovered from the CNS. Nevertheless the infected did develop a DENV-specific IgM and IgG response and had elevated hematocrit and lowered white blood cell count (Shresta, Kyle, Robert Beatty, et al., 2004). This non-physiological route of infection and/or involvement of the central nervous system, a characteristic that is controversial but generally agreed to be rarely involved in human DENV infections (Lum et al., 1996; Patey et al., 1993), limit the scope of immunocompetent mice as a useful model for DENV infection.

1.3.2.2 SCID Mice and Humanized Mice

Severe combined immunodeficient (SCID) mice have defective humoral and cellular immune responses since they lack functional T cells and B cells, and can easily be engrafted with tumor cells prior to infection with DENV. K562 cells, a human erythroleukemia cell line, can be engrafted onto SCID mice and K562-SCID mice displayed neurological signs of paralysis 2 weeks after PL046 DENV2 was injected into K562 cell masses (Y. L. Lin et al., 1998). Additionally, there was a high level of virus detectable in peripheral blood and brain, indicating the successful replication of

DENV2 in the K562-SCID mice (Y. L. Lin, et al., 1998). SCID mice transplanted with HuH-7 cells, a hepatocarcinoma cell line, and inoculated with DENV4 directly into the tumor developed a peak viremia of up to 8.0 $\log_{10}$ PFU/ml serum on day 6 to 7 p.i., with detectable virus in the brain and liver (Blaney et al., 2002). Similarly, SCID mice grafted with HepG2 cells and infected with DENV2 via the i.p. route had detectable viremia in the serum, liver and brain, developed paralysis 13 to 18 days p.i. at which point there was thrombocytopenia, elevated hematocrit and increased TNF-α which are associated with severe disease (An et al., 1999).

Since DENV can replicate better in human cells than mouse cells, several groups using different strategies have taken the approach of developing humanized mice, which are mice engrafted with human progenitor cells, as a potential small animal model for dengue infection. This was achieved by backcrossing SCID mice with non-obese diabetic (NOD) mice with defective natural killer cell function and deficient hemolytic complement response due to lack of complement C5, and subsequently with IL2Rγ –knock-out (KO) mice. The resulting NOD/SCID/ IL2RγKO mice are deficient both in their innate and adaptive immune responses, allowing them to better accept human cells and tissues (Zompi, et al., 2012). It has been previously established that NOD/SCID mice reconstituted with $CD34^+$ hematopoietic progenitor cells can develop functional human myeloid and plasmacytoid DCs (Cravens et al., 2005; Palucka et al., 2003). Immature DCs such as Langerhans cells in the skin are found to be one of the major replication sites of the virus after it has been delivered via a mosquito bite (Marovich et al., 2001) and a mouse model with high levels of functional human DCs would be useful. It was first demonstrated in 2005 that NOD/SCID mice engrafted with human $CD34^+$ cells and infected subcutaneously with K0049 DENV2 developed fever, rash and thrombocytopenia, all relevant clinical signs observed in humans (Bente et al., 2005). When such mice were infected with various genotypes of non mouse-adapted DENV2 subcutaneously, a significant drop in platelets 10 days post-infection was observed in most mice, coupled with elevated body temperature and erythema (Mota et al., 2009). Infection with a virulent strain of DENV2 resulted in infection in the bone marrow, spleen and blood, coupled with the detection of cytokines and chemokines such as TNF-α, IFN-γ, IL-2, sIL-2R, IL-6, IL-10, MCP-1 and VEGF which peaked at day 8 p.i. (Mota et al., 2011). However, these mice failed to generate a sustained antibody response (Kochel, et al., 2002; Mota, et

al., 2009, 2011). The most recent approach has been the "bone marrow liver thymus" BLT mice, whereby human immune system (HIS) BLT-NOD/SCID mice were infected intravenously with a clinical DENV2 isolate to result in sustained viremia and infection of leukocytes in lymphoid and non-lymphoid organs, increased levels of serum cytokine levels and IgM antibodies that are able to neutralize DENV2 (Frias-Staheli et al., 2014).

$RAG2^{-/-}\gamma_c^{-/-}$ mice engrafted with human $CD34^{+}$ hematopoietic stem cells (RAG-hu mice) were shown to develop multi-lineage human hematopoiesis, including T-cells, macrophages, B cells and dendritic cells (Kuruvilla et al., 2007). Viremia could be detected in infected RAG-hu mice, together with fever although no other clinical signs could be observed. Interestingly, human anti-DENV antibodies could be detected in the serum of infected mice, both IgM at 2 weeks and IgG at 6 weeks post infection (Kuruvilla, et al., 2007).

1.3.2.3 Immunocompromised Mice

Severely immunocompromised mice such as BALB/c athymic nude (nu/nu) (Hotta et al., 1981a)and $RAG1^{-/-}$ (Shresta, Kyle, Snider, et al., 2004) mice are permissive to DENV infection which is lethal in both. But death is primarily attributed to paralysis, a symptom irrelevant to human infection. Since it was proposed that interferon-mediated immunity is essential for resolving DENV infection in mice (Shresta, et al., 2004), the potential of STAT-deficient (S. T. Chen, et al., 2008; Shresta et al., 2005) and IFN-α/β and –γ receptor (A. J. Johnson et al., 1999; Shresta, et al., 2004) deficient mice were explored as potential models of DENV infection.

1.3.2.3.1 Athymic Nude Mice

Athymic nude (nu/nu) BALB/c mice that lack T cells were infected intraperitoneally with mouse adapted DENV1 (Mochizuki strain) did not exhibit viremia (Hotta, et al., 1981a). The mortality rate of these mice was at 40% 2 weeks post infection, with most of the mice euthanized upon exhibiting paralysis. In mice exhibiting paralysis, highest viral titers were detected in the brain followed by skeletal muscle and heart tissues (Hotta et al., 1981b). Replicating virus was also detected in the lymph node, lung, liver, spleen and kidney for some mice.

1.3.2.3.2 AG129 Mouse Model

AG129 mice of the 129/Sv(ev) background lacking both type I (alpha and beta) and type II (gamma) interferon (IFN) receptors was first described by Johnson and Roehrig in 1999 (A. J. Johnson, et al., 1999). They found that when these mice were infected with mouse-adapted NGC DENV2, 100% of the mice developed neurological abnormalities and succumbed to infection (A. J. Johnson, et al., 1999). Both IFN-α/β and IFN–γ receptor knockouts were found to be essential for viral replication in peripheral tissues and subsequent disease (Shresta, et al., 2004). The AG129 model of DENV infection has been found by numerous groups to recapitulate many relevant attributes of DENV infection in humans. AG129 mice have been found to be permissive to clinical isolates from all four serotypes, with effective replication of the virus in the spleen, lymph node, bone marrow, peripheral blood and liver to some extent (Calvert et al., 2006; A. J. Johnson, et al., 1999; Kyle et al., 2007; Schul et al., 2007; Shresta, et al., 2004; Shresta et al., 2006). Comparison of the tropism of DENV infection in humans and AG129 mice by immunostaining of autopsy samples and mouse tissue samples with anti-NS3 antibody revealed similarities in the cell types (macrophages, DCs, hepatocytes and bone marrow-derived myeloid cells) with active viral replication in humans and the AG129 model (Balsitis et al., 2009). Thrombocytopenia, a hallmark of sever dengue in humans, has also been found in the AG129 model (Schul, et al., 2007).

One major advantage of the AG129 model is its apparent functional adaptive immune response, although it should be noted that there is a lack of IFN-mediated responses between the innate and adaptive immunity (van den Broek et al., 1995) and the IgG subclass distribution is also heavily biased to IgG1 (van den Broek, et al., 1995). The role of humoral response in a secondary DENV infection was found to be greater than the cellular immune response since passive transfer of DENV-immune serum to naïve mice conferred greater protection against heterologous DENV challenge, as opposed to adoptive transfer of DENV-immune cells (Kyle, Balsitis, et al., 2008). The role of T cells in DENV infections was investigated in AG129 mice and it was determined that mice depleted of CD8+ T cells had significantly higher viral loads compared to non-depleted control mice (Yauch et al., 2009). These $CD8^+$ T cells produced IFN-γ, TNF-α and displayed cytotoxic activity *in vivo* (Yauch, et al., 2009).

One of the hallmarks of severe dengue disease in humans is shock brought about by increased vascular permeability. A lethal "vascular leak syndrome" was produced in AG129 mice upon infection with D2S10, a virulent strain of DENV2 generated by alternately passaging clinical isolate PL046 DENV2 between non-neuronal tissues of mice and mosquito cells (Shresta, et al., 2006). Two mutations in the E protein (N124D and K128E) were produced, causing weakened affinity for heparan sulfate leading to increased serum half-life, elevated systemic viral loads and TNF-α in the serum (Prestwood et al., 2008). D2S10 was found to increase vascular permeability in numerous tissues of infected mice and elevate circulating levels of TNF-α which was postulated to be a key mediator of D2S10-induced disease in AG129 mice since treatment with anti-TNF-α antibody prevented early D2S10-induced mortality (Shresta, et al., 2006). A non mouse-adapted strain D2Y98P inoculated subcutaneously into AG129 mice also induced vascular permeability but structurally intact blood vessels in moribund animals, as reported in patients with DHF/DSS (G. K. Tan et al., 2011).

The effect of ADE was also investigated in the AG129 model. Passive administration of serotype-specific and serotype cross-reactive sera and anti-DENV monoclonal antibodies resulted in antibody-enhanced lethal disease in AG129 mice infected with either mouse-adapted or clinical isolates of DENV2, with the manifestation of several hallmarks of severe disease in humans including thrombocytopenia, vascular leakage, elevated serum cytokine levels (TNF-α and IL-10) and a 20-fold increase in viremia (Balsitis et al., 2010). Notably, there was an increased infection in macrophages, DCs in numerous organs and endothelial cells in the liver, similar to the cell types infected by DENV in humans (Williams et al., 2009). It was observed that administration of the N297Q variant of anti-DENV antibody E60 that cannot bind FcγR diminished enhancement and instead promoted neutralization to reduce viral load, indicating that FcγR interaction was required for ADE *in vivo* (Balsitis, et al., 2010).

Recently, an AG129 model of ADE was described, where progeny mice of DENV1-immune mothers demonstrated higher viremia, increased vascular leakage that correlated with earlier death time points when they were infected with a lethal strain of DENV2, as compared to progeny mice from DENV naïve mothers (Ng et al.,

2014). Moreover, the disease enhancement observation correlated with age of these mice born from DENV-immune mothers that mirrored epidemiological observations in humans – 2-week old mice were protected from DENV2 challenge but 3,5 and 8-week old mice displayed disease enhancement. This was associated with serum levels of maternal anti-DENV1 antibodies and their neutralizing activity against DENV2. This model offers a new strategy to test the possibility of potential therapeutic antibodies competing with pre-existing circulating antibodies and their implication in disease enhancement.

2 Objectives of this Project

In view of the lack of an approved vaccine and specific treatment for dengue, we sought to develop an effective therapeutic for DENV infections. Since the induction of serotype-specific neutralizing antibodies is a key component for the resolution of DENV infection in humans, we postulate that the administration of neutralizing antibodies could be a therapeutic strategy for combating DENV infections.

The specific objectives of this project are:

1. Generate recombinant neutralizing antibodies specific for each serotype of DENV.
2. Carry out extensive *in vitro* characterization of the antibodies including their binding, neutralizing and enhancing characteristics.
3. Determine the binding epitopes of human antibodies on DENV.
4. Evaluate their *in vivo* neutralizing and/or protective capabilities as potential therapeutic candidates.

3 Materials and Methods

3.1 Cell Lines

Baby hamster kidney-21 (BHK-21) (ATCC CCL-10) cells were maintained in Roswell Park Memorial Institute 1640 (RPMI 1640) (Hyclone) supplemented with 10% fetal bovine serum (FBS) at 37°C with 5% CO_2. Human erythroleukemia K562 cells (American Type Culture Collection (ATCC) CCL-243) were maintained in RPMI 1640 supplemented with 10% FBS at 37°C with 5% CO_2. *Aedes albopictus* clone C6/36 (ATCC CRL-1660) cells were maintained in Leibovitz's L-15 (Gibco) supplemented with 10% FBS at 28°C. African green monkey kidney Vero cells (ATCC CCL-81) were propagated in Medium 199 (M199) (Gibco) supplemented with 10% FBS at 37°C with 5% CO_2.

3.2 Virus strains and virus propagation

All four DENV serotypes were used in this study, as summarized in Table 2.

Serotype	Strain	Source	Cell line virus propagated in
1	EHI	Professor Ng Mah Lee, NUS. Laboratory-adapted clinical isolate from Environmental Health Institute (EHI)	C6/36
	Westpac 74	Novartis Institute of Tropical Diseases (NITD), Singapore	C6/36
	16007	Michael Diamond, Washington University School of Medicine, USA	C6/36
	DC164D01	Environmental Health Institute (EHI)	C6/36
	3146SL	Michael Diamond, Washington University School of Medicine, USA	C6/36
	PVP159	Duke-NUS Graduate Medical School	C6/36
2	EHI	Environmental Health Institute (EHI)	C6/36
	New Guinea C (NGC)	Dr Brendon Hanson, Defense Science Organization (DSO) Singapore	Vero
	D2Y98P	Environmental Health Institute (EHI), 1998 DENV2 Singapore human isolate	C6/36
	MT5	A/Prof Sylvie Alonso, National University of Singapore	C6/36
	DC117D01	Environmental Health Institute (EHI)	C6/36
	DC186D01	Environmental Health Institute (EHI)	C6/36
	DC146D01	Environmental Health Institute (EHI)	C6/36
3	EHI	Environmental Health Institute (EHI)	C6/36
	802DK1	Environmental Health Institute (EHI)	C6/36
	791DK1	Environmental Health Institute (EHI)	C6/36
	2892DK1	Environmental Health Institute (EHI)	C6/36
4	EHI	Environmental Health Institute (EHI)	C6/36

Table 2 **List of virus strains, source and cell lines viruses were propagated in.**

To propagate virus stocks, a confluent monolayer of C6/36 cells grown in a non-vented T-75 flask (Nunc) was inoculated with 1ml of virus stock containing $1x10^7$ PFU of virus. Virus adsorption was carried out for 1 hour at 37°C, with flasks rocked every 15 minutes. 9ml of growth medium containing 2% FBS was added and incubated until cytopathic effect was observed. Culture supernatant was collected cellular debris cleared by centrifugation for 10 minutes at 10,000rpm. Viral supernatant was collected and stored at -80°C in 1ml aliquots.

3.3 Virus Quantification – Plaque Assay

A standard plaque assay was used to determine the number of infectious viral particles. BHK-21 cells were grown to approximately 80% confluency in 24-well plates (Nunc). Virus stock was serially diluted 10-fold, from 10^{-1} to 10^{-6} in RPMI 1640. 100µl of each dilution was added per well of BHK-21 monolayer in triplicates. Virus was allowed to infect for 1 hour at 37°C with gentle rocking every 15 minutes. Thereafter 1ml of overlay medium (1% (w/v) carboxymethylcellulose in RPMI supplemented with 2% FBS) was added per well. Cells were incubated for 4 to 6 days at 37°C with 5% CO_2 before overlay medium was discarded and cells fixed paraformaldehyde and stained with 1% (w/v) crystal violet dissolved in 25% (v/v) paraformaldehyde for 1 hour. Plaques were scored visually.

3.4 Hybridoma culture and antibody purification

Hybridoma cell lines D1-4G2-4-15 (ATCC HB-112), 3H5-1 (ATCC HB-46), D3-2H2-9-21 (ATCC HB-114) were cultured in RPMI 1640 supplemented with 10% FBS at 37°C with 5% CO_2. Culture supernatant was harvested after approximately 4 days and purified using Protein A sepharose columns on a fast protein liquid chromatography (FPLC) apparatus (GE Pharmagia). Buffer exchange to phosphate buffered saline (PBS) was carried out on the purified antibodies. Purity of antibodies were verified by SDS-PAGE and binding specificity assessed by ELISA. A standard Bradford assay was used to quantify antibody concentration.

3.5 Purification of DENV

DENV was purified by polyethylene glycol (PEG) precipitation. When cytopathic effect was observed, virus supernatant was harvested and clarified by centrifugation and passed through a 0.22µm filter. 20% w/v PEG6000 in 20mM NaCl (pH 7.4) was added to virus supernatant at a ratio of 1:1 and the mixture was stirred overnight at 4°C before it was centrifuged at 15,000 g for 30 minutes to obtain the pellet. Pellet was resuspended in HNE buffer (5mM Hepes, 150mM NaCl, 0.1mM EDTA) and layered onto a 20% sucrose cushion on top of a 30% sucrose cushion for further

purification by ultracentrifugation at 80,000g for 18 hours at 4°C. Purified virus was resuspended in HNE buffer incubated for 2 hours on ice then stored at -80°C. Infectious viral titer was determined on a standard plaque assay (Section 3.3).

3.6 Isolation of primary $CD22^+$ cells

Dengue patients were recruited from either National University Hospital (NUH) or Tan Tock Seng Hospital (TTSH). Informed consent was obtained from all patients recruited and all procedures carried out under an approved protocol from the National University Institutional Review Board. Blood sample was obtained from these patients approximately 60 days after disease onset. Peripheral blood mononuclear cells (PBMCs) were isolated on a Ficoll-Paque (GE Healthcare) differential density gradient. $CD22^+$ mature B cells were isolated by positive selection using human CD22 MicroBeads (Miltenyi Biotec) as per manufacturer's instructions. Briefly, PBMCs were incubated with CD22 MicroBeads before suspension was passed through a LS autoMACS column whereby $CD22^-$ fraction is eluted and $CD22^+$ fraction subsequently flushed out of the column.

3.7 Generation of anti-DENV antibodies using Epstein-Barr virus (EBV) -immortalized B cells

The generation of the anti-DENV1 antibody human mAb 14C10 used in this study is the subject of a United States Patent (US 2013/0259871 A1, published 3 October 2013). Briefly, approximately 30 $CD22^+$ B cells isolated were seeded per well in 96-well U-bottom plates (Nunc) in AIM-V medium (Gibco) containing 3g/ml polyclonal B cell activator CpG oligodeoxynucleotide 2006 (5'-TCGTCGTTTTGTCGTTTTGTCGTT-3'), human B cell growth factors Interleukin-2 and Interleukin-4 (1000U/ml each) in the presence of EBV (30% supernatant of HEK-293 transfected with a recombinant B95-8 strain genome) and $5x10^4$ cells per well of irradiated allogenic PBMCs. Culture supernatants were screened for the presence of antibodies specific for DENV using ELISA, CPE-assay or PRNT. B cell lines producing DENV specific antibodies were used as a source of mRNA for IgG heavy and light chains gene amplification. Sequences of heavy and light IgG genes were cloned into an in-house pCMV vector and transfected into Freestyle 293F cells to

produce recombinant antibody. Antibody expressed was purified on a Protein A column.

3.8 Generation of anti-DENV2 antibody 10.15

3.8.1 Construction of a Human Immune Library

Anti-DENV2 human mAb 10.15 was generated from an immune library was derived from the genetic material of an individual who recovered from dengue. A blood sample was taken approximately 2 months after disease onset and $CD22^+$ B cells obtained as described in Section 3.6. An immune library was generated based on the method by de Haard and colleagues (de Haard et al., 1999). Briefly, total RNA was isolated from $CD22^+$ B cells using the Purelink RNA Mini Kit (Invitrogen) followed by cDNA synthesis using the Superscript III 1^{st} strand cDNA synthesis kit (Invitrogen). Random hexamer primers were used to synthesize heavy chain while oligo dT primers for light chain synthesis. This is followed by 2 rounds of PCR to amplify the heavy and light (kappa and lambda) chains of IgG using primer sets specific for IgG light chain (kappa and lambda) and heavy chain. Restriction sites are then added with a second round PCR. Heavy chain PCR products are then ligated into pCES-1 MCS HC Pst plasmid vector and light chain PCR products into pCES-1 MCS LC Pst plasmid vector before they are electroporated into TG1 E coli. separately. The sequences are checked before heavy chain is cloned into light chain vector and then elctroporated into TG1 E coli. KM13 helper phage was added to TG1 containing both heavy and light chain at a MOI of 1:40 and phages are subsequently recovered for storage at -80°C. A detailed protocol is attached in the Appendix.

3.8.2 Panning of library against DENV2

Before the actual panning of the phage displayed Fab library against the antigen of interest, DENV2, additional steps were utilized to reduce non-specific phages. Firstly, an anti-prM antibody, D29, was used to bind any phages that target prM to eliminate potentially cross-reactive, non-neutralizing antibodies. prM protein is highly conserved between the 4 dengue serotypes Also, the library was allowed to bind to DENV4 as a depletion step for phages/antibodies that are potentially cross-serotype-

specific and to increase the percentage of DENV2 specific phages/antibodies. Unbound phages were finally panned against the desired antigen, DENV2.

The human immune phage library is panned against DENV2. Purified DENV2 diluted 1:20 in PBS was coated onto immune tubes and allowed to rotate for 1.5 hours at room temperature or at 4°C overnight. Tubes were then washed with PBS and blocked with 4% skim milk. Phage was also pre-blocked with 2% skim milk before added to the pre-blocked tube and incubated for 1.5 hours at rotating room temperature. TG1 E coli containing heavy and light chain plasmids (from overnight culture) were grown in 30ml of 2TY at 37°C shaking at 700 rpm till OD600 = 0.5 ± 0.05.

3.8.3 Conversion of Fab to IgG and Expression of IgG

Phages expressing Fab fragments with the desired binding specificity were selected for conversion into full length IgG using an in-house pCMV-Fab-IgG1 vector that encodes for the human IgG1 constant framework as previously described (Hanson et al., 2006). To express the full length human IgG, HEK293T cells were transfected with constructs encoding the IgGs using lipofectamine 2000 (Invitrogen) and cells were maintained at 37°C and 5% CO_2. Culture supernatant was collected 72 hours later and secreted IgG purified with Protein A sepharose.

3.9 Enzyme Linked Immunosorbent Assay (ELISA) Binding Assay

For the sandwich ELISA, 96-well Maxisorp plates (Nunc) were coated with 5µg/ml murine HB112 antibody overnight. Plates were washed thrice with PBS containing 0.01% Tween-20 (PBST). Plates were blocked using 5% skim milk at room temperature (RT) for 2 hours. $1x10^5$ PFU of DENV was added per well and incubated for 2 hours at RT, followed by three washes with PBST. For testing of binding activity of the antibodies to purified EDIII, Maxisorp plates were coated overnight with 0.25µg per well of TSV201 EDIII, kindly provided by Dr Lok Shi-Mei.

Primary antibodies serially diluted in 5% skim milk was added and incubated for 1 hour at RT then washed thrice using PBST. Secondary antibody goat anti-human IgG Fc conjugated with horse radish peroxidase (HRP) (Pierce) was diluted in 5% skim milk was added and incubated for 1 hour at RT, followed by three washes using PBST. 3,3',5,5'-tetramethylbenzidine (TMB) substrate (GE Healthcare) was added as indicated by the manufacturer and the reaction stopped using 1M sulphuric acid. Optical density was read at 450nm using a microplate reader (Bio-Rad).

3.10 Plaque Reduction Neutralisation Test (PRNT) Neutralisation Assay

PRNT was used to determine the degree of neutralization of an antibody. BHK-21 cells were grown to approximately 80% confluency in 24-well plates (Nunc). Serially diluted antibody was incubated with approximately 40PFU of DENV for 1 hour at RT. 100µl of each dilution was added per well of BHK-21 monolayer in triplicates. After incubating for 1 hour at 37°C with gentle rocking every 15 minutes, 1ml per well of overlay medium (1% (w/v) carboxymethylcellulose in RPMI supplemented with 2% FBS) was added. After 4 to 6 days incubation at 37°C with 5% CO_2, cell monolayers were fixed with paraformaldehyde and stained with 1% (w/v) crystal violet dissolved in 25% (v/v) paraformaldehyde for 1 hour. Plaques were scored visually. Percentage neutralization was calculated with the following formula.

$$Percentage\ plaque\ reduction = \frac{\#\ plaques\ in\ absence\ of\ antibody - \#\ plaques\ with\ certain\ antibody}{\#\ plaques\ in\ absence\ of\ antibody} \times 100\%$$

$PRNT_{90}$ (concentration of antibody needed to decrease the number of plaques to 10% of that in the absence of antibody) and $PRNT_{50}$ values (concentration of antibody needed to decrease the number of plaques to 50% of that in the absence of antibody) were calculated by nonlinear regression in GraphPad Prism v5.0 for Mac Os X (GraphPad Software, Inc).

3.11 Antibody-Dependent Enhancement (ADE) Assay

ADE assay was used to determine the enhancing effect of a neutralizing antibody at sub-neutralizing concentrations. Antibody was serially diluted 12 times and a fixed amount of DENV was incubated with each dilution for 1 hour at RT. K562 cells were added at a multiplicity of infection (MOI) of 0.1 and incubated for 2 hours at 37°C. Cells were washed twice with PBS and incubated with RPMI supplemented with 2% FBS for 72 hours before virus produced was quantified by a standard plaque assay (Section 3.3).

3.12 Pre- and Post-attachment Neutralization Assay

To determine if the anti-DENV antibodies inhibited viral infectivity at a pre- or post-attachment step, a modified PRNT assay was performed. In the post-attachment assay, BHK-21 cells cultured in 24-well plates were pre-chilled to 4°C before infected with 100 PFU of DENV per well. DENV was allowed to adsorb for 1 hour at 4°C before unbound virus was washed off using ice-cold PBS. The various anti-DENV antibodies were added at the specified concentrations. Virus-antibody complexes were allowed to form for 1 hour at 4°C before increasing the temperature to 37°C for infection to occur for 1 hour. 1ml per well of overlay medium (1% (w/v) carboxymethylcellulose in RPMI supplemented with 2% FBS) was added.

For the pre-attachment assay, DENV was mixed with specified concentrations of anti-DENV antibodies for 1 hour at 4°C before the virus-antibody complexes were added onto BHK-21 cells for 1 hour at 37°C. Cells were then washed thrice with cold PBS followed by the addition of 1ml per well of overlay medium (1% (w/v) carboxymethylcellulose in RPMI supplemented with 2% FBS).

After 4 to 6 days incubation at 37°C with 5% CO_2, cells were fixed paraformaldehyde and stained with 1% (w/v) crystal violet dissolved in 25% (v/v) paraformaldehyde for 1 hour. Plaques were scored visually.

3.13 Immunoprecipitation pull down

Vero cells were infected with NGC DENV2 at a MOI of 0.5 and incubated for 48 hours at 37°C and 5% CO_2. Infected cells were washed once with PBS, scraped and lysed with 1ml of ice-cold lysis buffer (1% Triton X-100 with Complete Protease inhibitor cocktail (Roche)) for 30 minutes on ice. Cell suspension was centrifugated at 12000g for 2 minutes to remove cell debris. Supernatant containing the cell lysate was then pre-cleared with 50% Protein A sepharose beads (Pierce) for 1 hour at 4°C and rotating at 15rpm. Cell lysate was immunoprecipitated with anti-DENV2 human antibodies 10.15, 12.17 and 14.19 and humanized hu4G2 as a positive control at 4°C overnight. DENV-antibody complexes were pulled down using 50% Protein A sepharose beads by incubating for 2 hours at 4°C and rotating at 15rpm. Suspension was centrifugated for 1 minute at maximum speed and washed thrice with wash buffer (lysis buffer diluted 10 times with PBS). Supernatant was discarded and SDS loading buffer was added to the pellet containing sepharose beads bound to DENV-antibody complexes. Samples were boiled at 100°C for 5 minutes, centrifugated at maximum speed for 1 minute and supernatant resolved on 12% polyacrylamide gel. Proteins were transblotted onto nitrocellulose membrane and subjected to western blot analysis. Blots were incubated with murine antibody 3H5 to detect E protein followed by secondary antibody goat anti-mouse IgG (H+L), conjugated to HRP (Pierce, Catalogue Number 31430). Western Lightning Plus ECL (Perkin Elmer, Catalogue Number NEL103001EA) was added to the membrane, incubated for 1 minute before being exposed to an X-ray film and developed.

3.14 Dot Blot

2µg of purified and properly refolded EDIII protein (strain TSV01, kindly provided by Dr Lok Shee-Mei) was dotted on nitrocellulose membrane and allowed to air dry. Non-specific sites were blocked with 5% skim milk in PBST for 1 hour at room temperature before incubating with anti-DENV antibodies for 1 hour at room temperature. Primary antibodies tested include anti-DENV human antibodies 10.15, 12.17, 14.19, murine 3H5, humanized 3H5 and humanized 4G2, used at a concentration of 1µg/ml. Membrane was washed with PBST 3 times before

incubating with secondary antibody for 1 hour at room temperature. Secondary antibodies include goat anti-human IgG Fc cross adsorbed secondary antibody, conjugated to HRP (Pierce, Catalogue Number 31413) and goat anti-mouse IgG (H+L), conjugated to HRP (Pierce, Catalogue Number 31430). Western Lightning Plus enhanced chemiluminescence substrate (ECL) (Perkin Elmer, Catalogue Number NEL103001EA) was added to the membrane, incubated for 1 minute before being exposed to an X-ray film and developed.

3.15 Sodium dodecyl – polyacrylamide gel (SDS-PAGE) and Western Blot

25µl of purified NGC DENV2 was diluted with 5x loading dye and boiled for 10 minutes at 95°C before loading onto 12% SDS-PAGE gels and separated. Resolved SDS-PAGE gels were transblotted onto PVDF membranes, blocked with 5% skim milk and probed with either hu3H5 or 10.15 followed by goat-anti-human HRP-conjugated secondary antibody. Bands were developed with Western Lightning ECL before being exposed to an X-ray film and developed.

3.16 Time-Lapse Confocal Live Cell Imaging

Time-lapse live cell microscopy was performed on an inverted A1Rsi confocal microscope (Nikon, Japan) fitted with Plan-Apochromat 100X 1.4 numerical aperture (N.A.) lens. Live cell imaging was performed using live BHK cells grown on 25 mm glass coverslips (Marienfeld GmbH, Germany) mounted on a chamber holder (Nikon, Japan). BHKs were cultured at a density of $4x10^4$ per well 24 hours prior to the start of the experiment. To detect Alexa Fluor (AF)-488 labeled antibodies and AF-647 labeled DENV1 viruses at the same time, the 488nm line of an argon ion laser and the light of a 633nm helium neon laser were directed over an HFT UV/488/633 beam splitter. A NFT 545 beam splitter combined with a 505–530 band pass filter were utilized to detect fluorescence from AF-488 while a 650 long pass filter was used to detect AF-647. Microscopic images were taken every 30 seconds for 30 to 60 minutes at an interval of 1 frame per second (fps). The entire live cell imaging experiment was performed using live BHK cells incubated in a microscope cage incubator system

(OkoLab, Italy) maintained at 37°C with 5% CO_2. Acquired images were analyzed and processed using Nikon Imaging Software (NIS) Elements software (Nikon, Japan).

3.17 Quantification of Intracellular Fluorescence

To determine how the presence of antibodies (14C10, hu4G2 or isotype control) affects the endocytosis of DENV1, the relative level of fluorescence from AF-647 labeled DENV1 within the live BHK cells was measured. Following treatment with the respective antibodies, images of a minimum of 100 cells were randomly captured using the A1Rsi confocal microscope from three independent experiments. Intracellular regions of each BHK cell were delineated manually with the "region of interest" (ROI) function of NIS Elements software (Nikon, Japan). Relative fluorescence level of AF-488 inside each BHK cell was determined using ROI statistics function of the software. Microsoft Excel was utilized to calculate the average, standard deviation and significance using a Student's t-test for each cell population. The fluorescence from cell populations infected with DENV1 only without antibody treatment was normalized to 100% and used to compare with DENV1 infected cell populations with antibody treatment.

3.18 Cryo-electron Microscopy (Cryo-EM)

PVP159 DENV1 was incubated with Fab 14C10 at a molar ratio of one Fab molecule to every E protein at 37°C for 30 minutes followed by 2 hours at 4°C. Following which, the DENV1-Fab complex was flash-frozen in liquid ethane on lacey carbon grids coated with a thin layer of continuous carbon. Images of DENV particles were captured using a 300-kV FEI Titan Krios. The following parameters were used: 16 e^{-}/Å2 electron dose, 47,000x magnification and 1 to 3 mm defocus range. A 4K by 4K Gatan charge-coupled device camera was used capture images with a pixel size of 1.9 Å per pixel. Programs boxer of the EMAN program suite was used to box 5566 particles and the contrast transfer function parameters determined using ctfit. Multipath simulated annealing protocol (Liu et al., 2007) was used to determine orientation of the DENV particles and the program make3d in EMAN (Ludtke et al.,

1999) was used to create a three-dimensional map with an ultimate resolution of 7Å as determined by the Fourier shell coefficient of 0.5. E protein domains were fitted separately since it was found that the crystal structure of DENV1 E protein does not fit well on to the cryoEM density map. The "fit-in-map" function of Chimera was used to optimize the fit of the molecules into the cryoEM map set at 4s contour level (Pettersen et al., 2004). The epitope was mapped by determining the connecting densities to the Fab molecules at 2.5s contour level. A homology model of the variable region of 14C10 was created on the program Modeller, based on the Fab crystal structure (PDB code 2GHW) with the highest sequence identity with 14C10. Heavy and light chains of this model were fitted into the 3s contour level cryoEM map individually in the two possible orientations of the Fab molecule.

3.19 *In Vivo* experiments

3.19.1 Infection of Mice

AG129 mice (129/SvEv) mice lacking alpha/beta interferon (IFN-α/β) and gamma interferon (IFN-γ) receptors were obtained from B&K Universal. They were kept in individually ventilated cages under specific pathogen-free conditions. All experimental procedures were performed under standards set by the National University of Singapore Institutional Animal Care and Use Committee (IACUC). 8 to 9 week old mice were used in all experiments. Mice were administered with DENV either via the intraperitoneal (i.p.) or the subcutaneous (s.c.) route.

3.19.2 Virus Quantification of Plasma of Infected Mice

Blood was obtained from 5 mice from each group at every time point for the quantification of viremia in infected mice. Mice were anaesthetized using a cocktail of ketamine, atropine and diazepam administered i.p before retro-orbital puncture was performed to obtain blood samples. Collected samples were centrifuged for 10 minutes at 6000*g* to get plasma. Plasma samples were stored at -80°C and thawed to assess for infectious viral titer on a standard plaque assay (Section 2.3). Viremia titers obtained were plotted as the logarithm with base 10 of the mean and standard

deviation of 5 mice in each group at every time point (log_{10} (mean ± SD) PFU/ml). Results represent the trend observed in at least 2 independent experiments.

3.19.3 Virus Quantification of tissues of Infected Mice

To assess the quantities of infectious viral particles in various tissues of infected AG129 mice, various tissues and organs were collected. These include the brain, intestine, liver, spleen, kidney and bone marrow. 5 mice per group (antibody treated and control) were sacrificed and extensively perfused with PBS. The entire tissues for the brain, intestine, liver, spleen and kidney were harvested from each mouse. Harvested tissues were kept on ice and their wet weights recorded. Tissues samples were then thoroughly homogenized on ice with a mechanical homogenizer (Omni) for 5 minutes in 1ml of RPMI 1640 to obtain a thorough homogenate. Homogenates were centrifuged at 14000rpm for 10 minutes at 4°C to clarify them. Pelleted debris was discarded and the supernatant passed through a 0.22μm pore sized syringe filter. The volume of the filtrate was measured and considered to be representative of the total amount of infectious virus in the harvested tissue. The filtrate was assayed on a standard plaque assay (Section 3.3) using 10-fold serial dilutions ranging from 10^{-1} to 10^{-4}. Data was plotted as log_{10} (mean ± SD) PFU/ml of 5 mice per treatment group. Results are representative of 2 independent experiments.

3.20 Statistical Analysis

Results were analyzed using the two-tailed unpaired Student T-test. Results were considered statistically significant (*) when the *p* value obtained was less than 0.05. All calculations were performed using GraphPad Prism v5.0 for Mac Os X (GraphPad Software, Inc).

4 Results Chapter One - Anti-DENV1 Antibody 14C10

14C10 is a serotype-specific neutralizing fully human monoclonal antibody against DENV1, induced by a natural dengue infection. The antibody was generated from screening EBV-immortalized B cells isolated from a patient who recovered from a natural infection, based on the method described by Traggiai and colleagues (Traggiai, et al., 2004). A brief outline of the generation of this antibody is outlined in Figure 3.

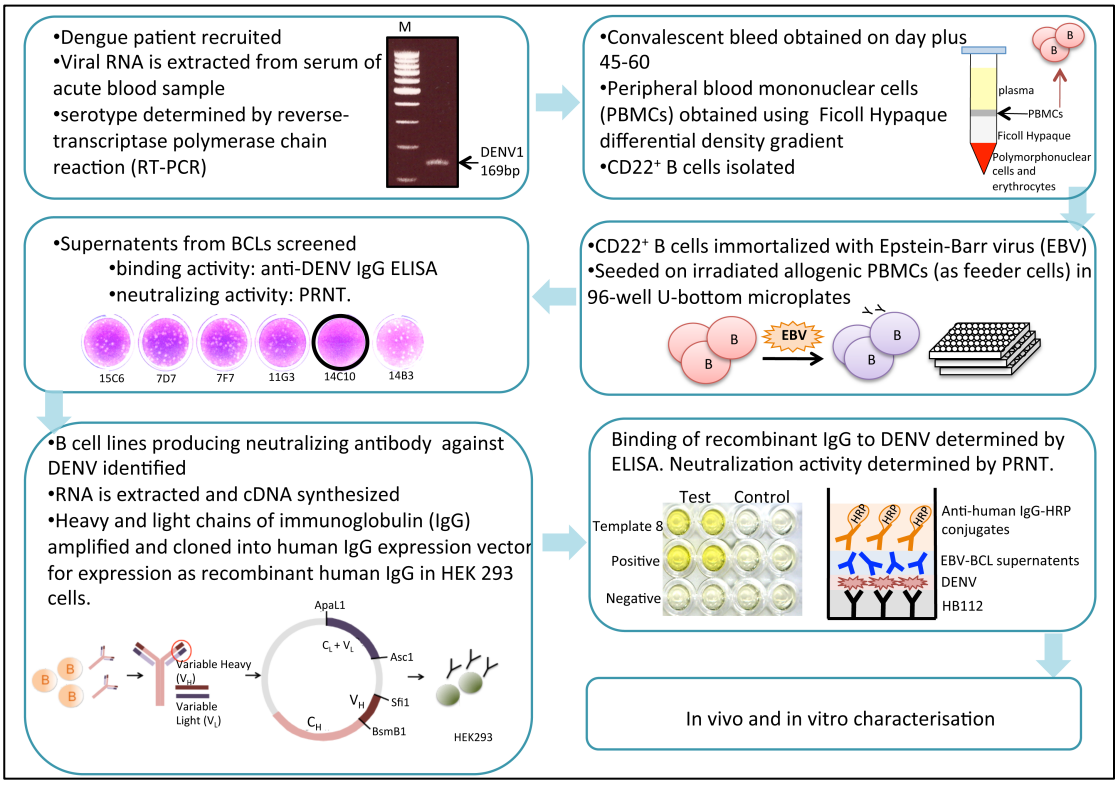


Figure 3 **Experimental outline of the generation of human anti-DENV1 mAb 14C10.** A convalescent DENV1 patient was recruited into our study. A blood sample was collected and total PBMCs isolated using a Ficoll-Hypaque differential density gradient. $CD22^+$ memory B cells were separated using MACS anti-human CD22 magnetic beads following manufacturer's instructions. $CD22^+$ B cells were immortalized using EBV and seeded onto irradiated allogenic PBMCs as a feeder layer in U-bottom 96-well microplates. Supernatants secreted by these B cell lines (BCLs) were screened for their binding activity to DENV1 using ELISA and neutralizing activity for DENV1 using PRNT. Clone 14C10 was identified for its neutralizing activity for DENV1 converted into a recombinant protein. To achieve this, total RNA was extracted from this clone and cDNA synthesized. Heavy and light chains of IgG were amplified using primers specific for human IgG, amplified and subsequently cloned into a human IgG expression vector for expression of recombinant human IgG in HEK293 cells. Expressed protein was isolated from cell supernatant on a Protein A column. Expressed 14C10 IgG was tested for its specific binding and neutralizing activity before subsequent *in vitro* and *in vivo* characterization was carried out.

4.1 Binding activity of 14C10 in comparison to humanized 4G2 (hu4G2)

The binding affinity of 14C10 in comparison to humanized mouse monoclonal antibody 4G2 (hu4G2) was compared on a sandwhich ELISA (Figure 4A). The five DENV1 isolates tested include EHI.D1 (Genotype 1), 16007 DENV1 (Genotype 2), DC164 D01 (Genotype 3), Westpac74 (Genotype 4) and 3146SL (Genotype 5). Each DENV1 isolate was tested against serially diluted concentrations of antibodies. The results demonstrated inter-genotype differences in binding activity, with binding activity directly proportional to antibody concentration. Additionally, hu4G2 bound more strongly than 14C10 to all genotypes except for genotype 5. This suggests that strongly neutralizing antibodies such as 14C10 may not necessarily demonstrate better binding activities. Binding of both 14C10 and hu4G2 were significantly reduced for genotype 2 DENV1 DC164D01 (Figure 4B).

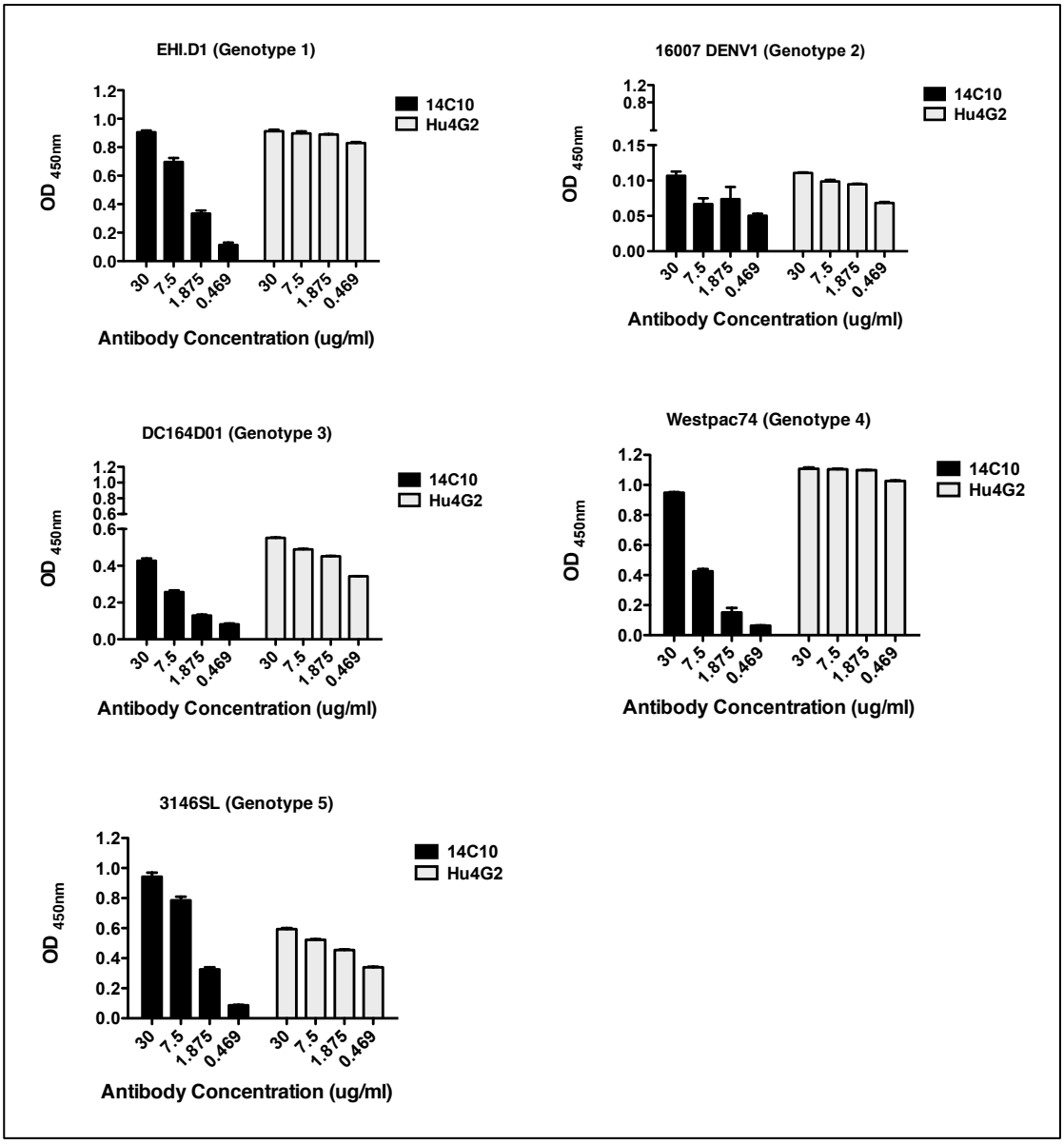


Figure 4A **Comparison of binding activity of 14C10 to hu4G2.** Serially diluted concentrations of 14C10 and hu4G2 binds to all five genotypes of DENV 1 on a sandwhich ELISA in a concentration dependent manner. Inter-genotype differences in binding activity can be observed. Hu4G2 is used as a positive control. Results are representative of 3 independent experiments.

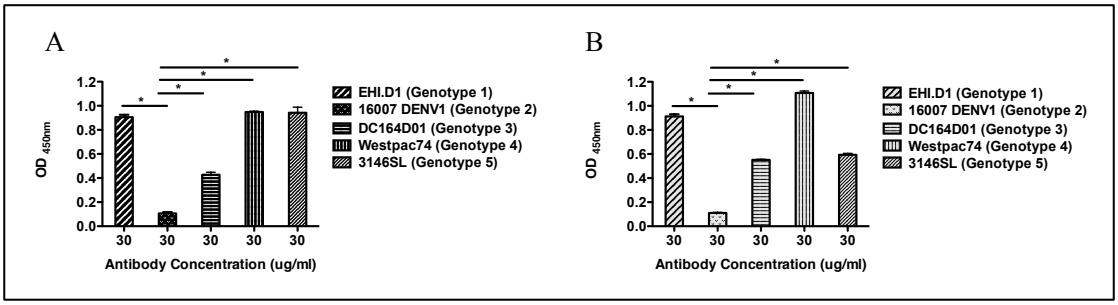


Figure 4B **Comparison of the binding of 30 μ g/ml of (A) 14C10 and (B) Hu4G2 across 5 genotypes of DENV1.** The binding of 14C10 and Hu4G2 were significantly lower for 16007 DENV1 (Genotype 2) compared to EHI.D1 (Genotype 1), DC164 D01 (Genotype 3), Westpac74 (Genotype 4) and 3146SL (Genotype 5). *p<0.0001

4.2 Neutralizing activity of 14C10 on 5 genotypes of DENV1

The neutralizing activity of 14C10 was tested against EHI.D1 (Genotype 1), 16007 DV1 (Genotype 2), DC164 D01 (Genotype 3), Westpac74 (Genotype 4) and 3146SL (Genotype 5) as shown in Figure 5. PRNT was performed on 4- fold serially diluted 14C10, with a starting concentration of 30μg/ml. Results were expressed as percentage neutralization as compared to the positive control with no antibody present. 14C10 exhibited neutralization activity for all five genotypes tested. The $PRNT_{50}$ values of 14C10 with each genotype of DENV1 determined by nonlinear regression in GraphPad Prism are as follows: EHI.D1-$PRNT_{50}$ = 0.0897μg/ml; 16007 DV1-$PRNT_{50}$ = 1.051μg/ml; DC164D01-$PRNT_{50}$ = 0.105μg/ml; Westpac74-$PRNT_{50}$ = 0.566μg/ml and 3146 SL-$PRNT_{50}$ = 0.0381μg/ml. $PRNT_{50}$ value of an antibody represents the concentration of antibody necessary to decrease the number of plaques by 50% when compared to the number of plaques in the absence of antibody. A lower $PRNT_{50}$ value would indicate a more potent neutralizing antibody, or in this case, an isolate of DENV1 that can be more strongly neutralized by 14C10. 14C10 had slightly better neutralizing activity for genotypes 1, 3, and 5 compared to genotypes 2 and 4.

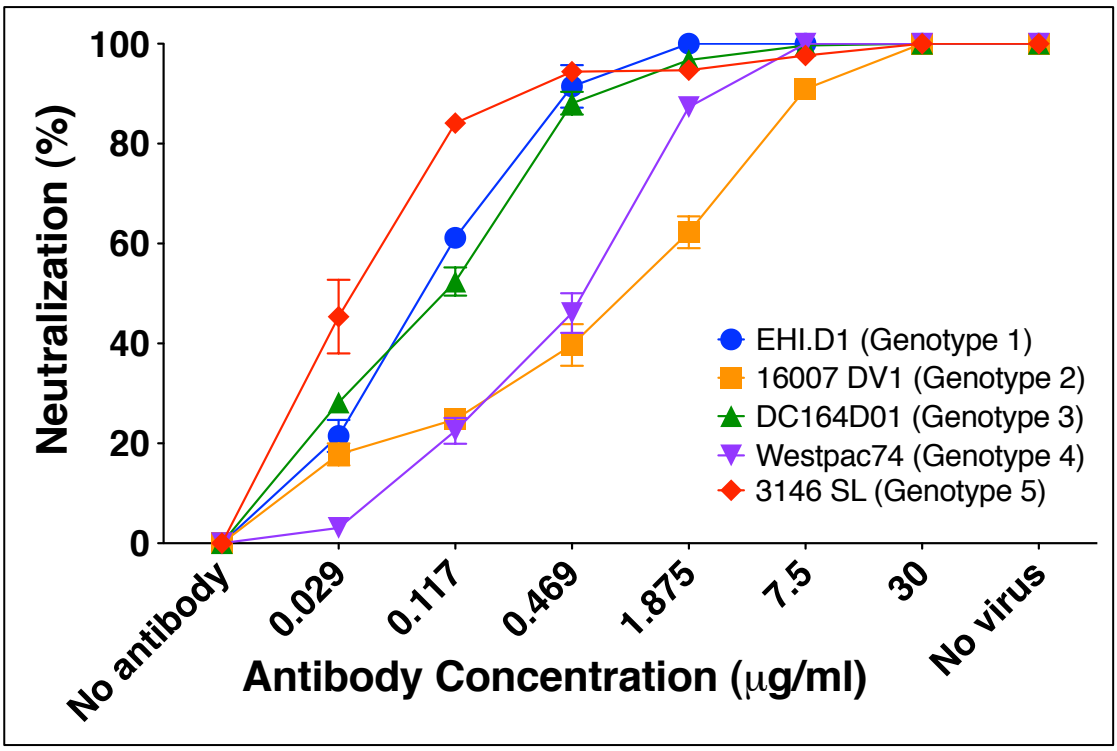


Figure 5 **Neutralising activity of 14C10 for DENV1 isolates representing all five DENV1 genotypes.** 14C10 is neutralizing for all 5 DENV1 genotypes to varying degrees. The genotypes are indicated with each DENV1 isolate. Results are representative of 3 independent experiments. Error bars represent SDs of triplicate samples.

4.3 Antibody Dependent Enhancement (ADE) of 14C10

ADE activity associated with severe forms of dengue had been postulated to occur when sub-neutralizing concentrations of antibodies form complexes with DENV. These complexes can attach and enter cells expressing Fc receptors such as monocytes. This leads to greatly increased uptake of virus and subsequent secretion of pro-inflammatory cytokines and chemokines (Halstead, 2003). 14C10 has been previously shown to exhibit homotypic ADE with DENV1 but not heterotypic ADE with DENV2, 3 and 4.

4.3.1 ADE effect of various subclasses of 14C10

To find out if antibody subclass affects the degree of enhancement of 14C10, 14C10 was expressed as 14C10 IgG1, 14C10 IgG2, 14C10 IgG3 and 14C10 IgG4. ADE activity was determined by an established *in vitro* assay which utilizes cells bearing

Fcγ receptors IIA (FcγRIIA), the myelomonocytic cell line K562 (Littaua et al., 1990). As shown in Figure 5, ADE activity can be ranked as follows: IgG3 > IgG1 > IgG2 > IgG4. IgG3 exhibits the highest enhancing activity whilst IgG4 displays the lowest enhancing activity. This partially correlates with the binding activities for FcγRIIA expressed by K562 cells previously described (Chiofalo et al., 1988). Thus, the ADE activity of 14C10 appeared to be FcγRIIA binding dependent. However, the influence of Fcγ receptors I (FcγRI) and complement components on virus neutralization and/or enhancement were not addressed in this assay (Mehlhop et al., 2007).

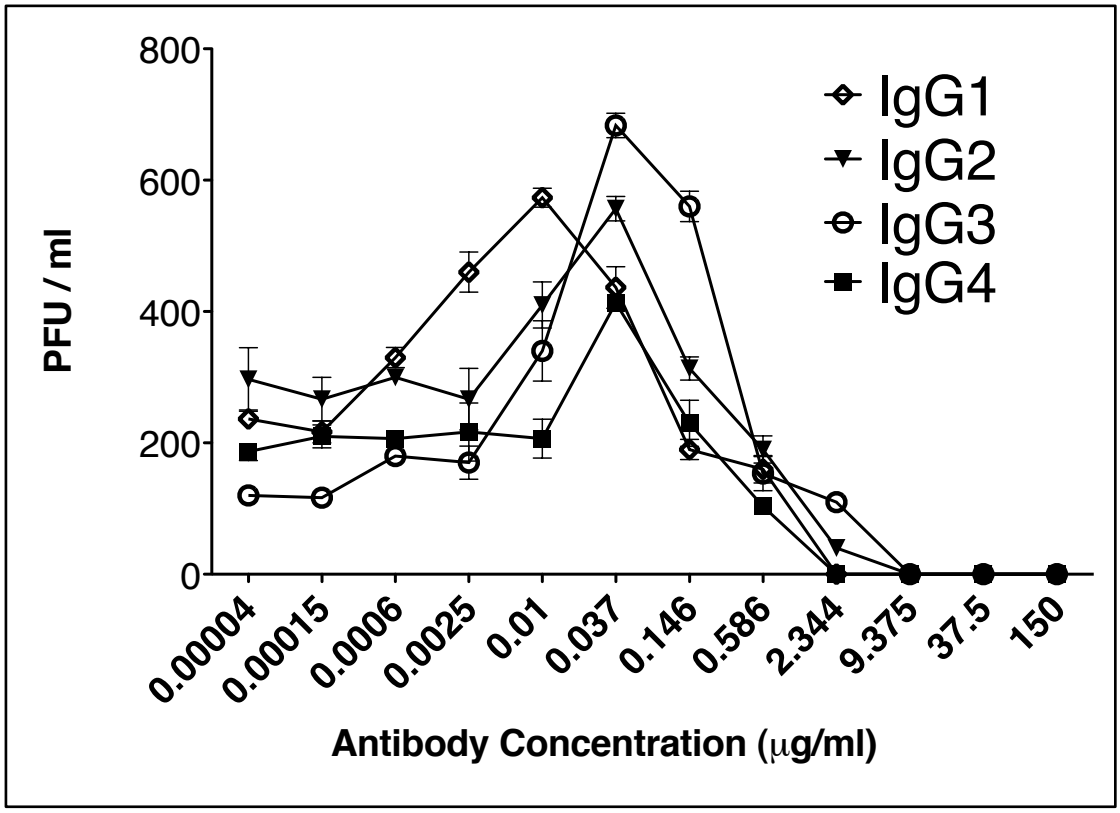


Figure 6 **Homotypic ADE of the various subclasses of 14C10**. The various subclasses of human IgG (HM14c10) exhibit differential levels of homotypic ADE, with IgG3 > IgG1 > IgG2 > IgG4. Results represent the average of 3 independent experiments.

4.3.2 Effect of Fcγ receptor binding on ADE

14C10 was expressed as an antigen binding fragment (Fab fragment) and a N297Q mutant to study the contribution of FcγRIIA expressed by K562 on homotypic ADE. The N297Q mutant 14C10 had the asparagine residue (N) at position 297 substituted for glutamine (Q), abolishing the glycosylation site on human IgG1 and hence

reducing FcγR binding (Tao et al., 1989). As shown in Figure 7, the Fab fragment of 14C10 and N297Q mutant both displayed lowered homotypic ADE activity compared with the controls of wild-type IgG1 antibodies.

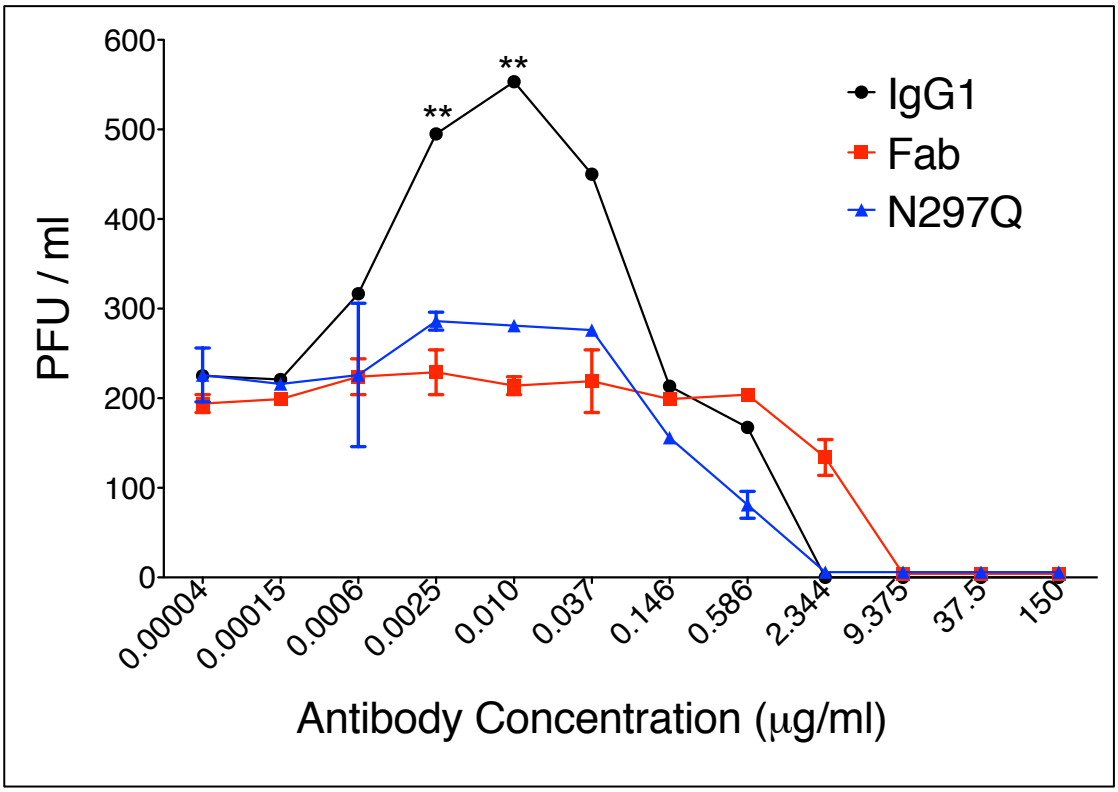


Figure 7 **Fc receptor binding mediates homotypic ADE.** The Fab fragment of 14C10 and N297Q mutant reduced homotypic ADE in an *in vitro* ADE assay using K562. Results are the average of 3 independent experiments. **p<0.01

4.4 Cryoelectron Microscopy (cryoEM) structure of 14C10 Fab-DENV1 Complex

The cryoEM structure of 14C10Fab-DENV1 complex was resolved to a 7Å resolution in collaboration with our collaborators Assistant Professor Lok Shee-Mei and Dr Petra Kukkaro at Duke-NUS Graduate Medical School. Our collaborators conducted the actual fitting of the 14C10 Fab onto DENV1 and cryoEM imaging. We provided 14C10 Fab, and helped with the analysis of the data.

A cryoEM structure of 14C10 Fab - DENV1 complex was resolved to 7Å resolution as seen in Figure 8. 120 copies of 14C10 Fab bound to all 180 available copies of E protein on DENV1 surface at full occupancy.

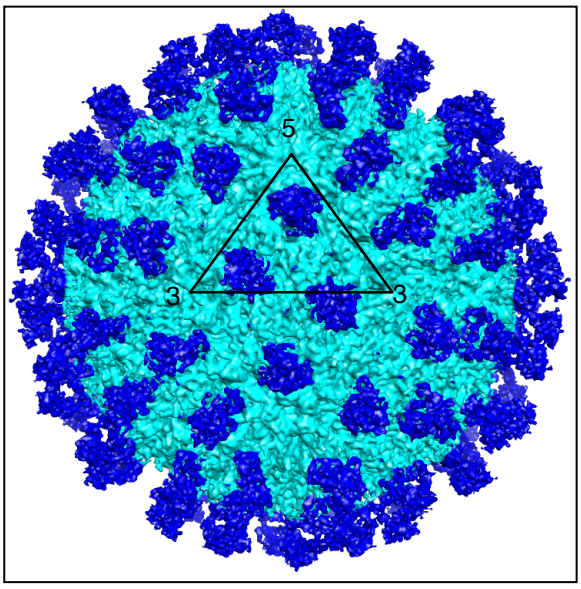


Figure 8 **CryoEM map of a complex of 14C10 Fab-DENV1.** 120 copies of Fab (blue) bind 180 copies of E proteins on the surface of the virus (cyan). Each asymmetric unit is represented by the black triangle.

The crystal structure of DENV1 E protein was fitted on to the cryoEM density map (Figure 9) to identify the footprint of 14C10 on DENV1 E protein.

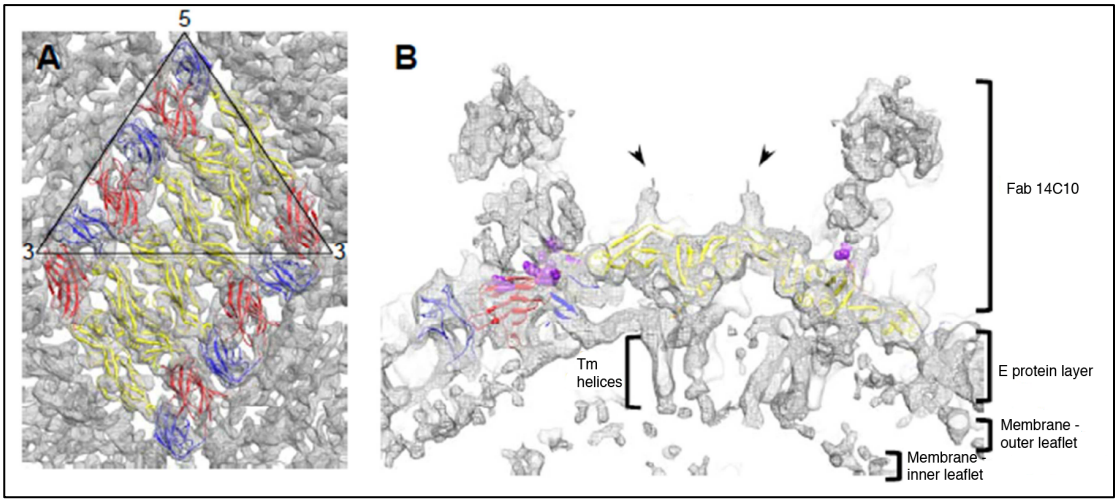


Figure 9 **The post-fusion crystal structure of DENV1 E proteins fitted on to the cryoEM map of 14C10 Fab – DENV1 complex.** (A) Top view of the fitted DENV1 E proteins. The cryoEM map is displayed at a high contour level of 5.5σ for to enable the outline of E protein densities to be observed clearly. At this contour level, the disappearance of Fab densities indicates that not all available E protein epitopes are occupied by Fab molecule on the virus surface. To interpret the electron densities of the virus surface, the crystal structure of the post-fusion structure of DENV1 E protein was fitted onto the virus. The three domains of the E protein had to be fitted separately because the crystal structure of the DENV1 post-fusion E protein does not fit well into the cryoEM map as a whole rigid body. Domains I, II and III of the E protein are indicated in red, yellow and blue respectively. E proteins from two asymmetric units are shown here with one asymmetric unit indicated with a triangle. (B) Side view of the fitted E proteins on the surface of DENV1. Densities of the Fab molecules, E protein ectodomain and transmembrane (Tm) helices can be observed. Densities corresponding to glycans at position Asn159 on two adjacent E proteins are marked with arrowheads and the position of outer and inner leaflet of the lipid bilayer are indicated. The cryoEM map is shown at 2.5σ contour level.

The identification of E protein residues at the interacting surface was made possible by the 7Å resolution cryoEM map, which showed clear density connections between the 14C10 Fabs and the envelope proteins (Figure 10).

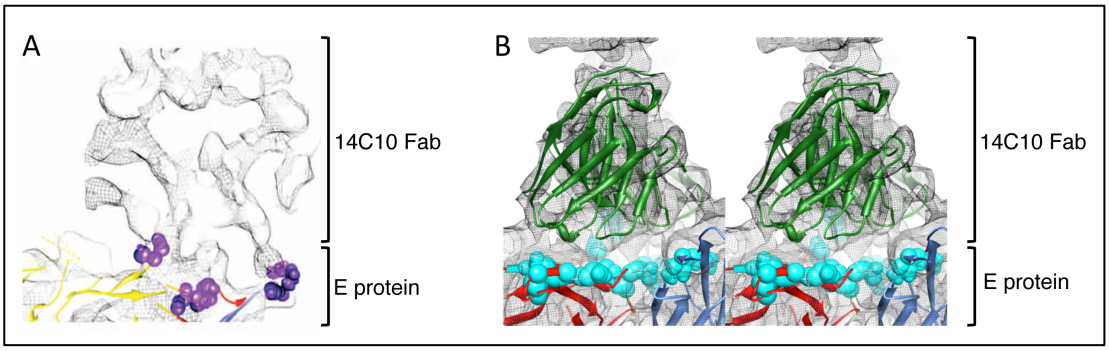


Figure 10 **Densities connecting 14C10 Fab to the E protein epitope.** EDI, EDII and EDIII are indicated as red, yellow and blue respectively. (A) View exhibiting connecting densities of one 14C10 Fab to E protein epitope (purple spheres). (B) Density of 14C10 Fab shows clear connections to E proteins on DENV1 surface. The fitted 14C10 variable region homology model is shown in green. Contact residues are indicated with cyan spheres. CryoEM density is shown at 2.5σ contour level

14C10 recognized an epitope that was dependent on the quaternary structure of DENV1. Two Fabs of 14C10 bound to three E proteins in the virus asymmetric unit (Figure 11). One single 14C10 antibody binds across two neighboring E proteins with one half of the epitope on EDIII and the other half on EDI as well as the EDI–EDII hinge of a neighboring E protein.

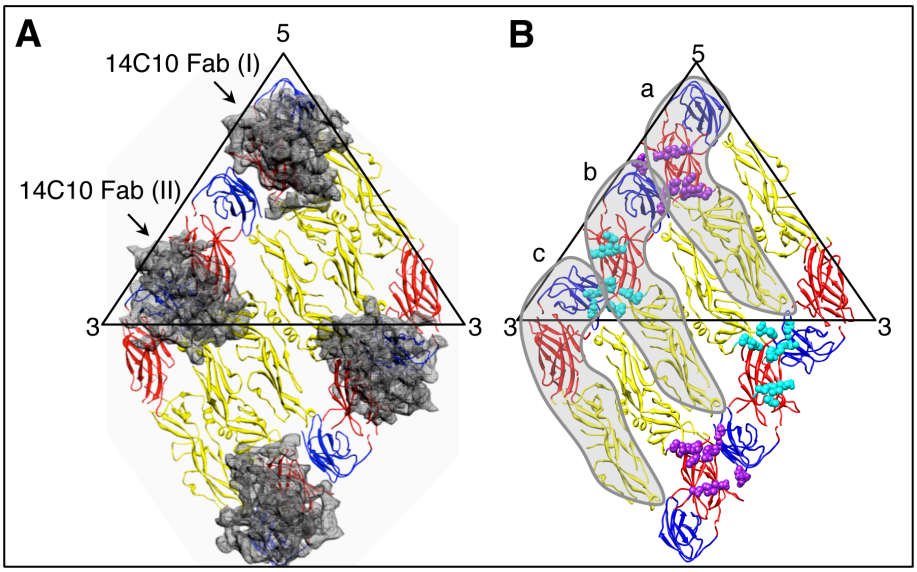
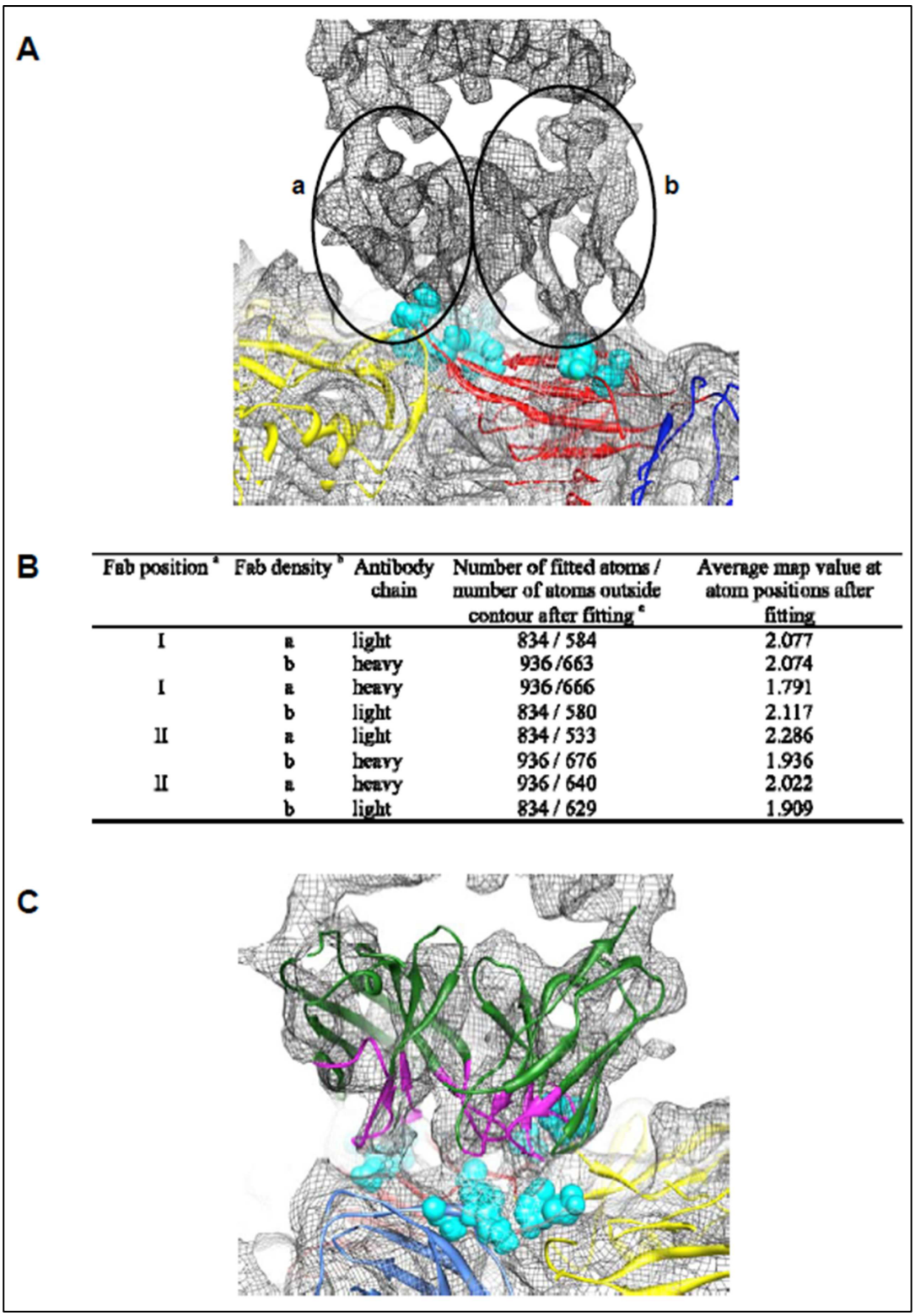


Figure 11 **Two 14C10 Fabs bind three E proteins in each virus asymmetric unit.** (A) Diagram depicting the densities of 14C10 Fab molecules on E protein Cα chains in two asymmetric units. 14C10 Fab (I) and 14C10 Fab (II) are the two independent molecules in the asymmetric unit. (B) Epitopes of Fab 14C10 Fab (I) (purple spheres) and 14C10 Fab (II) (cyan spheres) on the three E proteins (gray areas) in one asymmetric unit.

The Modeller program (Eswar et al., 2006) was used to generate a homology model of the variable region of 14C10 based on a reference human antibody structure (Protein Data Bank (PDB) code 2GHW) and this was utilized to elucidate the interaction of 14C10 Fab with DENV1 E protein. The variable heavy and light chain regions of the homology model were then fitted into the cryoEM density map. One distinctive fit was found to correlate better to the density despite the structures of both heavy and light chains being similar, as shown in Figure 12A and 12B. All complementarity-determining regions (CDRs) of the heavy and light chains were found to be involved in the interaction (Figure 12C).



Fab position [a]	Fab density [b]	Antibody chain	Number of fitted atoms / number of atoms outside contour after fitting [c]	Average map value at atom positions after fitting
I	a	light	834 / 584	2.077
	b	heavy	936 /663	2.074
I	a	heavy	936 /666	1.791
	b	light	834 / 580	2.117
II	a	light	834 / 533	2.286
	b	heavy	936 / 676	1.936
II	a	heavy	936 / 640	2.022
	b	light	834 / 629	1.909

Figure 12 **Homology model depicting the fitting of the variable region of 14C10 into 14C10-DENV1 cryoEM density map.** (A) The densities corresponding to the individual chains (a and b) of the antibody variable region are circled from the cryoEM map. The contact residues of the fitted E protein are indicated with cyan spheres. E-DI, E-DII and E-DIII are colored in red, yellow and blue, respectively. (**B**) The homology model light and heavy chains were fitted separately into the variable region of the Fab cryoEM densities. The fits of the homology model into the 14C10:DENV1 cryoEM map (3σ contour level) were optimized using Chimera's fit-in-map function. (**C**) The fitted 14C10 variable region homology model (green) showing the CDRs in magenta. The fit shown has light chain in Fab density a, and heavy chain in Fab density b.

Within each asymmetric unit, the binding footprints of the two 14C10 Fabs were not identical. As seen in Table 3, there were 4 unique amino acids in each interface whilst 12 amino acids were common.

Fab	Envelope protein molecule in the asymmetric unit	Envelope protein domain	Envelope protein residues*
14c10 (I)	A	I	**T51**, L135, K136, **G159**, **T160**, **T165**, **P166**, **Q167**, **E172**, **I173**, **T275**
	A	II	**N52**, **G274**
	B	III	**K310**, E384, K385
14c10 (II)	B	I	**T51**, Q131, Y132, **G159**, **T160**, **T165**, **P166**, **Q167**, **E172**, **I173**, L175, **T275**
	B	II	**N52**, **G274**
	C	III	L308, **K310**

*Residues common in both epitopes bound by Fab HM14c10 (I) and HM14c10 (II) are indicated in bold.

Table 3 **Epitope of 14C10 Fab on DENV1 E protein**. Observation of the E protein residues in the epitope to 14C10 Fab molecules at 2.5σ contour level enabled the identification of connecting densities.

Sequence comparison of the epitope residues between various DENV1 genotypes (I to V) indicated the conservation of all residues (Figure 13). The amino acid sequences of the epitopes recognized by Fab 14C10 were conserved within all DENV1 genotypes. This is consistent with the observation that 14C10 binds to all tested DENV1 genotypes. However, there were differences in the neutralization activities of 14C10 when tested against the various DENV1 genotypes (Figure 5), suggesting that target residues present on the surface of the virus amongst the various genotypes were differentially exposed.

Figure 13 **The epitope of 14C10 on DENV1 (PVP159) as compared with the epitope of other DENV1 genotypes.** Common amino acid residues between the epitopes recognized by Fab 14C10(I) and Fab 14C10(II) in one asymmetric unit are highlighted in green. Residues that are uniquely

recognized by Fab 14C10(I) or Fab 14C10(II) are indicated highlighted in purple and cyan, respectively.

When residues in the epitope were compared between DENV serotypes and West Nile Virus (WNV), we found that the epitope was not conserved (Figure 14). This observation was consistent with the finding that 14C10 binds specifically to DENV1 (Figure 3).

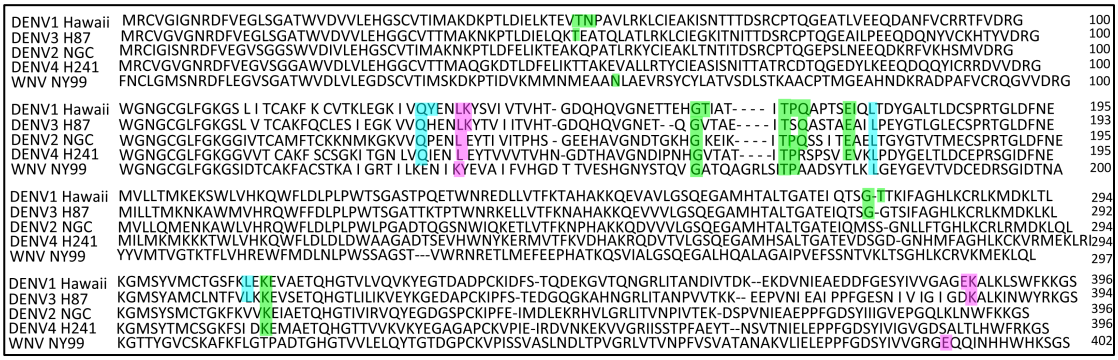


Figure 14 **Epitope of 14C10 on DENV1 (Hawaii) compared to the epitope with other DENV serotypes and WNV.** Common amino acid residues between the epitopes recognized by Fab 14C10(I) and Fab 14C10(II) in one asymmetric unit are highlighted in green. Residues that are uniquely recognized by Fab 14C10(I) or Fab 14C10(II) are highlighted in purple and cyan, respectively.

4.5 Pre- versus Post- Attachment Assay

To further elucidate the mechanism of 14C10, a pre- and post-attachment neutralization assay was performed. As seen in Figure 15, 14C10 is able to inhibit the infection of DENV1 after DENV1 has bound to its host cell – at a post-attachment step. However, the concentration of 14C10 required for this to occur was significantly higher than the concentration required for pre-attachment inhibition whereby 14C10 was allowed to complex with DENV1 before adding to host cells.

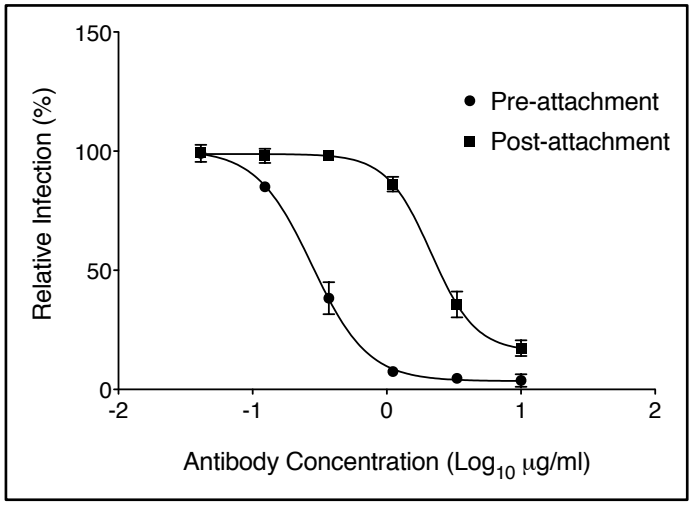


Figure 15 **Neutralization of DENV1 by 14C10 at a pre- and post-attachment step.** 14C10 is able to inhibit virus attachment (pre attachment neutralization) at 1 μg/ml. 14C10 can also inhibit a post-attachment step of infection at higher concentrations. Results represent two independent experiments performed with duplicates. SDs are represented by error bars.

In order to investigate if 14C10 is able to prevent entry of DENV after binding, we inhibit the entry of DENV by using Compound 6, which is a known entry inhibitor of DENV. Compound 6 prevents infection of host cells with DENV1 at both the pre- and post-attachment step, to a slightly higher extent when incubated with DENV1 first (pre-attachment inhibition) (Figure 16). NITD008 is a known RNA synthesis inhibitor. It does not prevent infection of host cells with DENV1 when pre-incubated with DENV1, nor does it prevent infection when added after host cells have been exposed to DENV1 (Figure 16).

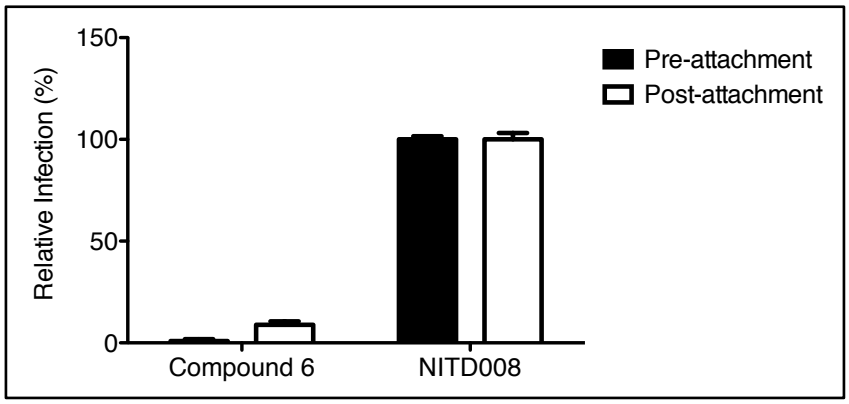


Figure 16 **Controls performed for pre- and post-attachment neutralization assay.** Compound 6 (entry inhibitor) and NITD008 (RNA synthesis inhibitor) were included as controls. The antiviral effects were measured by plaque assay. Results represent two independent experiments performed with duplicates. SDs are represented by error bars.

4.6 Time-Lapse Confocal Microscopy

To investigate how 14C10 neutralizes DENV1, high-resolution time-lapse confocal microscopy was used to visualize the real-time interaction between fluorescently labeled live DENV1 and 14C10 real time during an infection. The course of infection of target BHK cells was followed for an hour, with 6 random fields of view recorded as videos. The results are presented as picture stills extracted from the recordings.

An isotype control, hu4G2 and 14C10 tagged to AF488 (green) were tested. DENV1 fluorescently tagged to AF647 (red) was first incubated with each antibody before BHK cells were infected with the antibody-DENV1 complexes (yellow). The course of infection was recorded as a video. When BHK cells were infected with DENV1 complexed to an isotype control antibody that does not bind DENV, DENV1 coalesced in numerous intracellular compartments, mostly outside the nucleus (Figure 17, top panel). DENV1 complexed to neutralizing concentration of hu4G2 led to DENV1 aggregates forming in the extra-cellular region. These viral aggregates were internalized into BHKs as well, which indicates that hu4G2 does not inhibit viral attachment or internalization (Figure 17, middle panel). 14C10 led to the formation of smaller viral aggregates, effectively blocking their attachment to BHKs. This resulted in the retention of majority of the viral aggregates within the extracellular space after one hour (Figure 17, bottom panel).

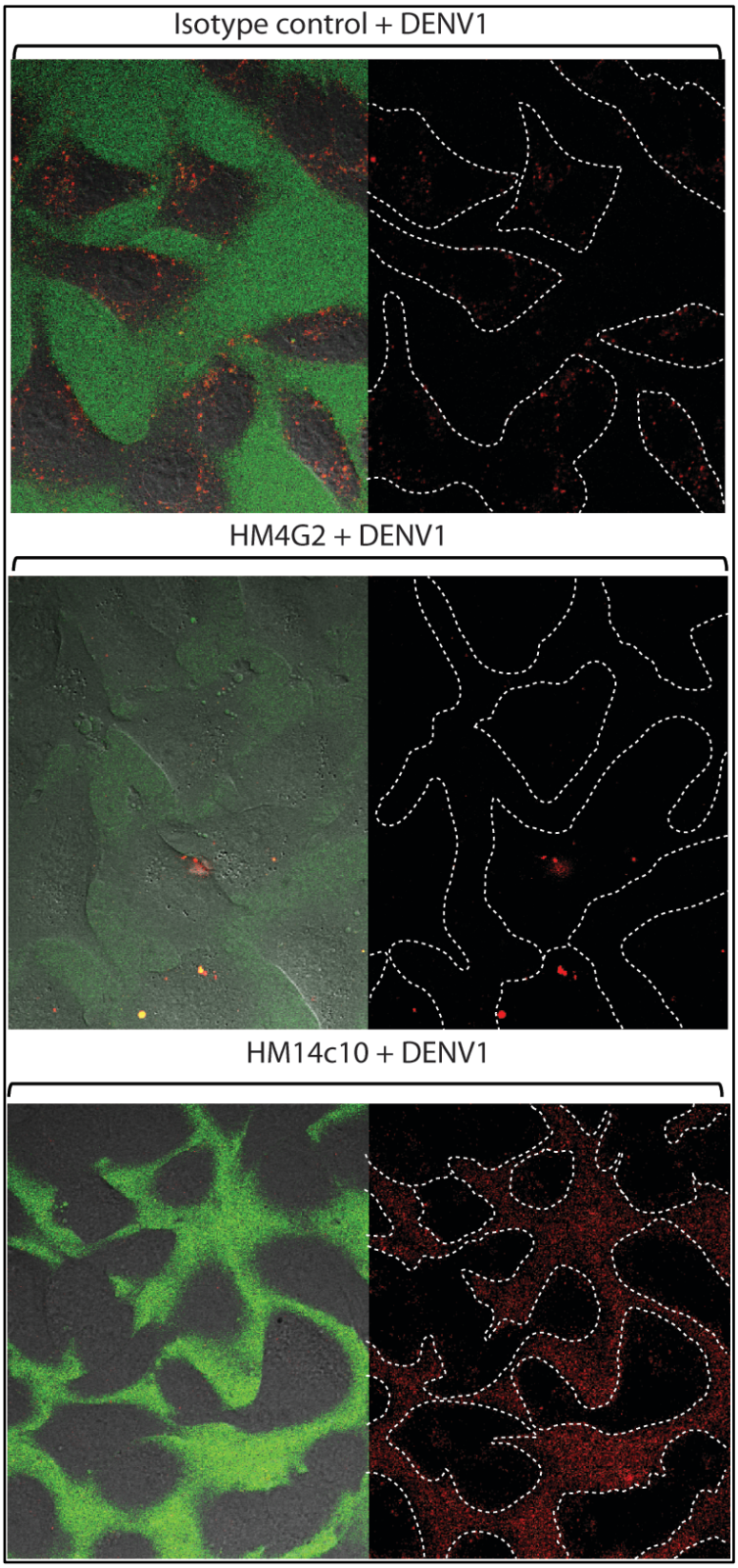


Figure 17 **Time-lapse confocal microscopy illustrating the live infection of BHK target cells with DENV1.** Fluorescently tagged DENV1 (AF647, red) was incubated with either an isotype control antibody (top panel), hu4G2 (middle panel) or 14C10 (bottom panel), (all tagged to AF488, green) before infecting BHKs. White dashed lines demarcate cell boundaries on right panels, to indicate disribution of DENV1 within target cells. All stills were captured 1 hour post-infection.

The kinetics by which DENV1 enters BHK cells differ significantly between the isotype control antibody and hu4G2. With the isotype control, DENV1 rapidly accumulates in the intracellular space of BHK cells. The accumulated DENV1 were visible within the target cells at 18 minutes post-infection (Figure 18A). Comparatively, hu4G2 led to the formation of viral aggregates but did not prevent viral entry. Hu4G2-DENV1 complexes (yellow) can be seen to enter target BHK cells by 28 minutes post infection (Figure 18B). 14C10 induces small numbers of viral aggregates. These aggregates were seen to deflect from target cell surfaces and were not internalized (Figure 18C). This suggests that 14C10 is a classical viral entry inhibitor whereby its primary mechanism of neutralization is to bind to DENV1. Although 14C10 did not bind DENV as strongly as hu4G2, it prevented the subsequent entry of DENV1 into target cells. This is in contrast to hu4G2, a fusion inhibitor, which binds strongly to DENV and efficiently induces virus aggregation, but fails to prevent virus entry.

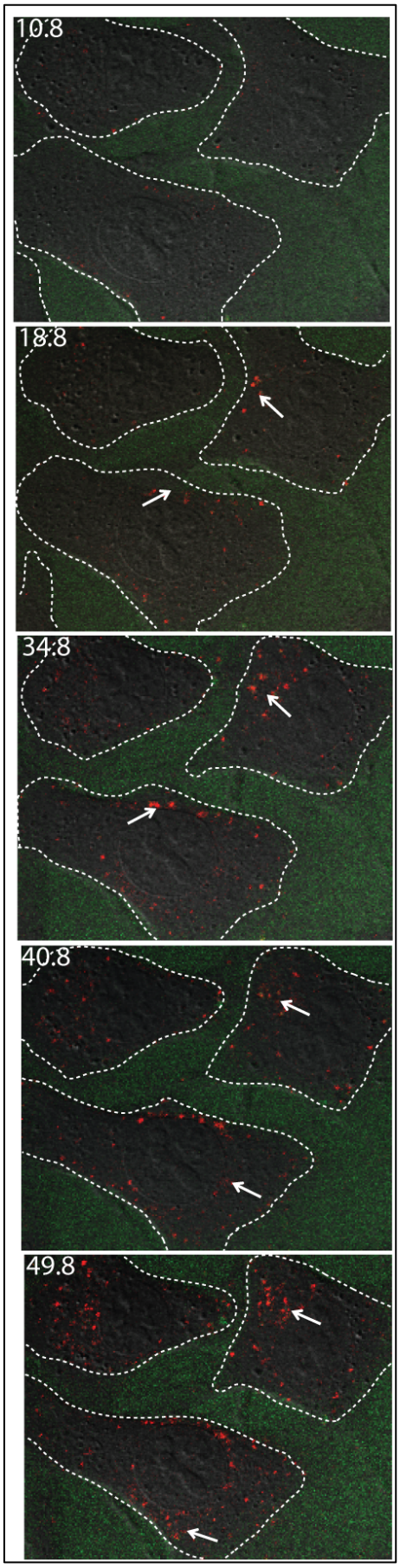


Figure 18A **Series of stills depicting live infection of BHK cells with DENV1 (labeled with AF647, red) in the presence of an isotype control antibody (labeled with AF488, green).** DENV1 can be observed within BHK cells from 18 minutes post-infection with the isotype control antibody. White arrows indicate the location of fluorescently labeled DENV1 within BHK cells. Boundaries of BHK cells are demarcated with dashed white lines. Time (minutes) from start of infection is indicated on the top left corner of each image. Stills were taken from a 60-minute time-lapse confocal microscopy imaging video.

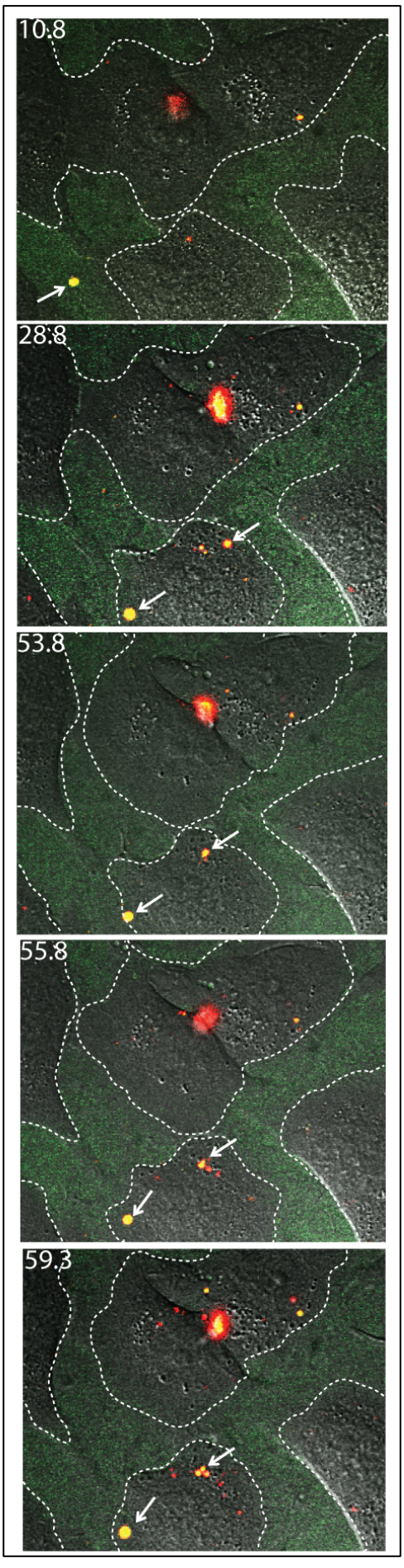


Figure 18B **Series of stills depicting live infection of BHK cells with DENV1 (labeled with AF647, red) in the presence of hu4G2 (labeled with AF488, green).** DENV1-hu4G2 complexes (yellow) can be seen within BHK cells 28 minutes post-infection, with the direction of motion of complexes indicated with white arrows. Boundaries of BHK cells are demarcated with dashed white lines. Time (minutes) from start of infection is indicated on the top left corner of each image. Stills were taken from a 60-minute time-lapse confocal microscopy imaging video.

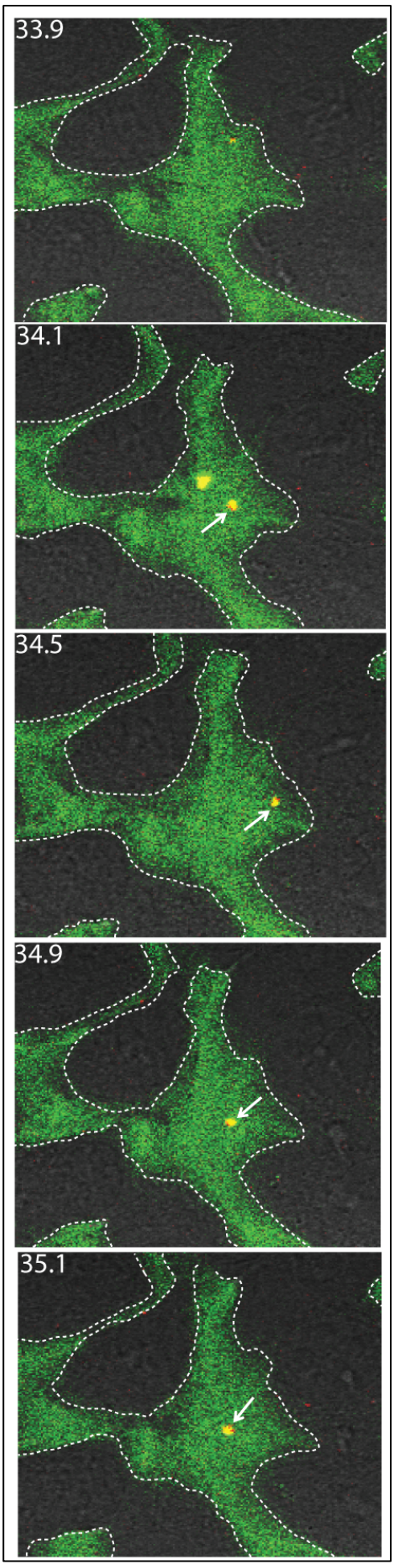


Figure 18C **Series of stills depicting live infection of BHK cells with DENV1 (labeled with AF647, red) in the presence of 14C10 (labeled with AF488, green).** DENV1-14C10 complexes (yellow) were unable to attach to and enter target BHK cells and can be seen to be deflecting from cell boundaries, as seen from the direction of motion of the complexes indicated by white arrows. Boundaries of BHK cells are demarcated with dashed white lines. Time (minutes) from start of infection is indicated on the top left corner of each image. Stills were taken from a 60-minute time-lapse confocal microscopy imaging video.

To quantify the degree of DENV1 internalization after 1 hour in the presence of the 3 test antibodies, the relative intensity of fluorescence derived from internalized virus was measured from at least 100 randomly selected cells per condition. As seen from Figure 18, there is significantly less fluorescence intensity in target cells in the presence of 14C10, indicating that 14C10 significantly reduced the amount of DENV1 infection of target cells.

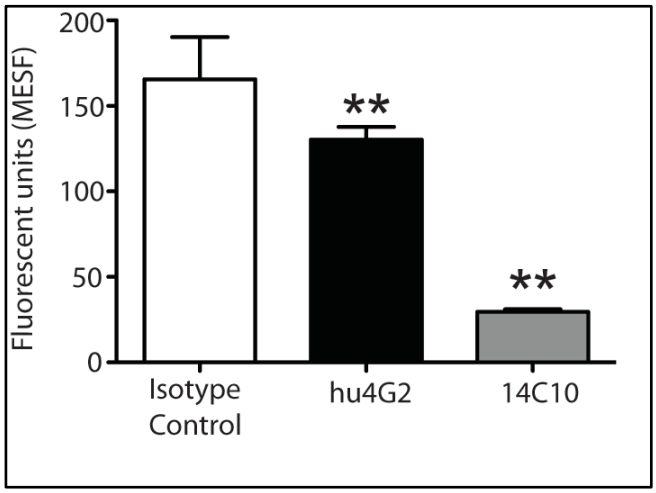


Figure 19 **Quantification of DENV1 within target BHK cells.** Number of viruses within target BHK cells was quantified by the red fluorescence intensity within at least 100 randomly selected cells 1 hour post-infection for each condition. (**p < 0.0001, Student's t test)

4.7 *In vivo* efficacy of 14C10

To investigate the efficacy of 14C10 as both a prophylactic and therapeutic agent *in vivo*, we tested 14C10 in a DENV viremia model in AG129 mice. AG129 mice are deficient for both type I and II interferons and are susceptible to DENV infection. In this study, two different models were used. These two models differed in terms of the route of infection and the strains of DENV1 used to infect the mice.

4.7.1 Subcutaneous (s.c.) infection of EHI DENV1 (EHI.D1)

The first model utilized the subcutaneous (s.c.) route of infection with a non mouse adapted strain of DENV1, EHI.D1. EHI.D1 (genotype 1) is a laboratory adapted clinical isolate obtained from the Environmental Health Institute, Singapore. Infection

with this strain of DENV1 resulted in a non-lethal transient viremia in AG129 mice. Mice were infected with $1x10^6$ PFU of EHI.D1 per mouse (Day 0). 14C10 was administered intraperitoneally (i.p.) either 24 hours before (Day -1) DENV infection (prophylactic treatment) or 48 hours after (Day +2) DENV infection (therapeutic treatment). The amount of 14C10 administered ranged from 0.04µg/mouse to 15µg/mouse for the pre-infection (prophylactic treatment) group (Figure 19A) and 0.6µg/mouse to 16µg/mouse for the post-infection (therapeutic treatment) group (Figure 19B). Blood was collected from the mice via retro-orbital puncture on Day +4 and viremia was assessed by plaque assay. For the pre-infection treatment, 4µg/mouse of 14C10 rendered plasma viremia in the mice undetectable using plaque assay. 2µg/mouse treatment given therapeutically significantly lowers plasma viremia, with viremia undetectable at a dose of 16µg/mouse.

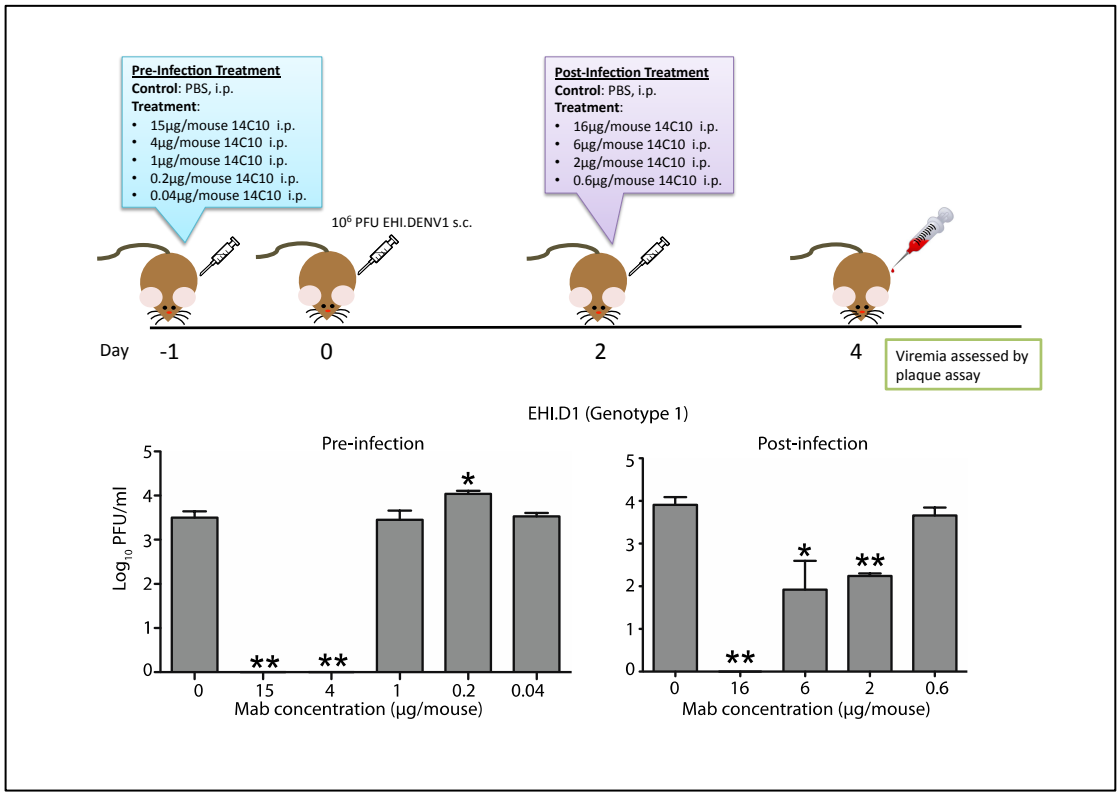


Figure 20 **14C10 was tested for *in vivo* efficacy in an AG129 mouse model of subcutaneous DENV infection.** 14C10 was administered to DENV1-infected (s.c.) mice on days -1 before and days +2 after infection with $1x10^6$ PFU/mouse of EHI.D1 DENV1 respectively and viremia assessed on days +4 after infection. Data is representative of 2 independent experiments with n=5 mice per condition. *p<0.05; **p<0.0001 compared to PBS controls, using a Student's t-test.

4.7.2 Intraperitoneal (i.p.) infection of Westpac74 DENV1

The second model utilized the intraperitoneal (i.p.) route of infection with a non mouse adapted strain of DENV1, Westpac74 (genotype 4) to give a non-lethal transient viremia in AG129 mice. Mice were infected with $1.25x10^7$ PFU of Westpac74 DENV1 (i.p.) per mouse (Day 0). 14C10 was administered intraperitoneally (i.p.) either 24 hours before (Day -1) DENV infection (prophylactic treatment) or 48 hours after (Day +2) DENV infection (therapeutic treatment). The amount of 14C10 administered ranged from 0.04μg/mouse to 15μg/mouse for the pre-infection (prophylactic treatment) group (Figure 21A) and 0.6μg/mouse to 16μg/mouse for the post-infection (therapeutic treatment) group (Figure 21B). Blood was collected from the mice on Day +3 and viremia was assessed by plaque assay. For the pre-infection treatment, there was a slight reduction in plasma viremia with 1μg/mouse and 2μg/mouse of 14C10, while 15μg/mouse of 14C10 resulted in undetectable plasma viremia in the mice using plaque assay. 0.6μg/mouse of 14C10 administered post-infection significantly lowers plasma viremia, with viremia undetectable at 6μg/mouse. The minimum amount of 14C10 to significantly reduce plasma viremia was observed to be 0.6μg/mouse, indicating that 14C10 has strong efficacy *in vivo*.

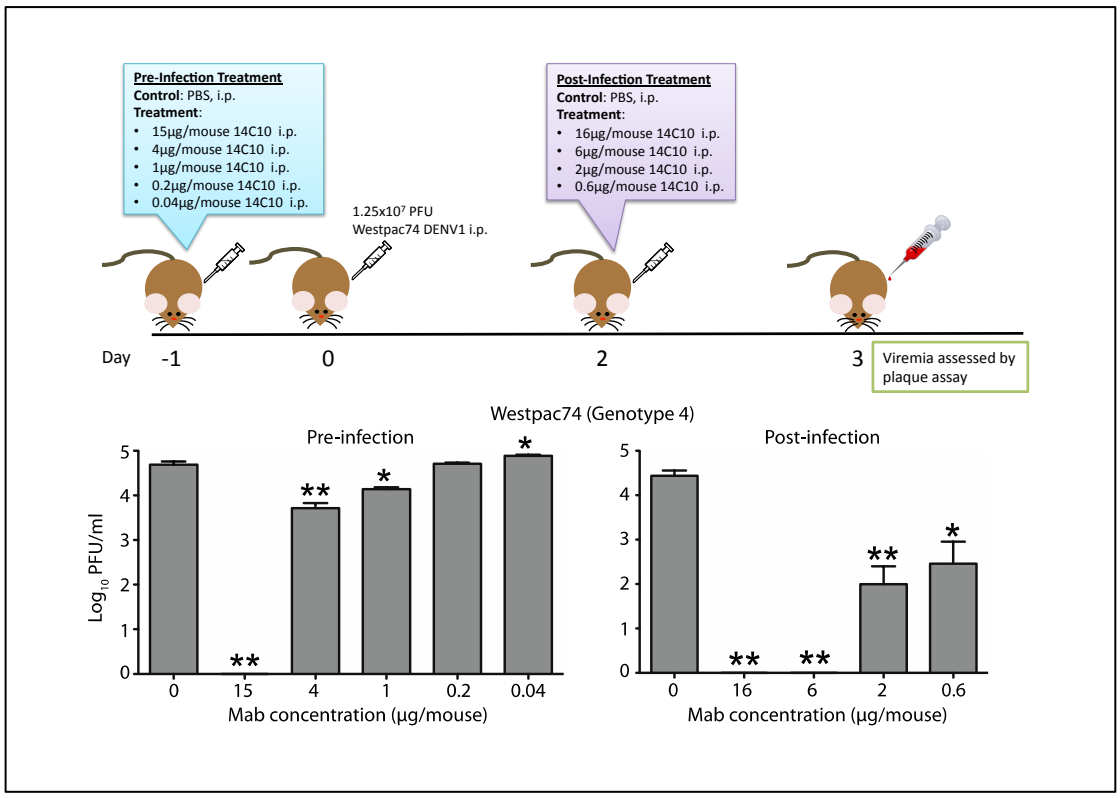


Figure 21 **14C10 was tested for *in vivo* efficacy in an AG129 mouse model of intraperitoneal DENV infection.** 14C10 was administered (i.p.) to DENV1-infected (i.p.) mice on days -1 before and days +2 after infection with $1.25x10^7$ PFU/mouse of Westpac74 DENV1 respectively and viremia assessed on days +3 after infection. Data is representative of 2 independent experiments with n=5 mice per condition. *p<0.05; **p<0.0001 compared to PBS controls, using a Student's t-test.

5 Results Chapter Two - Anti-DENV2 Antibodies

10.15, 12.17 and 14.19 are anti-DENV2 antibodies derived using a slightly different method as compared to 14C10. A human immune library was constructed from purified B cells isolated from a patient who recovered from DENV2 infection, followed by subsequent panning of a phage displayed human immune library for binding activity specific for DENV2. A brief outline of the generation of these anti-DENV2 antibodies are outlined in Figure 22. 10.15 exhibited the strongest neutralizing activity out of the three anti-DENV2 antibodies, and was selected for subsequent for *in vivo* characterization.

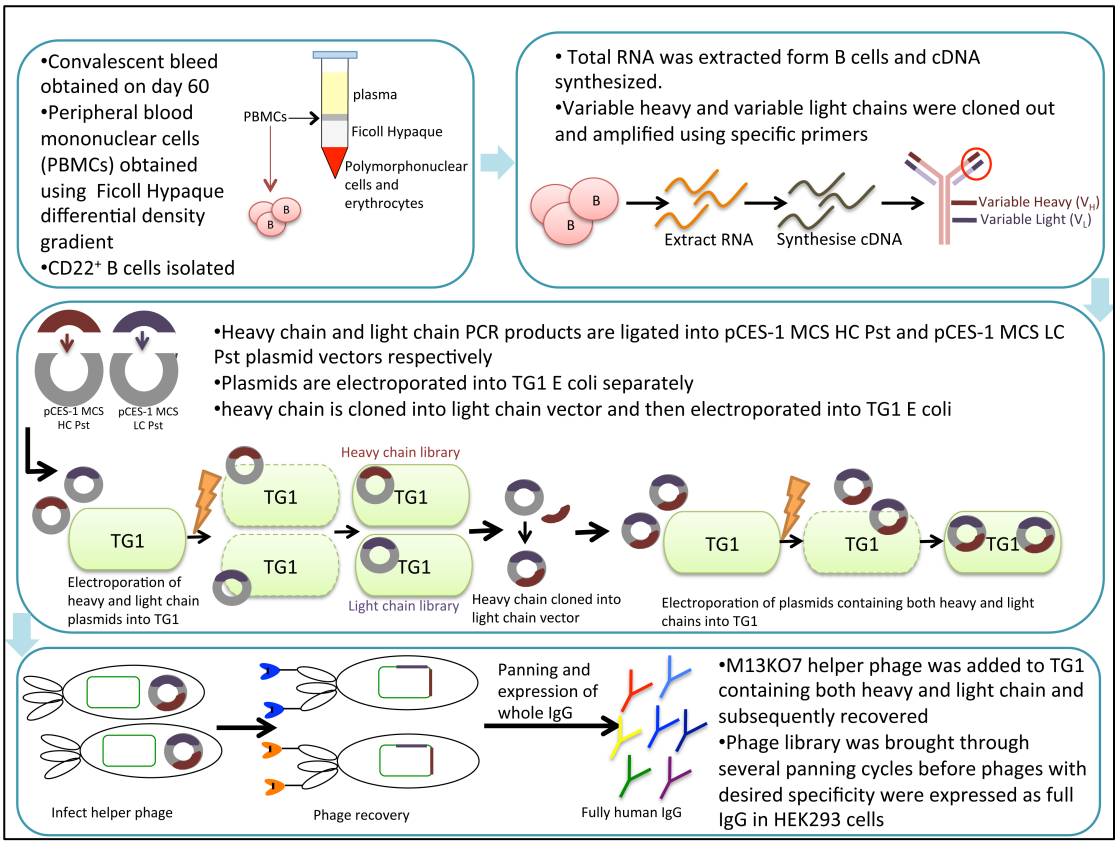


Figure 22 **Schematic of the generation of anti-DENV2 antibodies using a phage displayed human immune library.**

5.1 Binding activity of 10.15, 12.17 and 14.19

The binding activities of the three anti-DENV2 antibodies were tested on a sandwhich ELISA as described in Section 3.9. As seen from Figure 23, 10.15 bound exclusively to DENV2 but not DENV1, 3 and 4. The binding activity of 10.15 was comparable to the positive control of hu4G2 for the majority of the DENV2 strains tested, with the exception of DC186 DENV2. Similarly, 12.17 and 14.19 bound exclusively to DENV2 (Figures 24 and 25 respectively) but not DENV 1,3 and 4. Binding activities of 12.17 to DC117 DENV2, DC186 DENV2 and DC146 DENV2 were comparable to hu4G2, but slightly weaker to DC151 DENV2 and NGC DENV2 (Figure 24). With respect to hu4G2, 14.19 showed comparable binding to DC117 DENV2 and DC146 DENV2, slightly better binding activity to DC186 DENV2 and DC151 DENV2 and slightly weaker binding to NGC DENV2 (Figure 25).

Comparing the binding of 10.15, 12.17 and 14.19 to the various DENV2 strains (Figure 26), we observed that 10.15 and 14.19 showed marginally better binding to DC117 and DC186 DENV2 compared to 12.17 and hu4G2, whilst all three antibodies showed comparable binding activity to DC151 and DC146 DENV2. All three antibodies showed slight binding activity to NGC DENV2 compared to hu4G2, with 14.19 binding most weakly.

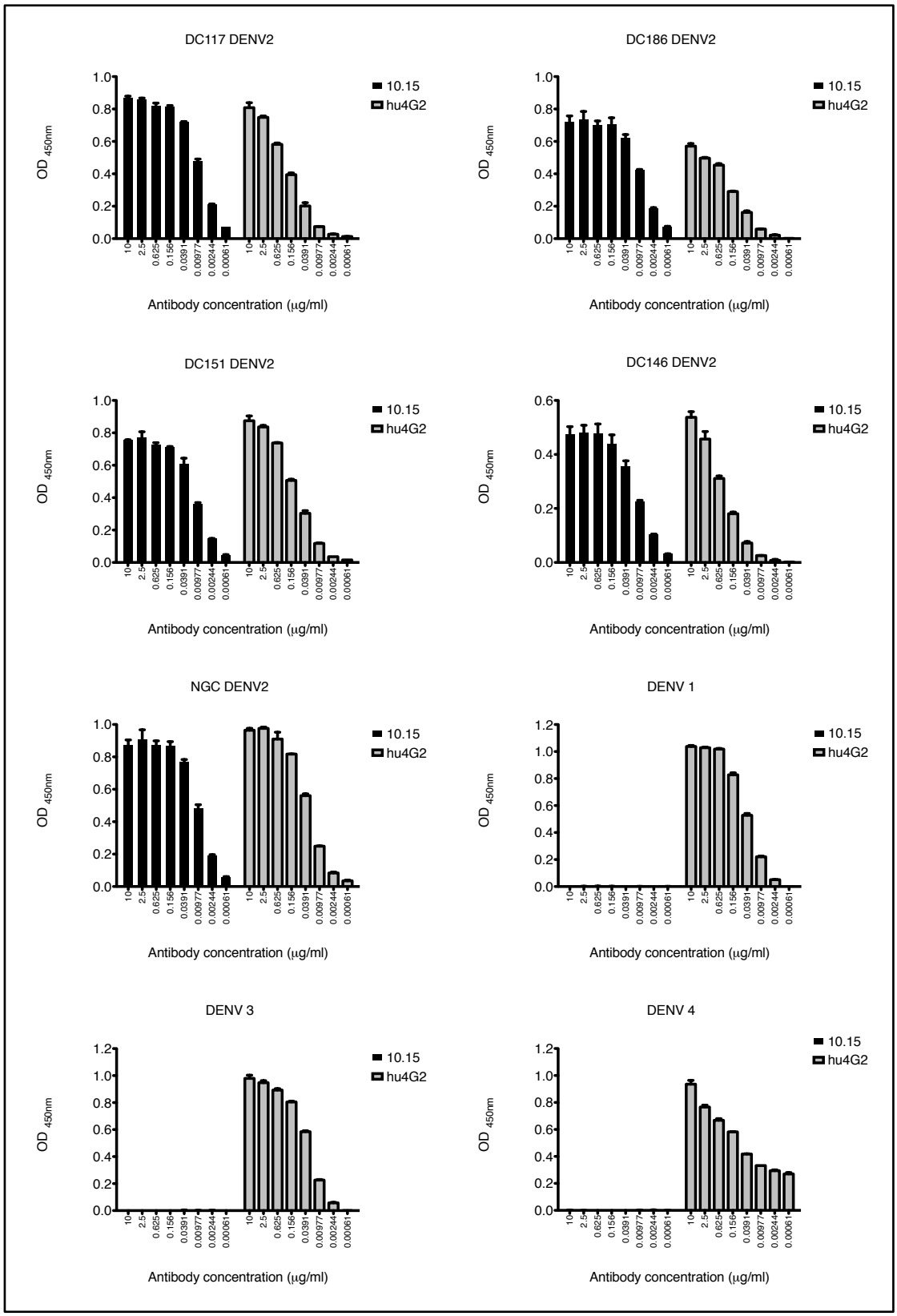


Figure 23 **Binding activity of 10.15 to various strains of DENV2 and DENV1, 3 and 4.** 10.15 binds exclusively to DENV2 and binding activity was comparable to hu4G2 (positive control) for the majority of the DENV2 strains tested except for DC186, with slightly higher binding with 10.15. Results represent the average of 3 independent experiments performed in triplicates. Error bars represent SDs.

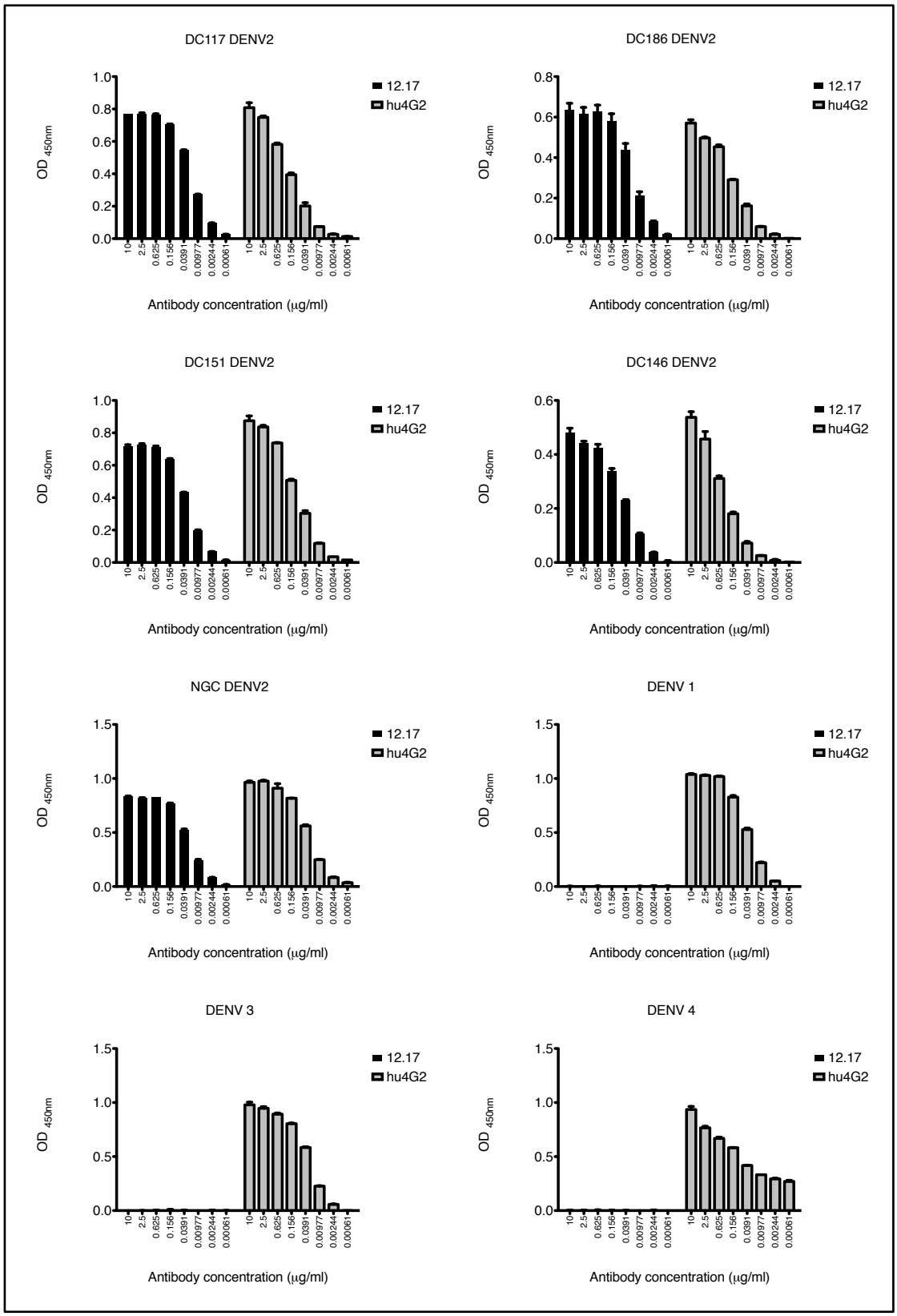


Figure 24 **Binding activity of 12.17 to various strains of DENV2 and DENV1, 3 and 4.** Binding activity of 12.17 to DC117 DENV2, DC186 DENV2 and DC146 DENV2 was comparable to hu4G2, but slightly weaker to DC151 DENV2 and NGC DENV2. Results represent the average of 3 independent experiments performed in triplicates. Error bars represent SDs.

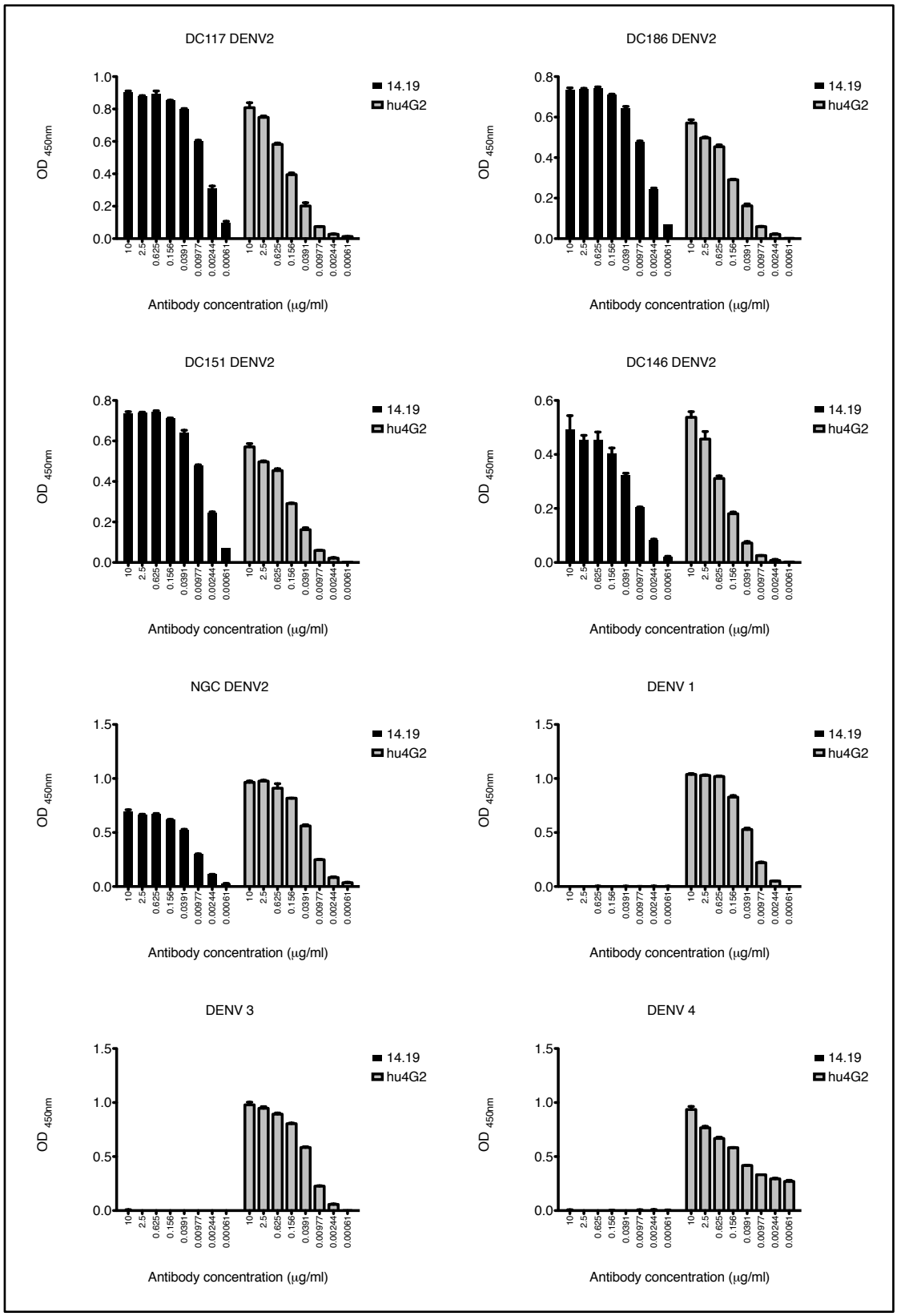
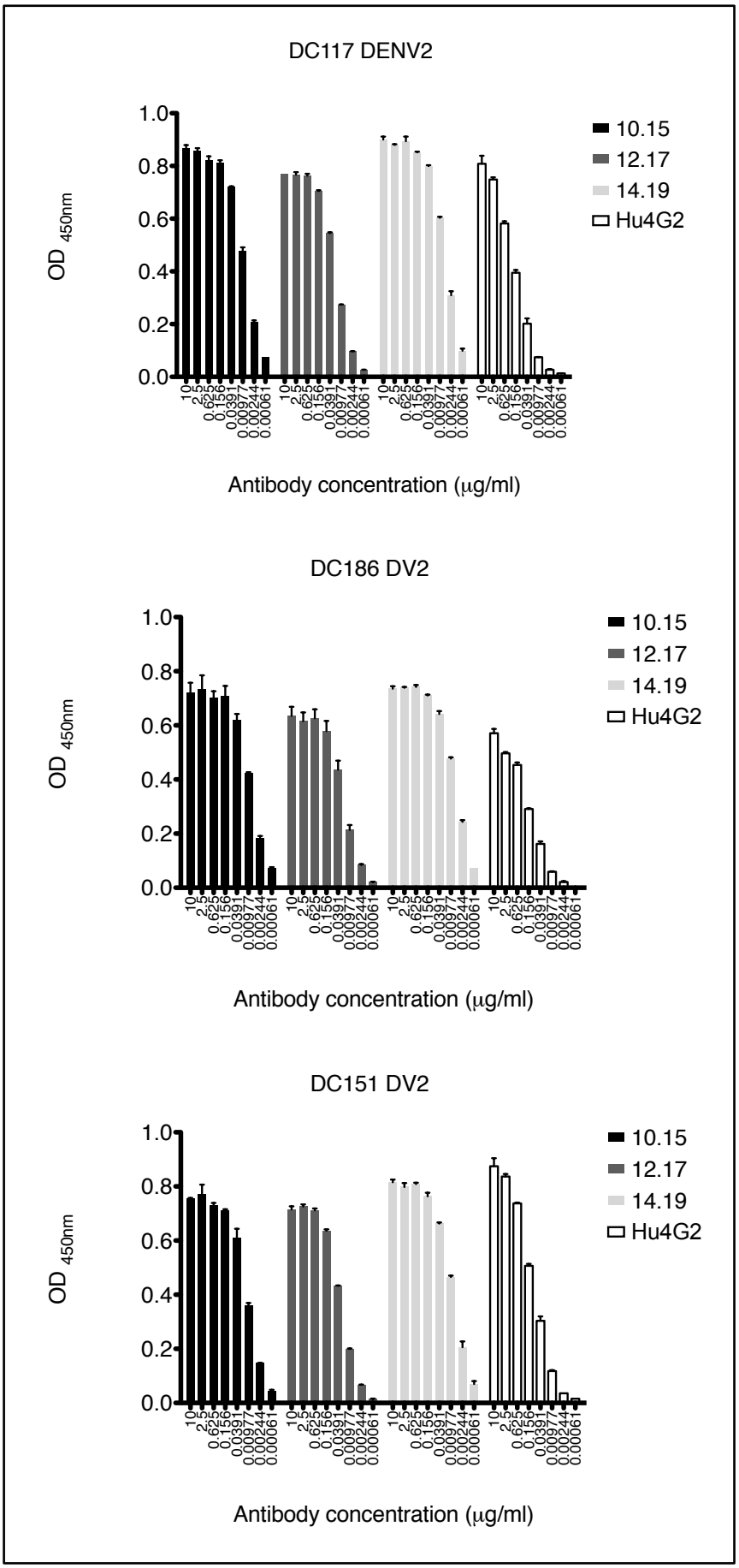


Figure 25 **Binding activity of 14.19 to various strains of DENV2 and DENV1, 3 and 4.** 14.19 shows comparable binding to DC117 DENV2 and DC146 DENV2, slightly better binding activity to DC186 DENV2 and DC151 DENV2 and slightly weaker binding to NGC DENV2 as compared to hu4G2. Results represent the average of 3 independent experiments performed in triplicates. Error bars represent SDs.


DC117 DENV2
OD 450nm
1.0
0.8
0.6
0.4
0.2
0.0
10.15
12.17
14.19
Hu4G2
10
2.5
0.625
0.156
0.0391
0.00977
0.00244
0.00061
Antibody concentration (μg/ml)
DC186 DV2
OD 450nm
10.15
12.17
14.19
Hu4G2
Antibody concentration (μg/ml)
DC151 DV2
OD 450nm
10.15
12.17
14.19
Hu4G2
Antibody concentration (μg/ml)

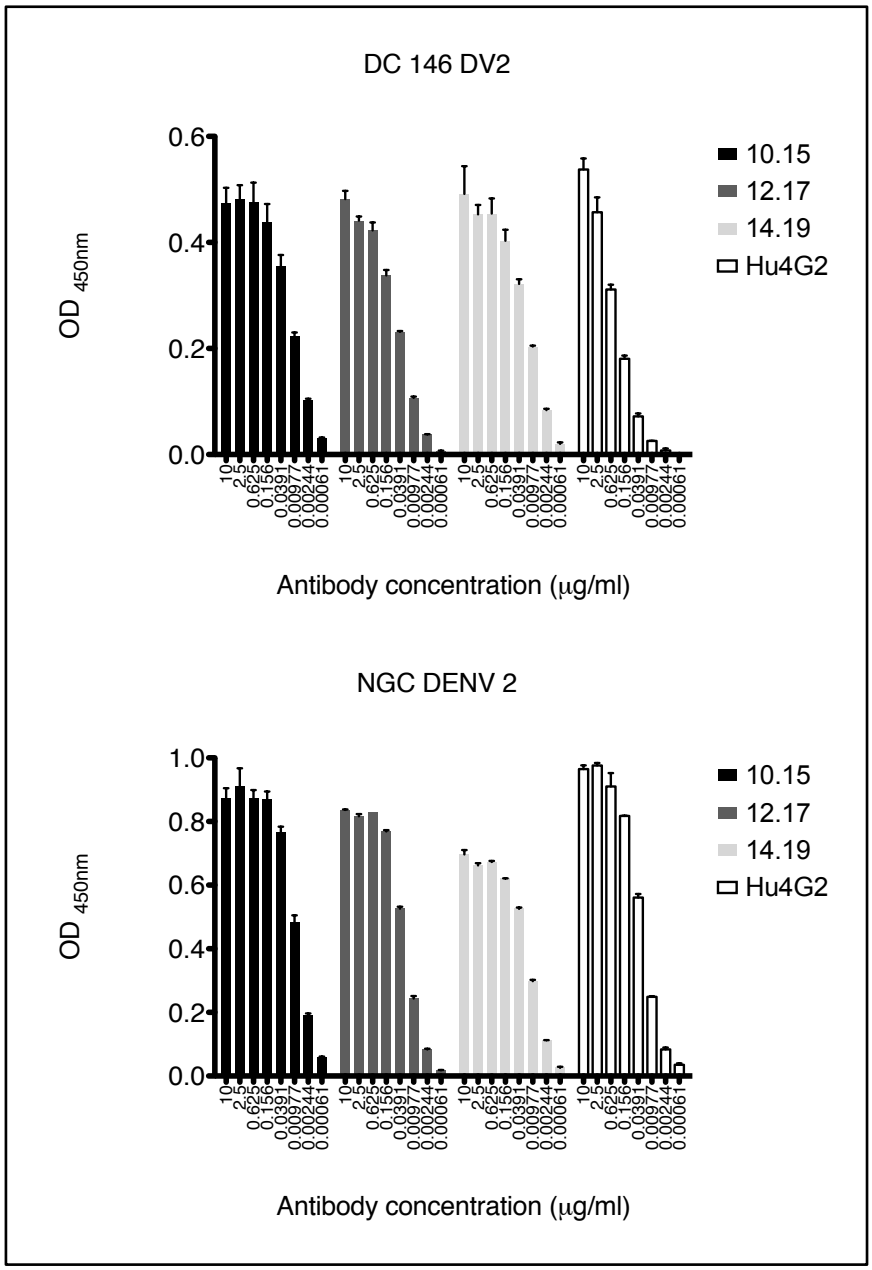


Figure 26 **Comparison of binding activities of 10.15, 12.17 and 14.19 to various DENV2 strains.** 10.15 and 14.19 bind marginally better binding to DC117 and DC186 DENV2 compared to 12.17 and hu4G2, whilst all 3 antibodies show comparable binding activity to DC151 and DC146 DENV2. All 3 antibodies show slightly binding activity to NGC DV2 compared to hu4G2, with 14.19 binding most weakly. Results represent the average of 3 independent experiments performed in triplicates. Error bars represent SDs.

5.2 Neutralizing Activity of anti-DENV2 Antibodies

After establishing that 10.15, 12.17 and 14.19 demonstrated binding activity to DENV2 on a sandwich ELISA, we seek to explore the potential of these antibodies in neutralizing DENV *in vitro*. A standard PRNT was used (Section 3.10). The results were plotted as the percentage neutralization against $\log_{10}$ antibody concentration with a nonlinear fit. $PRNT_{90}$ and $PRNT_{50}$ were computed using nonlinear regression using Graphpad Prism. As seen from Figure 27, 10.15 was the most neutralizing antibody with a $PRNT_{90}$ value of 0.2μg/ml, followed by 14.19 with a $PRNT_{90}$ of 5.77μg/ml and 12.17 with a $PRNT_{90}$ of 10.8μg/ml.

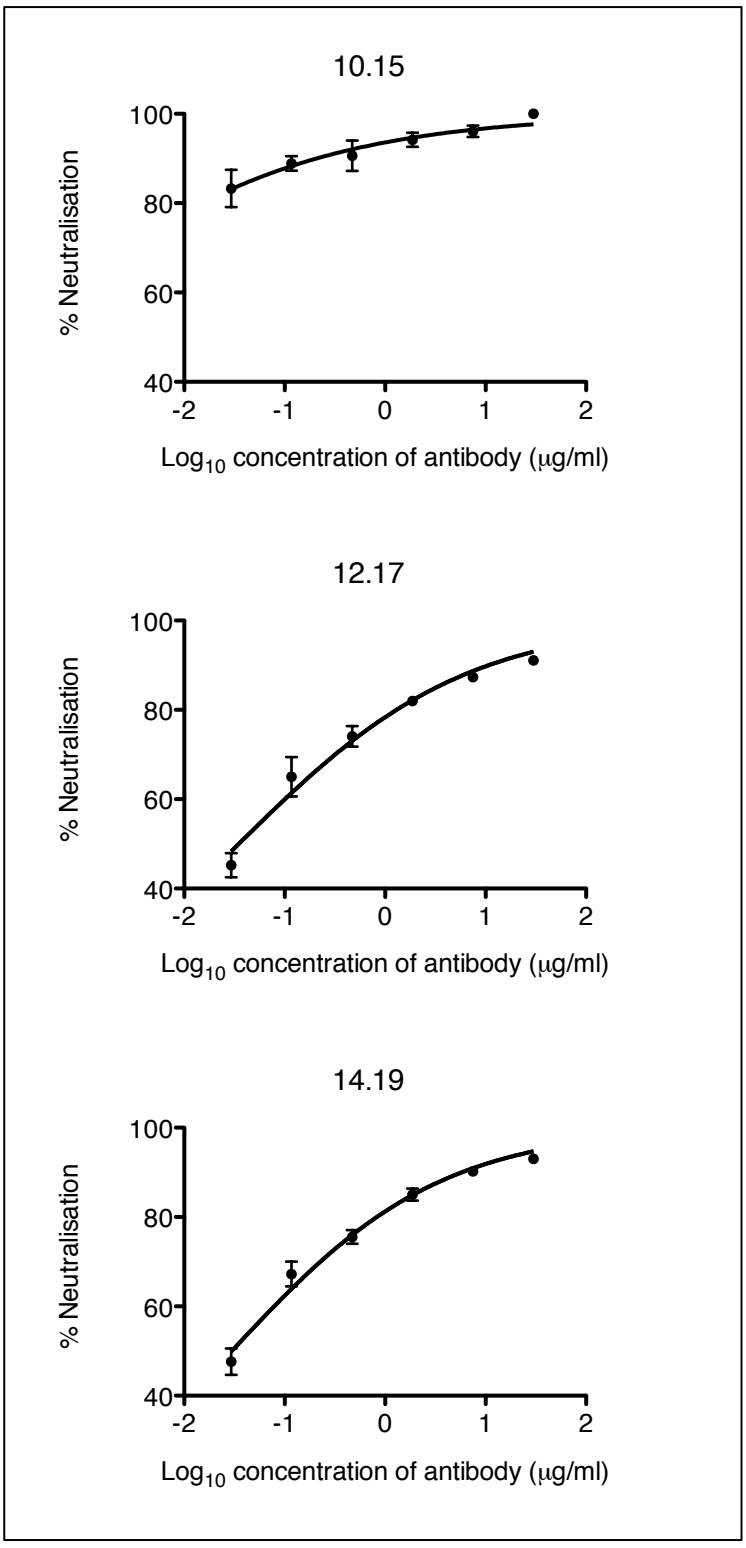


Figure 27 **Neutralization profile of anti-DENV2 antibodies.** Neutralization activity of 10.15, 12.17 and 14.19 was assessed using a standard PRNT. Serially diluted antibody was incubated with EHI DENV2 for 1 hour at room temperature before infecting onto a BHK monolayer for an additional hour at 37°C. Overlay medium was added and incubated for 5 days at 37°C. BHK monolayer was fixed and stained before plaques formed were scored visually. The $PRNT_{90}$ and $PRNT_{50}$ values were computed using nonlinear regression on GraphPad Prism. The values are as follows: 10.15 - $PRNT_{90}$=0.2μg/ml; 12.17 - $PRNT_{90}$=10.8μg/ml, $PRNT_{50}$=0.0349μg/ml; 14.19 – $PRNT_{90}$=5.77μg/ml, $PRNT_{50}$=0.0297μg/ml. Results represent the average of at least 3 independent experiments performed in triplicates. Error bars represent SDs.

5.3 Neutralization profile of 10.15 with various strains of DENV2

As 10.15 was the most neutralizing antibody, subsequent experiments were conducted using this antibody candidate. The neutralizing efficacy of 10.15 was tested against 5 strains of DENV2 (Figure 28). DC117, DC146 and DC186 were acquired from the Environmental Health Institute, Singapore. All three strains belonged to the Cosmopolitan genotype but they are of different lineages. DC117 belongs to clade I, DC146 belongs to clade III and DC186 is of the Indian subcontinent lineage. New Guinea C (NGC) DENV2 belongs to the Southeast Asian genotype and is a commonly used reference DENV2 strain. EHI DENV2 is a local clinical isolate. A standard PRNT was used to assess the neutralizing activity of 10.15 with the various DENV2 strains, as described in Section 3.10. The various strains of DENV2 were incubated with 4-fold serially diluted antibody at a starting concentration of 30µg/ml for a total of 12 dilutions, allowed to form virus-antibody complexes for 1 hour at room temperature before infected onto BHK cells. Results were expressed as percentage reduction in neutralization compared to positive controls with no antibody. The $PRNT_{50}$ values were obtained using linear regression with GraphPad Prism. The $PRNT_{50}$ values of 10.15 with the various strains are as follows: DC117- $PRNT_{50}$ = 0.00782µg/ml, DC146- $PRNT_{50}$=0.159µg/ml, DC186- $PRNT_{50}$ = 0.0136µg/ml, NGC- $PRNT_{50}$ = 1.09µg/ml, EHI DENV2 - $PRNT_{50}$ = 0.0214µg/ml.

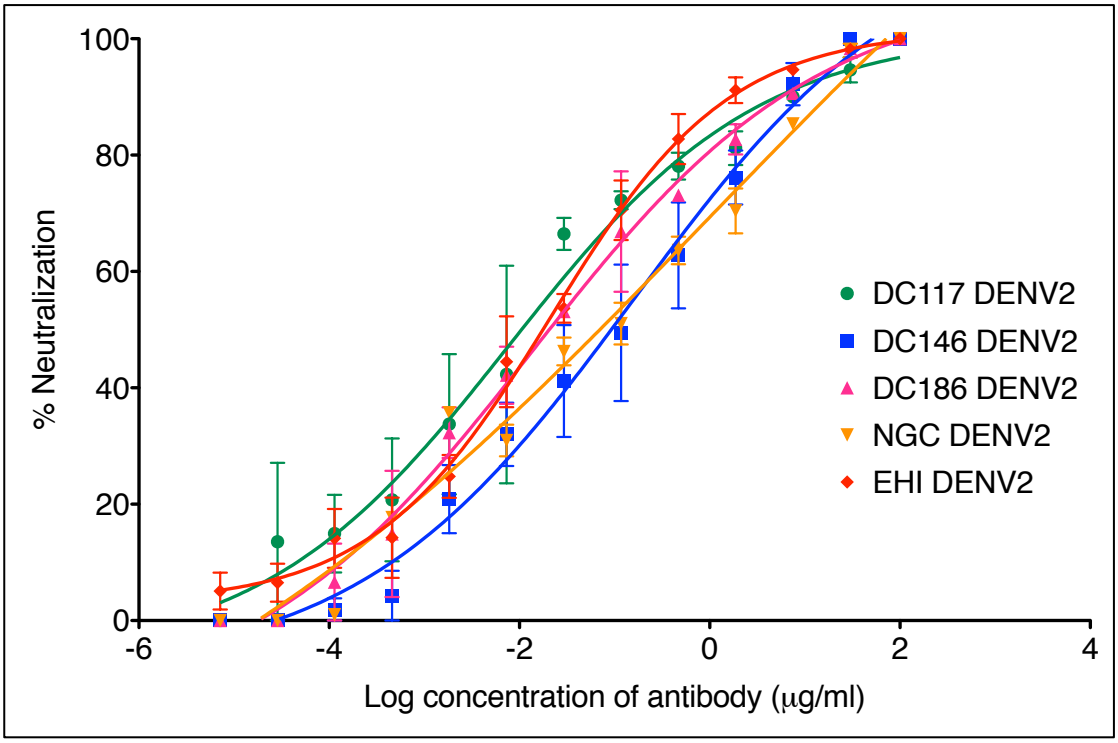


Figure 28 **Neutralization activity of 10.15 across various strains of DENV2.** The neutralizing activity of 10.15 was compared between various strains of DENV2. 10.15 neutralizes all strains of DENV2 tested but to varying degrees. The $PRNT_{50}$ values of 10.15 with the various strains are as follows: DC117- $PRNT_{50}$ = 0.00782μg/ml, DC146- $PRNT_{50}$=0.159μg/ml, DC186- $PRNT_{50}$ = 0.0136μg/ml, NGC- $PRNT_{50}$ = 1.09μg/ml, EHI DENV2 - $PRNT_{50}$ = 0.0214μg/ml. Results represent the average of at least 2 independent experiments performed in triplicates. Error bars represent SDs.

5.4 Comparison of Neutralizing activity of 10.15 at RT versus 37°C

There is suggestion that 10.15 recognizes a cryptic epitope that is more exposed at 37°C compared to at RT. Hence we decided to compare the difference in neutralizing activity of 10.15 when incubated with EHI DENV2 at 37°C versus incubation at RT for 1 hour before the virus-antibody complexes are allowed to infect onto BHK cells (Figure 29). We would expect that if indeed the epitope is more exposed at 37°C, the neutralizing activity of 10.15 would be higher at 37°C compared to at RT. The $PRNT_{50}$ value of 10.15 at 37°C ($PRNT_{50}$ = 0.00419µg/ml) is approximately 4 fold less than that at RT ($PRNT_{50}$ = 0.0161µg/ml). These results suggest that the epitopes recognized by 10.15 may be slightly more preferentially exposed at 37°C.

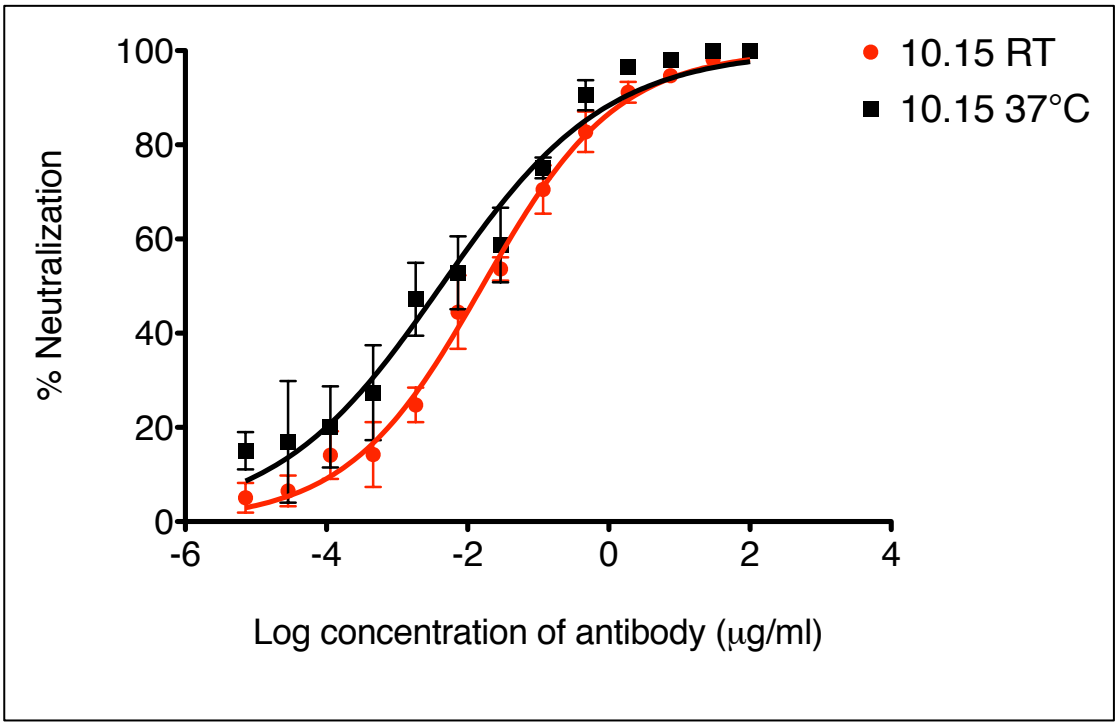


Figure 29 **Comparison of neutralizing activity of 10.15 at RT versus 37°C.** The neutralization activity of 10.15 for EHI-DENV2 was compared between incubating 10.15 with DENV2 at RT versus incubation at 37°C. The $PRNT_{50}$ value of 10.15 at 37°C ($PRNT_{50}$ = 0.00419µg/ml) is approximately 4 fold less than that at RT ($PRNT_{50}$ = 0.0161µg/ml). Results represent the average of 3 independent experiments performed in triplicates. Error bars represent SDs.

5.5 Pre- and post-attachment neutralization assays of anti-DENV2 antibodies

As a first step towards elucidating the mechanism of action of the anti-DENV2 antibodies, we performed pre- and post-attachment neutralization assays. This is to determine if the antibodies work by preventing attachment of DENV2 to the target cells or hindering the attachment of DENV2 to target cells.

As seen in Figure 30, all three mAbs showed lower neutralizing activity in the post-attachment neutralization assay. 12.17 showed the greatest reduction in neutralization activity in the post-attachment ($PRNT_{90}$ = 26.0μg/ml, $PRNT_{50}$ = 1.12μg/ml) compared to that in the pre-attachment assay ($PRNT_{90}$ = 4.55μg/ml, $PRNT_{50}$ = 0.203μg/ml). 14.19 also displayed reduced neutralizing activity but to a lesser extent ($PRNT_{90}$ = 68.2μg/ml, $PRNT_{50}$ = 0.337μg/ml for post-attachment assay compared to $PRNT_{90}$ = 8.52μg/ml, $PRNT_{50}$ = 0.203μg/ml in the pre-attachment assay). 10.15 showed the least reduction in neutralizing activity, with a reduction from $PRNT_{90}$ = 0.638μg/ml in the post-attachment assay compared to $PRNT_{90}$ = 0.290μg/ml in the pre-attachment assay.

There was an approximate 10-fold increase in the $PRNT_{50}$ values of 12.17 and 14.19 in this pre-attachment neutralization assay compared to the PRNT assay described in Section 4.2.2. One possible explanation for this observation is that the mAbs were incubated with DENV2 at 4°C instead of RT in the pre-attachment assay. This was necessary so as to ensure consistency with the post-attachment assay in which the mAbs were added to DENV2 coated on the surface of target cells. It might be possible that epitopes on EHI-DENV2 recognized by the mAbs become more exposed at RT compared to 4°C. Less mAb is required for neutralization at RT since the epitopes are more accessible at RT.

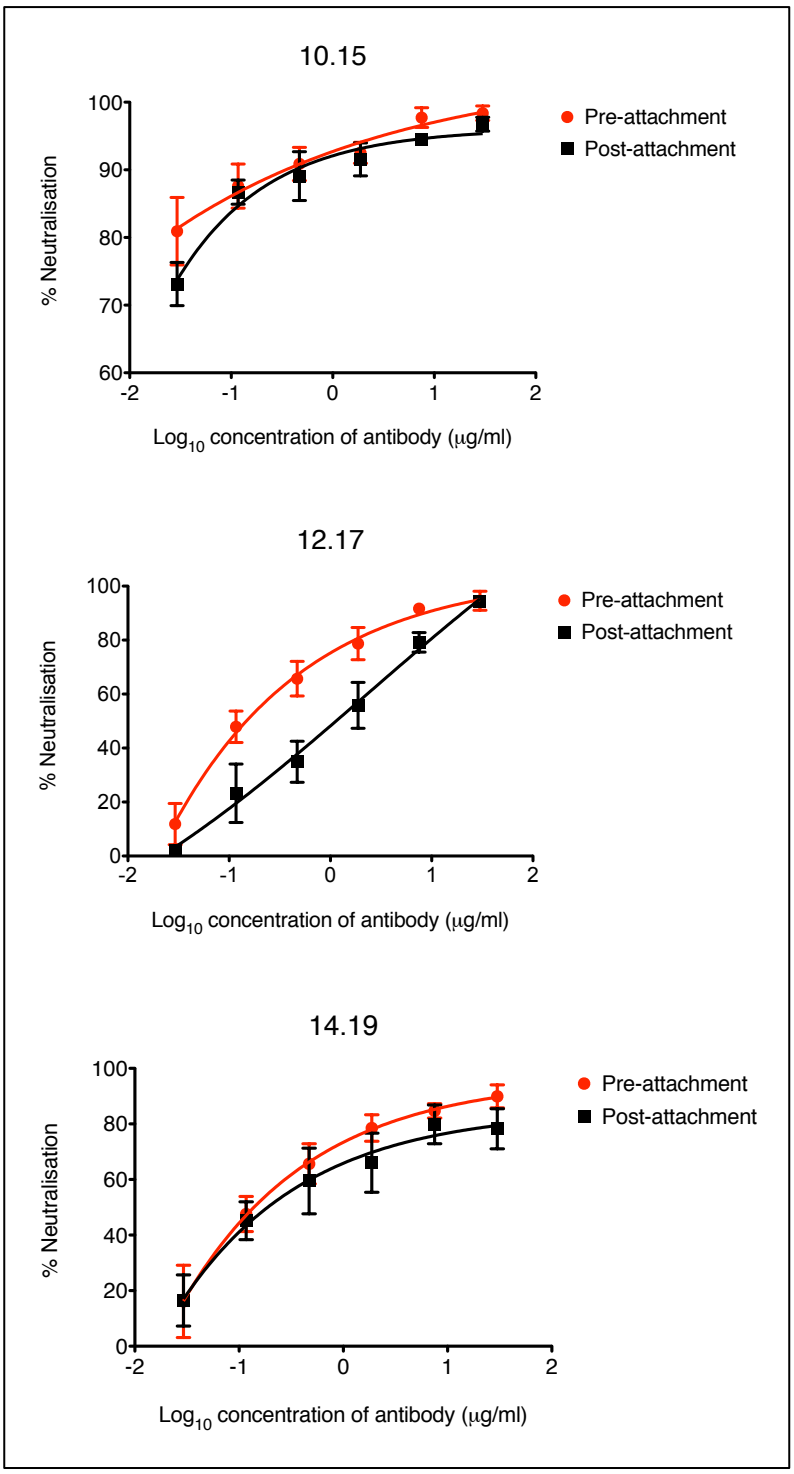


Figure 30 **Pre- versus post-attachment neutralization assays of 10.15, 12.17 and 14.19.** The ability of the mAbs to neutralize DENV2 was compared between incubating them with DENV2 before infecting onto BHK cells (pre-attachment assay) and coating DENV2 onto BHK cells before adding mAbs (post-attachment assay). For the pre-attachment neutralization assay, the $PRNT_{90}$ values of 10.15, 12.17 and 14.19 are 0.290μg/ml, 4.55μg/ml and 8.52μg/ml while $PRNT_{50}$ values of 12.17 and 14.19 are 0.203μg/ml and 0.203μg/ml respectively. For the post-attachment neutralization assay, the $PRNT_{90}$ values of 10.15, 12.17 and 14.19 are, 0.638μg/ml, 26.0μg/ml and 68.2μg/ml while $PRNT_{50}$ values of 12.17 and 14.19 are 1.12μg/ml and 0.337μg/ml respectively. Results are averaged from 3 independent experiments and error bars represent SD between experiments.

5.6 Immunoprecipitation assay with 10.15, 12.17 and 14.19

Since the majority of neutralizing flavivirus antibodies target the E protein, we wanted to investigate if these three anti-DENV2 mAbs recognize the E protein using an immunoprecipitation assay with NGC DENV2-infected Vero cells. Protein A sepharose beads were used to pre-clear Vero cell lysates before immunoprecipitation was carried out with the three anti-DENV2 mAbs. hu4G2 that recognizes the flavivirus group-specific fusion loop at the tip of flavivirus EDII was used as a positive control. Blots were probed with mu3H5 for detection of DENV2 E protein. As seen from Figure 31, all three mAbs were able to pull down E protein prepared from DENV2-infected Vero cell lysates as indicated by the band at approximately 53kDa recognized by primary antibody mu3H5. The mAbs pulled down non-reduced E protein in these lysates, suggesting that they may recognize conformational epitopes. The epitope of 4G2 is sensitive to reduction (Crill et al., 2004) and it is able to pull down non-reduced E protein in the cell lysate.

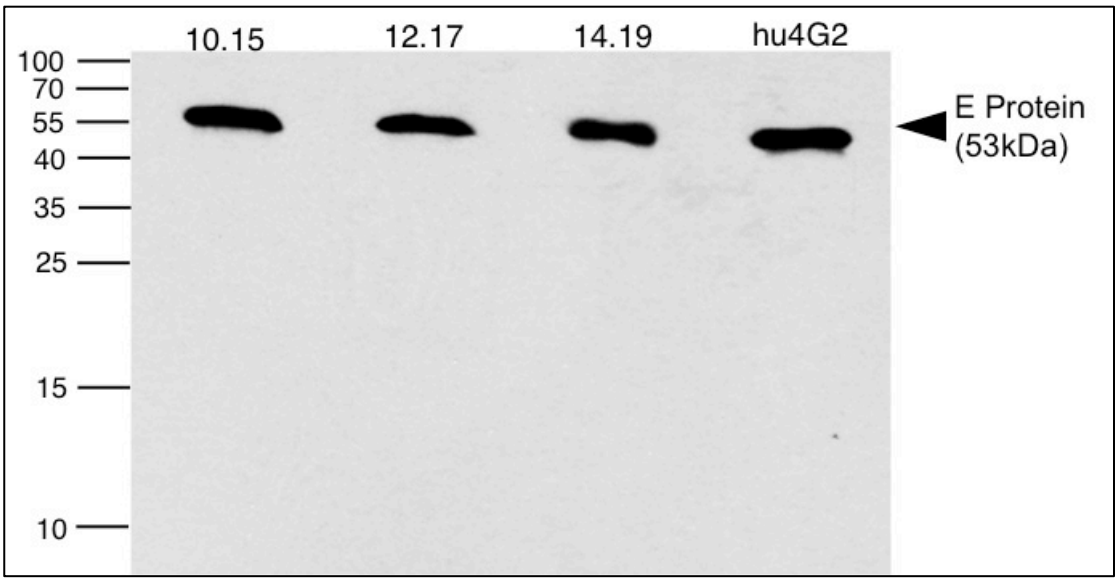


Figure 31 **Immunoprecipitation of DENV2 E protein using 10.15, 12.17 and 14.19**. Pre-cleared NGC DENV2-infected Vero cell lysates were incubated with each mAb. hu4G2 which binds flavivirus fusion loop located at the tip of EDII was used as the positive control. DENV2-mAb complexe were pulled down by protein A sepharose, resolved on reducing SDS-PAGE and analyzed by western blot. Membranes were probed with mu3H5 that recognizes DENV2 E protein to give bands at ~53kDa, indicating that all mAbs pulled down non-reuduced E protein. Results are representative of 2 independent experiments

5.7 Comparison of binding ability of hu3H5 and 10.15 to purified DENV2 on a reducing SDS-PAGE

To determine if 10.15 recognized a conformational or linear epitope on DENV2 E protein, we purified NGC DENV2 by PEG precipitation and subsequently pelleted on a sucrose cushion as described in Section 3.5. Purified DENV2 was reduced with the addition of β-mercaptoethanol, boiled and resolved on a reducing SDS-PAGE. Proteins were transblotted onto a nitrocellulose membrane and probed with either hu3H5 or 10.15, both used at a concentration of 1μg/ml/ As seen from Figure 32, hu3H5 recognized the E protein band at ~53kDa while 10.15 showed significantly weaker binding to the reduced E protein. 3H5 is able to bind to the non-reduced form of DENV2 E protein (A. H. Chan et al., 2012). 10.15 did not recognize the reduced and denatured form of DENV2 E protein, suggesting that the epitope it recognizes may be conformation sensitive. Also, it suggests that 10.15 binds to an epitope distinct from that of 3H5.

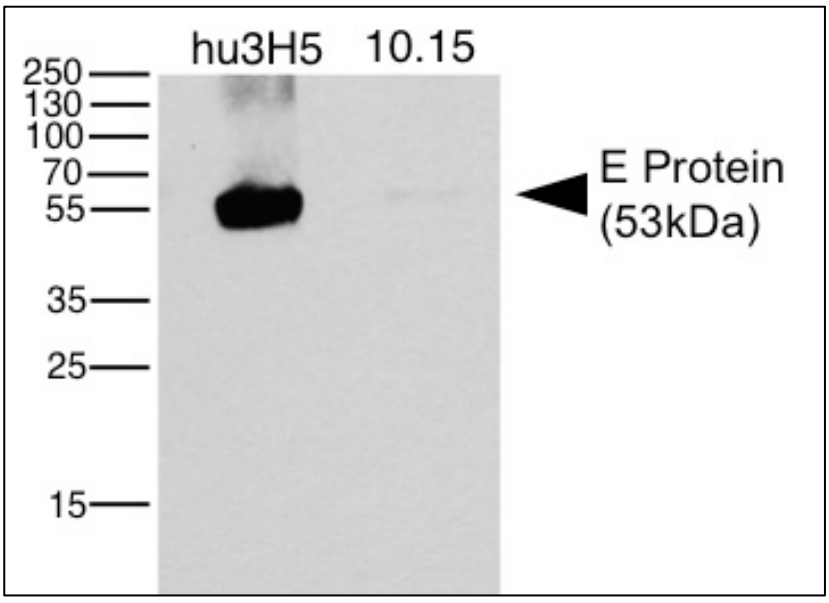


Figure 32 **Comparison of the ability of 10.15 and hu3H5 to bind purified DENV2 under reducing conditions.** Purified NGC was reduced with β-mercaptoethanol and boiled before resolved on a reducing SDS-PAGE. Proteins were transblotted onto nitrocellulose membranes and subjected to Western blot analysis. Blots were probed with 1μg/ml of hu3H5 or 10.15 and detected with anti-human HRP secondary antibody. Hu3H5 recognizes E protein band at ~53kDa while 10.15 binds weakly to E protein. Results are representative of 2 independent experiments.

5.7.1 Binding of DENV2 mAbs to recombinant EDIII

After establishing that all three anti-DENV2 antibodies recognize DENV2 E protein, we want to further elucidate the region on DENV2 E protein that the antibodies bind to. Many anti-flavivirus antibodies have been shown to bind EDIII. To determine if these antibodies bind to EDIII, 2μg of purified TSV01 DENV2 EDIII protein was dotted onto nitrocellulose membrane and probed with the test antibodies 10.15, 12.17 and 14.19. Both the murine and humanized versions of 3H5, which recognizes DENV EDIII, were used as positive controls. hu4G2 that binds to flavivirus group specific fusion loop located at the tip of EDII was used as a negative control. All primary antibodies were used at a concentration of 1μg/ml. As seen from Figure 33, 10.15 and 14.19 both recognized EDIII but 12.17 did not. This indicates that 12.17 recognizes an epitope on E protein that is distinct from EDIII.

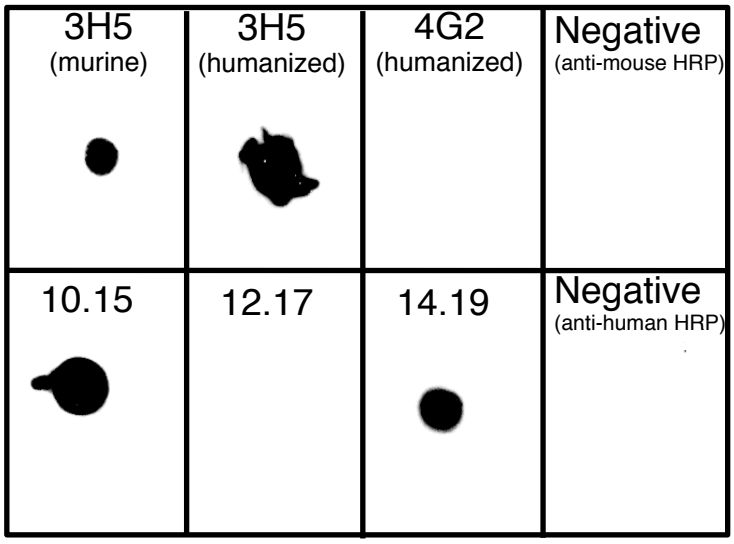


Figure 33 **Binding of 10.15, 12.17 and 14.19 to recombinant EDIII.** 2μg of purified TSV01 DENV2 EDIII protein was dotted onto nitrocellulose membrane and probed with the 10.15, 12.17 and 14.19. Anti-DENV2 EDIII mAb 3H5 (both murine and humanized forms) was used as the positive control while anti-flavivirus fusion loop EDII mAb 4G2 was used as the negative control. All primary antibodies were used at a concentration of 1μg/ml. 10.15 and 14.19 bind to DENV2 EDIII but 12.17 does not. Results are representative of 3 independent experiments.

5.8 Binding of 10.15, 12.17 and 14.19 to recombinant DENV2 EDIII on ELISA

The ability of 10.15, 12.17 and 14.19 to bind recombinant EDIII protein was also investigated using ELISA. 0.25μg per well of TSV201 EDIII coated onto Maxisorp plates were detected with four-fold serially diluted mAbs with a starting concentration of 10μg/ml. hu3H5 (anti-DENV2 EDIII) and hu4G2 (flavivirus group specific, targeting fusion loop located at the tip of EDII) were used as positive and negative controls respectively. As seen in Figure 34, 10.15, 14.19 and hu3H5 bind strongly to DENV2 EDIII. However, 12.17 exhibited very weak binding to EDIII, with signals only slightly higher than that of hu4G2, which does not bind to EDIII. This confirms that 10.15 and 14.19 recognize DENV2 EDIII while 12.17 does not.

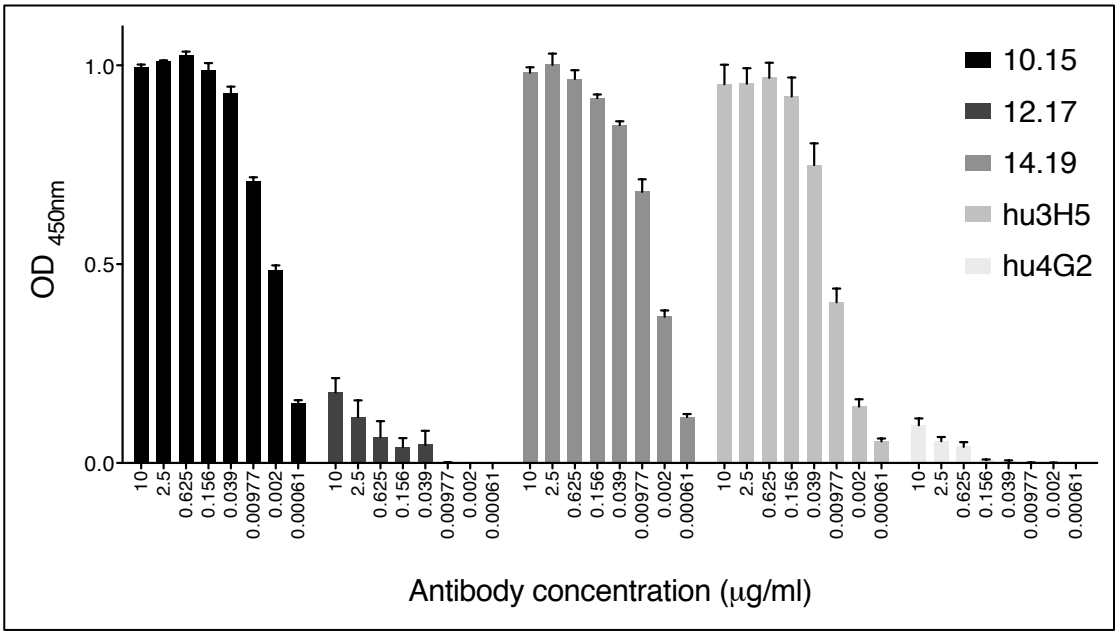


Figure 34 **Binding activity of 10.15, 12.17 and 14.19 to recombinant DENV2 EDIII protein.** 0.25μg per well of TSV201 EDIII was coated onto Maxisorp plates and probed with the respective serially diluted mAbs. Hu3H5 was used as the positive control while hu4G2 was used as a negative control. 10.15 and 14.19 recognize recombinant EDIII protein while 12.17 exhibits almost non-existent binding activity. Results are averaged from three independent experiments and error bars represent SD between experiments.

5.9 *In vivo* efficacy of 10.15

5.9.1 Survival rates of 10.15-treated AG129

The *in vivo* efficacy of 10.15 was investigated using a previously described AG129 model (G. K. Tan, et al., 2011). AG129 mice were subcutaneously (s.c.) infected with a non mouse adapted strain of DENV2, D2Y98P-PP1. Subcutaneous route of administration was used since it mimics the bite of an infected mosquito and is closer to the natural route of infection. Infection with 10^4 PFU/mouse of D2Y98P led to a range of clinical manifestations in the animals including ruffled fur with hunched posture. The observed manifestation developed into lethargy, watery stools and finally moribundity whereby the animals were euthanized. 100% mortality rate was observed at the doses of 10^4 PFU/mouse of D2Y98P. Notably, there were no signs of paralysis observed in the infected mice. 8-week old AG129 mice were infected with 10^4 PFU/mouse of D2Y98P and treated 24 hours post-infection intraperitoneally (i.p.) with 100µg/mouse or 250µg/mouse of 10.15 and PBS as a control, 24 hours post-infection. The survival of the mice was monitored for 32 days and the percentage survival for each group was recorded (Figure 35). Treatments with 100µg/mouse and 250µg/mouse of 10.15 significantly ($p=0.0033$ and $p<0.0001$ respectively) prolonged the survival of AG129 mice compared to PBS control mice when the survival curves were analyzed with a Log-Rank test. 100% of the control mice succumbed to the infection while 90% of the mice treated with 250µg/mouse of 10.15 survived and the percentage survival dropped to 70% by the experimental end point at Day 32. Treatment with 100µg/mouse of 10.15 resulted in slightly lower survival rates, with 70% survival when all the control mice succumbed to infection and 60% at the experimental end-point.

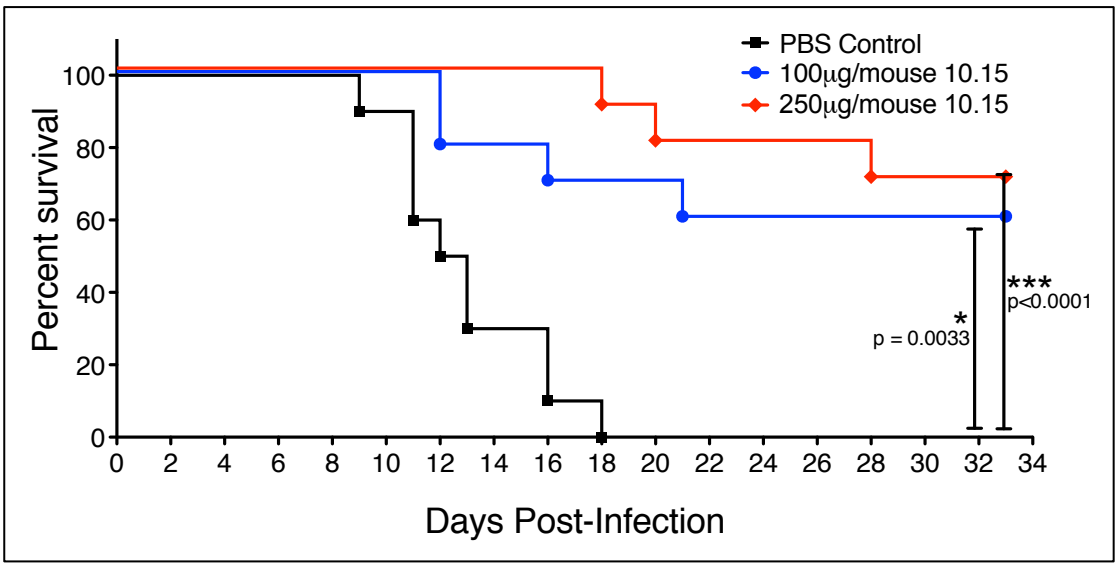


Figure 35 **Survival of AG129 mice**. Mice were treated with 100μg/mouse or 250μg/mouse of 10.15 with PBS as a control, 24 hours after infection with 10^4 PFU/mouse D2Y98P DENV2. Percentage survival was monitored over 32 days. 8-week old AG129 mice were used. There were 10 mice in each group. Results were analyzed with a Log-Rank test and the p values are indicated.

5.9.2 Survival rates of AG129 mice treated with 300µg/mouse of 10.15

After the preliminary investigation of survival rates of D2Y98P-PP1 DENV2 – infected mice treated with 10.15, we decided to monitor the survival of the mice over a longer period of time. 10 mice per group of 8-week old AG129 mice were infected s.c. with 10^4 PFU/mouse of D2Y98P-PP1. 24 hours following infection, the mice were treated with either 300µg/mouse of 10.15 or PBS as a control i.p. Surprisingly, 3 mice (30%) in the 300µg/mouse 10.15 – treated group reached moribundity and were euthanized 6 days p.i (Figure 36). The percentage survival remained at 70% until day 24 p.i. whilst percentage survival in the PBS control group decreased to 0% by day 19 p.i. The survival of the 10.15 treated mice was followed till day 41 p.i. where the survival rate dropped to 20%. Treatment with 300µg/mouse of 10.15 significantly (p=0.0141) prolonged the survival of the majority of mice which were treated with a lethal dose of D2Y98P-PP1 DENV2 when the survival curves were analyzed with a Log-Rank test.

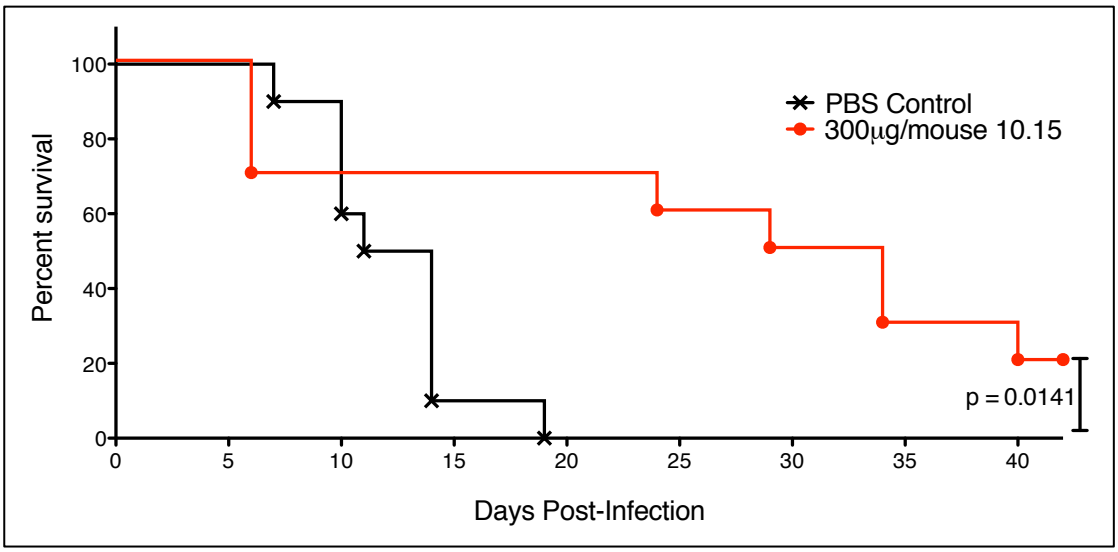


Figure 36 **Survival of AG129 mice**. Mice were treated with 300µg/mouse of 10.15 with PBS as a control, 24 hours after infection with 10^4 PFU/mouse D2Y98P-PP1 DENV2. Percentage survival was monitored over 41 days. 8 week old AG129 mice were used, with 10 mice per group. Results were analyzed with a Log-Rank test and the p value is indicated.

5.9.3 Effect of 10.15 on plasma viremia

We wanted to investigate the effect 10.15 on plasma viremia levels in infected mice. Five mice per group of 8-week old AG129 mice were infected s.c. with 10^4 PFU/mouse of D2Y98P-PP1 DENV2 and treated with either 300μg/mouse of 10.15 i.p. or PBS as a control. Plasma viremia has been previously found to peak at day 5 to 6 p.i. It was previously reported that viremia peaked at day 5 to 6 p.i. before decreasing to undetectable levels by day 12 p.i. corresponding to the time of death of the animals in this infection model (G. K. Tan, et al., 2011). Hence, blood samples were collected from the mice via retro-orbital puncture on days 3, 6, and 9 post-infection. Plasma viremia titers corresponding to the number of infectious virus particles were assessed by plaque assay. As seen from figure 37, viremia levels between the treated and control groups were comparable at day 3 p.i.. However at day 6 p.i., plasma viremia in treated mice was 2.5 $\log_{10}$PFU/ml below that of control mice. At day 9 p.i., plasma viremia in treated mice were undetectable by plaque assay whilst viremia in control mice dropped slightly to 4.8 $\log_{10}$PFU/ml. This suggests that treatment with 300μg/mouse of 10.15 24 hours p.i. was able to significantly lower plasma viremia in infected mice 5 days after treatment (on day 6 p.i.) and reduce it to undetectable levels by plaque assay 8 days after treatment (day 9 p.i.).

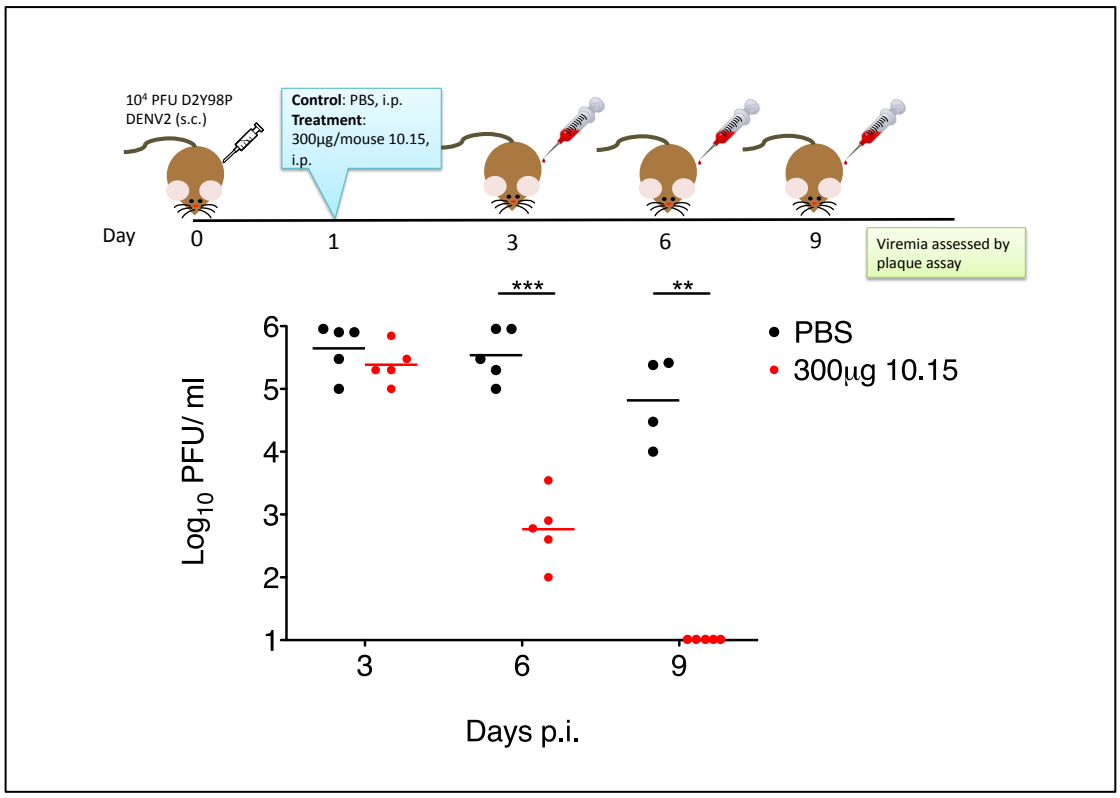


Figure 37 **Plasma viremia titers upon treatment with 10.15.** Mice were treated with either 300µg/mouse of 10.15 i.p. or PBS as a control, 24 hours p.i.. Plasma viremia was assessed on days 3, 6, and 9 p.i.. Viremia titers are expressed as $\log_{10}$ (mean ± SD) PFU/ml of 5 mice per group per time point. Results are representative of 2 independent experiments. ***p<0.0001, **p<0.001.

5.9.4 Assessment of viremia titers in various organs and tissues after treatment with 10.15

It has been previously shown that in this D2Y98P-PP1 DENV2 AG129 infection model, infectious viral particles can be detected in various organs and tissues. Day 5 to 6 p.i. corresponded to the peak of virus concentration detected. Following which, virus in most of the tissues were cleared, except for the brain and spinal cord where the virus concentration increased continuously. Hence the harvesting of various tissues and organs for analysis was performed on day 6 p.i..

To investigate the effect of 10.15 on the levels of viremia in various tissues and organs in this DENV infection model, mice were infected (s.c.) with 10^4 PFU per mouse of D2Y98P-PP1 DENV2 before treated with 300 μg/mouse of 10.15 or PBS as a control 24 hours p.i. as shown in the schematic in Figure 38. The mice were euthanized on day 6 p.i. and extensively perfused with PBS to ensure that the number of viral particles detected in the organs and tissues was accurately represented. The liver, intestines, spleen, kidney, spinal cord and brain were harvested and their wet weights were recorded. The tissues and organs were homogenized individually in 1ml RPMI before cell debris was pelleted by centrifugation and supernatant sterile filtered with a 0.22μm syringe filter. The supernatant was immediately assayed for infectious virus particles using plaque assay.

As seen in Figure 38, there was a significant decrease in infectious viral particles detected when infected mice were treated with 300μg of 10.15 in the liver ($\log_{10}$ 5.49 PFU/ml to $\log_{10}$ 4.17 PFU/ml), intestines ($\log_{10}$ 5.44 PFU/ml to $\log_{10}$ 4.58 PFU/ml), spleen ($\log_{10}$ 5.96 PFU/ml to $\log_{10}$ 4.31 PFU/ml), kidney ($\log_{10}$ 6.05 PFU/ml to $\log_{10}$ 5.04 PFU/ml), spinal cord ($\log_{10}$ 5.40 PFU/ml to $\log_{10}$ 4.24 PFU/ml) and plasma ($\log_{10}$ 6.15 PFU/ml to $\log_{10}$ 3.73 PFU/ml) but not in the brain ($\log_{10}$ 4.55 PFU/ml to $\log_{10}$ 3.00 PFU/ml). The results suggest that administration of 300μg of 10.15 lowers the number of infectious particles not only in the plasma, but also in various other organs.

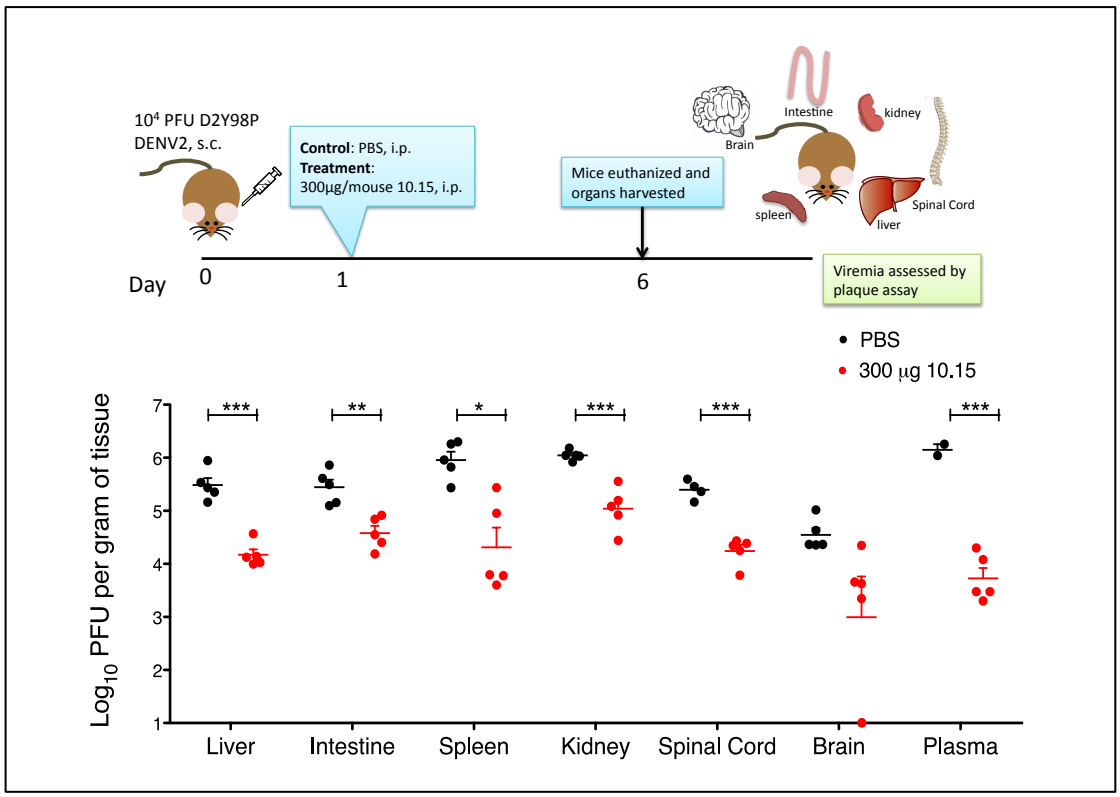


Figure 38 **Infectious viral particles present in organs and tissues from D2Y98P DENV2 – infected mice.** AG129 mice were infected (s.c.) with 10^4 PFU / mouse of D2Y98P DENV2. 24 hours p.i., mice were treated with 300µg/mouse 10.15 or PBS as control. On day 6 p.i., animals were euthanized, extensively perfused with PBS before the liver, intestines, spleen, kidneys, spinal cord, brain and blood harvested. The number of infectious viral particles in the various tissues was quantified on a standard plaque assay. The limit of detection was 10 PFU of virus per gram of tissue. Results are representative of 2 independent experiments. ***p<0.001, **p< 0.01, *p<0.05.

5.9.5 Viremia Kinetics of AG129 mice infected with MT5 DENV2

After establishing that administration of 300µg per mouse of 10.15 confers some degree of protection to mice infected with a lethal dose of DENV2 and lowers the quantity of infectious virus particles in both the plasma and various organs and tissues, we wanted to investigate if this effect is more marked in a non-lethal DENV2 infection model. It has been previously reported that a single amino acid change from Phenylalanine (Phe) to Leucine (Leu) at position 52 of NS4B in D2798P-PP1 DENV2 abolished its lethality in infected mice (Grant et al., 2011). The mutant virus, MT5, resulted in a transient viremia in infected mice. As shown in Figure 39, plasma viremia of 8-week old AG129 mice infected s.c. with 10^4 PFU/mouse of MT5 DENV2 increases gradually from 1 day p.i. to a peak at 4 days p.i. before decreasing to almost negligible levels by day 7 p.i..

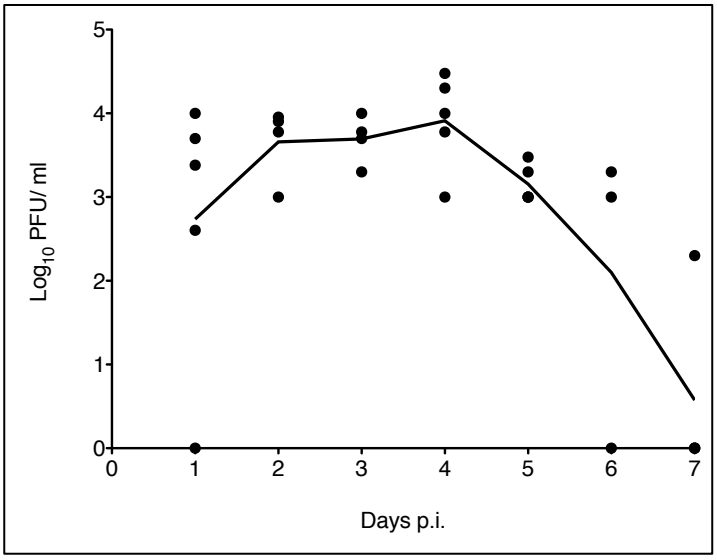


Figure 39 **Viremia kinetics of 8-week old AG129 mice infected s.c. with 10^4 PFU/mouse of MT5 DENV2.** Plasma viremia increases gradually from 1 day p.i. to a peak at 4 days p.i. before decreasing to almost negligible levels by day 7 p.i.. 5 mice were used to determine viremia kinetcs. Blood was obtained by retro-orbital puncture and infectious viral particles assayed on a standard plaque assay.

5.9.6 Assessment of effect of 10.15 in a non-lethal DENV2 infection model

We compared the effects of administering 10.15 prior to infection with MT5 (prophylactic treatment) with administration after infection with MT5 (therapeutic treatment), as outlined in the schematic in Figure 40. Briefly, five mice per group were treated with 300μg/mouse of 10.15 i.p. 24 hours before s.c. infection with 10^4 PFU/mouse of MT5. Another group of mice were treated with 300μg/mouse of 10.15 i.p. 24 hours after s.c. infection with 10^4 PFU/mouse of MT5. Blood was collected via retro-orbital puncture before plasma viremia was assayed on a standard plaque assay.

As seen in Figure 40, four out of five mice prophylactically treated with 300μg/mouse of 10.15 had undetectable plasma viremia on day 4 p.i. whilst there was no significant difference in plasma viremia levels between mice receiving therapeutic treatment compared to control animals. This is consistent with what was observed with administration of 300μg/mouse of 10.15 after infection with D2Y98P-PP1. On day 6 p.i., there is a significant reduction in plasma viremia levels in both the prophylactically and therapeutically treated groups. This suggests that administration of 10.15 prior to infection reduces plasma viremia in infected mice faster (by day 4 p.i.) as compared to post-infection treatment (by day 6 p.i.).

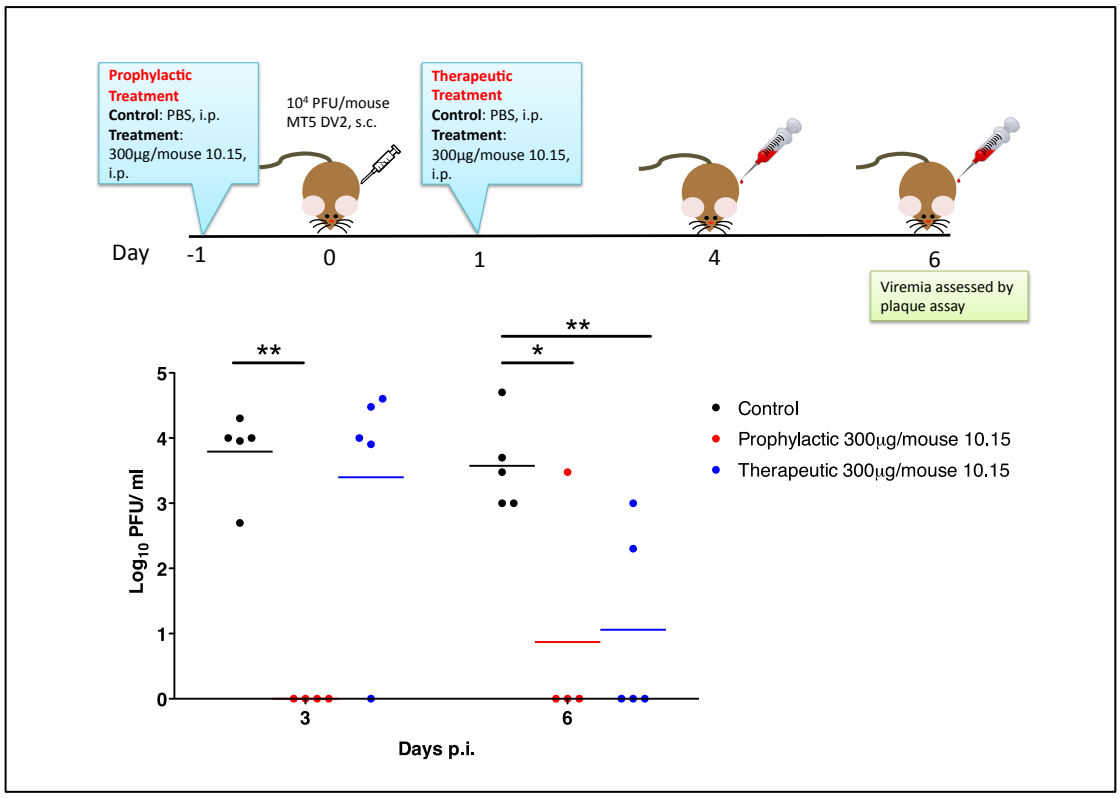


Figure 40 **Plasma viremia titers in a transient viremia model.** Mice were infected with MT5, a mutant strain of D2Y98P-PP1 that is non-lethal in mice. Mice were treated with either 300µg/mouse of 10.15 i.p. 24 hours prior to (prophylactic treatment) or 24 hours after (therapeutic treatment) infection with 10^4 PFU/mouse of MT5 s.c.. Treatment with PBS was used as a control. Plasma viremia was assessed on days 4 and 6 p.i.. Viremia titers are expressed as $\log_{10}$ (mean ± SD) PFU/ml of 5 mice per group per time point. Results are representative of 2 independent experiments. **p<0.01, *p<0.05

5.9.7 Assessment of viremia profile post-treatment

We seek to investigate how quickly plasma viremia decreases following administration of 10.15. As outlined in the schematic in Figure 41, 5 mice per group were infected s.c. with 10^4 PFU/mouse of MT5 24 hours before the administration of 300µg/mouse of 10.15. Mice in the control group were injected with PBS i.p.. Blood was collected via retro-orbital puncture on days 3,4,5 and 6 p.i. and plasma viremia was assayed by plaque assay. As seen in Figure 41, no difference in plasma viremia in the treated versus control groups was detected on day 3 p.i. while there was a non-significant decrease of plasma viremia in treated mice ($\log_{10}$ 3.87 PFU/ml) on day 4 p.i. compared to those in the control group ($\log_{10}$ 4.45 PFU/ml). On day 5 p.i., there was a significant decrease in plasma viremia for mice treated with 10.15 ($\log_{10}$ 2.15 PFU/ml) compared to the control group ($\log_{10}$ 3.92 PFU/ml). By day 6 p.i., plasma

viremia in the treated mice is not detectable by plaque assay while mice in the control group still exhibited viremia (log_{10} 4.35 PFU/ml). This indicates that plasma viremia of mice administered with 300µg/mouse of 10.15 did not decrease by 48 hours post-treatment and only started decreasing by 72 hours post-treatment. There was a significant decrease 98 hours post-treatment followed by a reduction to undetectable levels at 120 hours.

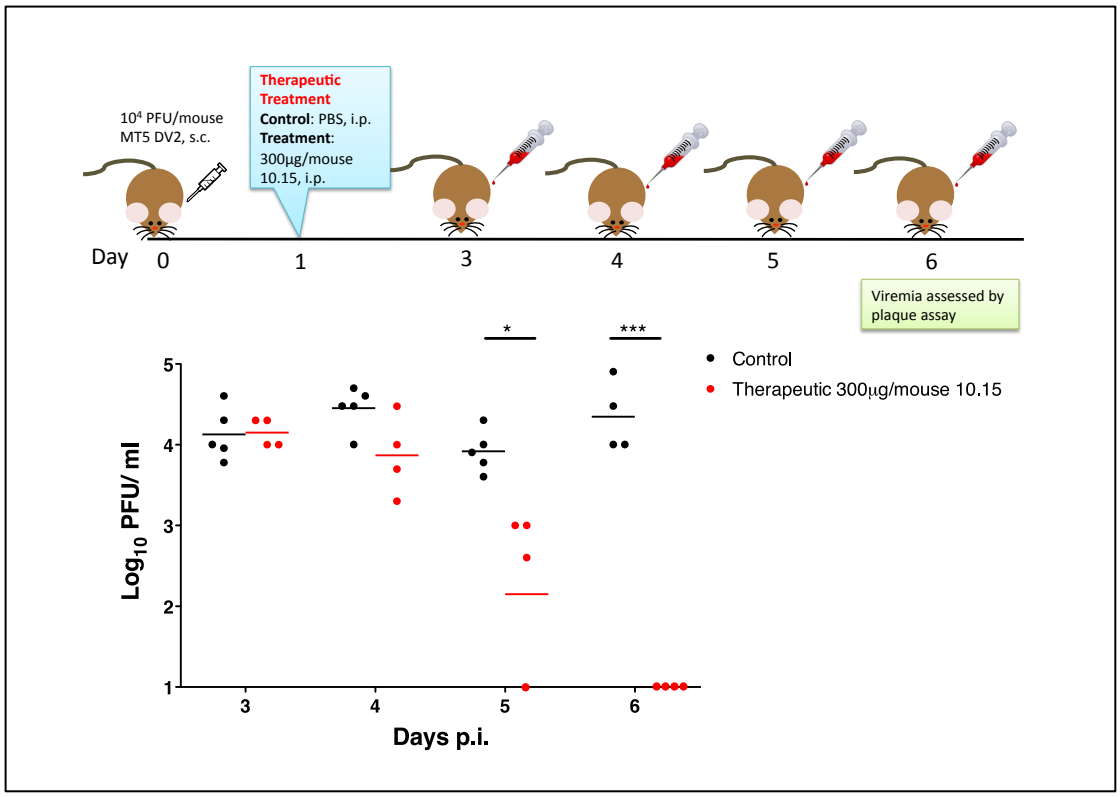


Figure 41 **Assesment of viremia profile following treatment with 10.15.** Mice were infected s.c. with 10^4 PFU/mouse of MT5. Mice were treated with 300µg/mouse of 10.15 i.p. 24 hours after infection. Treatment with PBS was used as a control. Plasma viremia was assessed on days 3, 4, 5 and 6 p.i.. Viremia titers are expressed as log_{10} (mean ± SD) PFU/ml of 5 mice in control group and 4 mice in the treatment group. Results are representative of 2 independent experiments. ***p<0.001, *p<0.05

5.9.8 Comparison of the effect of 10.15 with hu3H5 and hu4G2 treatment on plasma viremia

3H5 (HB46 3H5-1, ATCC) is a murine monoclonal antibody specific for DENV2. 4G2 (HB-112 D1-4G2-4-15, ATCC) is a murine monoclonal antibody against flavivirus group antigen. Both 3H5 and 4G2 exhibit binding activities and varying degrees of neutralization activity for DENV2 (Moi et al., 2010). The humanized versions of 3H5 (hu3H5) and 4G2 (hu4G2) made by swapping their murine Fc chains for human Fc chains, are kindly provided by Dr Brendon Hanson (DSO, Singapore). We wanted to compare the *in vivo* neutralization activity of 10.15, hu3H5 and hu4G2 in a transient viremia model of DENV2 infection.

As seen in Figure 42, there was a significant reduction in plasma viremia on day 4 p.i. in all the treated groups compared to the PBS control (log_{10} 4.34 PFU/ml) – slightly more than 2 log decrease in 10.15 treated mice (log_{10} 1.96 PFU/ml) and a slight decrease in the hu3H5 treated (log_{10} 3.59 PFU/ml) and hu4G2 treated (log_{10} 3.96 PFU/ml) mice. By day 6 p.i., plasma viremia is undetectable in all mice treated with 10.15 while there was no significant decrease in hu3H5 treated mice compared to PBS control (decrease from log_{10} 3.15 PFU/ml to log_{10} 2.70 PFU/ml) and s slight increase in hu4G2 treated mice (log_{10} 3.67 PFU/ml). Hence compared to the two other commercially available antibodies specific for DENV2, 10.15 seems to be more effective in decreasing plasma viremia *in vivo*.

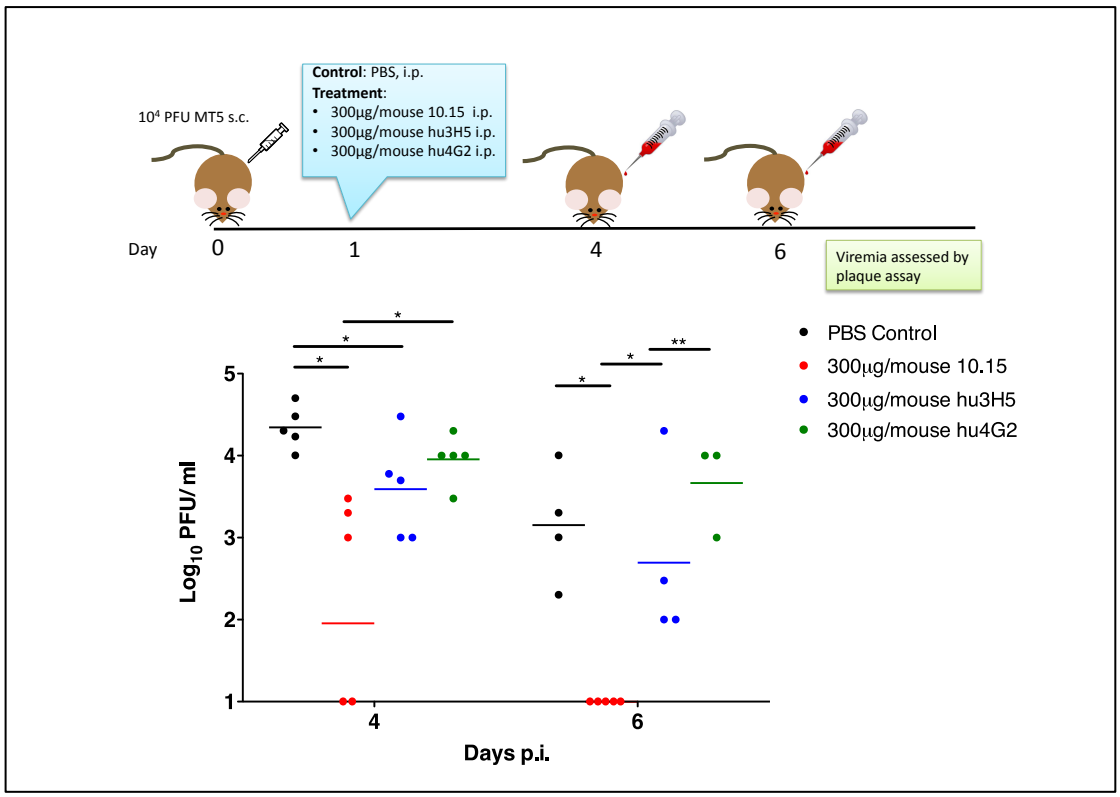


Figure 42 **Comparison of the effect of 10.15 with hu3H5 and hu4G2 *in vivo*.** Mice were infected with 10^4 PFU/mouse of MT5. 24 hours later, mice were treated with either 300µg/mouse of 10.15, hu3H5 or hu4G2 i.p.. Treatment with PBS was used as a control. Plasma viremia was assessed on days 4 and 6 p.i.. Viremia titers are expressed as $\log_{10}$ (mean ± SD) PFU/ml of 5 mice per group per time point. Results are representative of 2 independent experiments. 1 mouse in hu3H5 group and 2 mice in hu4G2 groups were euthanized on day 5 p.i. **p<0.01, *p<0.05

5.9.9 Titration of 10.15 *in vivo*

After establishing that treatment with 300μg per mouse of 10.15 can significantly decrease plasma viremia *in vivo*, we wanted to determine the lowest dosage of 10.15 required to do so. As shown in the schematic in Figure 43, mice were infected with 10^4 PFU per mouse of MT5 24 hours before treating with several doses of 10.15, ranging from 300μg, 150μg, 75μg to 30μg per mouse. Treatment with PBS was used as a control. Blood was collected via retro-orbital puncture on days 4 and 6 p.i. and plasma viremia was assayed with plaque assay.

There was a marginal dose-dependent decrease in plasma viremia for all treatment groups on days 4 p.i. compared to PBS control (Figure 43). Similarly on day 6 p.i., there was a significant decrease in viremia in treated mice. Compared to PBS control ($\log_{10}$ 4.37 PFU/ml), there was no detectable viremia in three out of five mice treated with 300μg/mouse of 10.15, approximately 3 log decrease in mice treated with 150μg/mouse of 10.15 ($\log_{10}$ 1.26 PFU/ml) 2.4 log decrease in mice treated with 75μg/mouse of 10.15 ($\log_{10}$ 1.66 PFU/ml) and a 1.6 log decrease in mice treated with 30μg/mouse of 10.15 ($\log_{10}$ 2.42 PFU/ml). Hence we are confident of the efficacy of the neutralizing activity of 10.15 *in vivo* since it reduces plasma viremia of infected mice in a dose-dependent fashion and even at the lowest dose of 30μg/mouse tested.

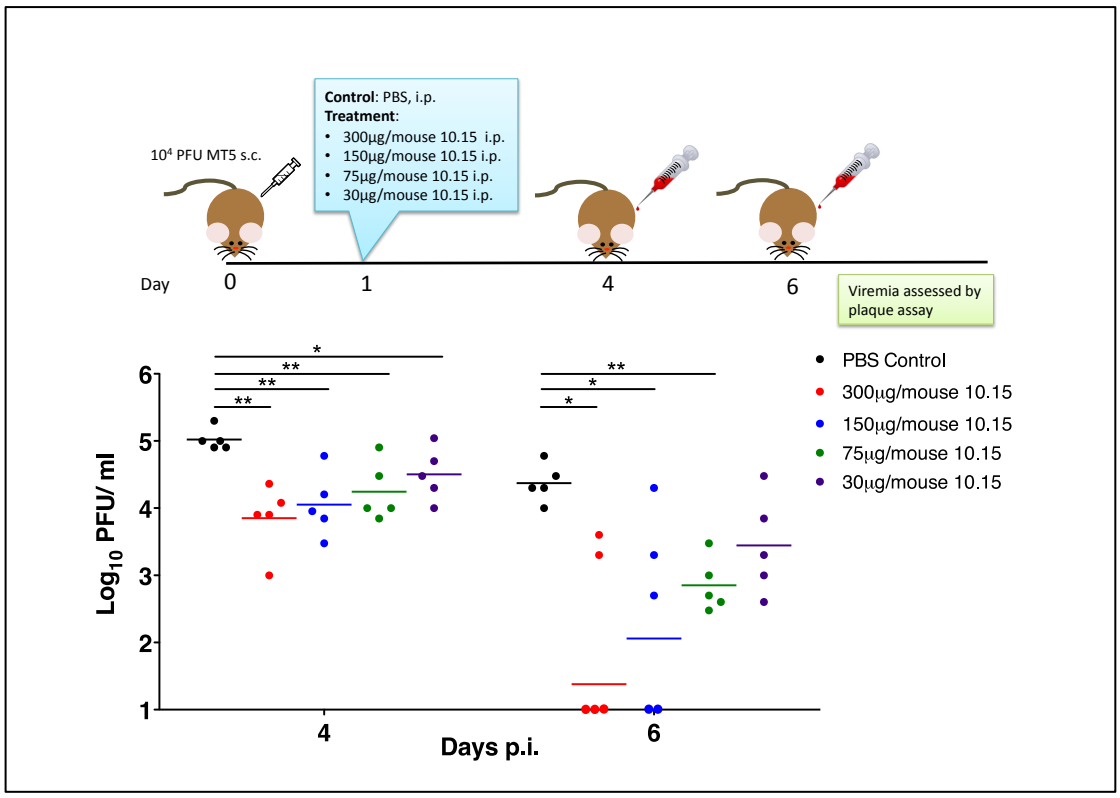


Figure 43 **Titration of 10.15 *in vivo*.** To find out the lowest possible dose of 10.15 required to decrease plasma viremia in MT5 infected mice, a range of doses of 10.15 was administered i.p. 24 hours after infection with 10^4 PFU/mouse of MT5. Viremia was assessed by plaque assay 4 and 6 days p.i.. A dose dependent decrease in plasma viremia was observed on day 6 p.i.. Viremia titers are expressed as $\log_{10}$ (mean ± SD) PFU/ml of 5 mice per group per time point. Results are representative of 2 independent experiments. **p<0.01, *p<0.05

6 Discussion

There has been a long history of using antibodies to treat viral infections. As early as 1888 when the toxin from diphtheria bacterium was isolated, von Behring and Kitasato from Robert Koch's laboratory injected the toxin into animals to generate serum containing antitoxin (or antibody as it is known today) for administrating to patients as a prophylaxis treatment for diphtheria (Waldmann, 2003). In the early 1900s, sera from individuals who have recovered from Rubeola infection were given as a prophylaxis treatment for Rubeola (Marasco, et al., 2007). This procedure became known as "serum therapy" whereby antibodies derived from the serum of immune donors (both humans and animals) were used to treat various infectious diseases (Casadevall et al., 1995). However, the use of polyclonal antisera was fraught with problems such as hypersensitive reactions caused by antigenic effects of foreign proteins and the risks associated with using human blood products.

Nevertheless, passive immunotherapy marked the beginning of the use of monoclonal antibodies (mAbs) as passive immunotherapeutics for the treatment of infectious diseases. mAbs are derived from one single clone of cells with an exquisite antigenic specificity. The use of mAbs as a viable alternative to hyperimmune sera for the treatment of infectious diseases was made possible with several technological advancements, pioneered by the development of the hybridoma technology in 1975 by Kohler and Milstein (Kohler, et al., 1975). It involved the fusion of murine B lymphocytes with a suitable fusion partner, usually non-secretory myeloma cell lines that are lacking the enzyme hypoxanthine guanine phosphoribosyl transferase (HGPRT). Cell fusion is made possible by the use of polyethylene glycol that enhances cell-to-cell adherence and nuclei exchange. Cells that have successfully fused are selected by culture in hypoxanthine, aminopterin and thymidine medium (HAT medium) where only hybrid cells are able to proliferate. The fused cells or hybridomas, are then screened for specificity and cloned by limiting dilution to obtain homogenous cell clones secreting mAbs (C. Zhang, 2012). With the advent of the hybridoma technology, large quantities of consistent and predictable antibody preparations with exquisite specificity against the desired antigen could be produced. However, murine mAbs had their limitations as successful therapeutic agents, as they could not sufficiently activate human effector functions and could possibly elicit

undesirable anti-murine protein response. These problems were addressed to a large extent with advances in genetic engineering to generate chimeric mouse-human or "humanized" antibodies and even fully human antibodies.

The first therapeutic antibody approved by the United States Food and Drug Administration (FDA) to be used as a drug in humans is the anti-CD3 mAb muromonab OKT3 in the year 1986 for the treatment of acute organ transplant rejection (Thistlethwaite et al., 1987). Further technological advancements accelerated the field of therapeutic mAbs, resulting in novel therapies for treating cancer, autoimmune disorders and inflammatory diseases. Notable successes were achieved with the following antibodies. The tumour necrosis factor (TNF)-specific antibodies infliximab and adalimumab for the treatment of rheumatoid arthritis and Crohn's disease, human epidermal growth factor receptor 2 (HER2)-specific antibody trastuzumab and vascular endothelial growth factor A (VEGFA)-specific antibody bevacizumab for treating several cancers and CD20-specific antibody rituximab used to treat rheumatoid arthritis and non Hodgkin's lymphoma (Leavy, 2010). mAbs and related products remain as the highest selling class of biologics in the United States in the year 2012 with total sales exceeding $24 billion and are dominated by mAbs for cancer and inflammatory disorders (Aggarwal, 2012). Although there have been numerous mAbs licensed for therapeutic purposes, their impact on infectious disease therapy is less marked (Brekke et al., 2003). To date, the only mAb licensed for an infectious agent is palivizumab, a humanized mAb that targets respiratory syncytial virus (RSV) (Group, 1998; S. Johnson et al., 1997) and is used in the prevention of RSV infections in high-risk infants (Groothuis et al., 2002).

While the production of a dengue vaccine remains the long-term goal for the eradication of the disease, the formulation of an effective vaccine proved to be more challenging than previously thought. The dengue vaccine that was furthest down the development pipeline, CYD-TDV from Sanofi Pasteur, proved to be disappointing in a recently completed Phase 2b clinical trial (Sabchareon, et al., 2012). The CYD-TDV vaccine candidate is a recombinant live attenuated tetravalent formulation that was theoretically be protective against all DENV serotypes. However, it failed to confer protection against DENV2, which coincidentally was the most prevalent serotype during the trial. Phase 3 trials are currently underway and the efficacy of this

candidate will be unveiled in late 2014. In the meantime, to address the pressing need for specific treatments for DENV infection, DENV-specific antibodies seem to be an attractive option.

In this study, we have generated and/or characterized two fully human anti-DENV antibodies against each specific serotype (DENV1 and DENV2), using two different methodologies. Anti-DENV1 mAb 14C10 was generated with an improved strategy of memory B-lymphocyte immortalization using EBV, as previously described for the generation of neutralizing antibodies that target severe acute respiratory syndrome (SARS) coronavirus (Traggiai, et al., 2004). It was shown that with this methodology, the immune repertoire of a convalescent patient could be interrogated to isolate antibodies generated as part of the immune response elicited in a natural infection. Antibodies with such affinities are particularly interesting since they constitute the neutralizing antibody repertoire that contributed to the clearing of the virus during the course of infection in the human host. The second antibody, anti-DENV2 10.15, was generated from the immune human Fab fragment phage library of a convalescent dengue patient. This strategy was adapted from a previously described methodology of rapidly generating high affinity human antibodies from a non-immunized human Fab fragment phage library (de Haard, et al., 1999). They devised a strategy to construct a very large antibody library using an improvised cloning strategy whereby heavy and light chain variable genes were separately cloned as digested PCR products. The V_H and V_L genes were combined by restriction fragment cloning to obtain a large library of 3.7×10^{10} independent Fab clones. The authors demonstrated that they could select specific antibodies to more than 20 different antigens, making this phage library methodology a potential source of antibodies to essentially any desired target. We adopted this methodology to generate an immune library from a convalescent dengue patient who was infected with DENV2 and screened the memory B cell repertoire against DENV2.

Most of the early knowledge of anti-DENV antibodies was obtained from studies performed using murine antibodies. These studies on DENV and other related flaviviruses led to the general consensus that the major antigenic target of neutralizing antibodies on the exposed surface of the virion is the E protein. Studies with mouse mAbs determined that anti EDI/EDII antibodies are generally more serotype cross-

specific and with low to moderate neutralizing activity (Crill, et al., 2004; Oliphant et al., 2006). On the other hand, mouse mAbs against EDIII, which protrudes from the surface of virions, are usually serotype-specific with potent neutralizing activity (Crill, et al., 2001; B. Lin et al., 1994; Sukupolvi-Petty et al., 2010). However, it should be noted that not all antibodies that target EDIII are serotype-specific. A number of groups showed that the residues recognized by the type-specific antibodies that recognize only DENV2 and subcomplex-specific antibodies that recognize more than one DENV serotype are different (Gromowski et al., 2007; Gromowski et al., 2008; Rajamanonmani et al., 2009; Sukupolvi-Petty et al., 2007). Type-specific mAbs target the BC, DE and FG loops on the lateral ridge of EDIII whilst subcomplex-specific mAbs target an adjacent epitope on the A strand of EDIII, specifically residues K305, K307 and K310 (Sukupolvi-Petty, et al., 2010).

Crill and Roehrig provided the earliest direct proof that EDIII encodes a host cell receptor-binding motif by demonstrating that EDIII specific mAbs were the most potent blockers of virus adsorption to target Vero cells compared to EDI and EDII specific antibodies (Crill, et al., 2001). This finding was consistent with previous studies with several flaviviruses that propose EDIII to contain the generalized flavivirus host cell-binding anti-receptor (Lee et al., 2000; Mandl et al., 2000; Rey, et al., 1995; van der Most et al., 1999). It also suggests that neutralizing antibodies act at least in part by interfering with the virus's ability to bind to cellular receptors on mammalian host cells.

3H5 is a murine DENV2 specific mAb that binds to the lateral ridge on EDIII. An early study to elucidate its antigenic site using mutational analysis revealed it to be the Glu-Pro-Gly sequence at positions 383-385 of DENV E protein, located on the lateral surface of EDIII (Hiramatsu, et al., 1996). However, a subsequent study by Gromowski and colleagues did not fully support this finding. They proposed that only residue P384 was critical while E383 is on the periphery and G385 was not part of the epitope. They also proposed that the epitopes recognized by 3H5 and 6 other DENV2-specific mAbs were conformational epitopes since the two critical residues they identified, K305 and P384, lie on adjacent and discontinuous sections of EDIII (Gromowski, et al., 2007). They predicted that 3H5 recognized an epitope that span at least 3 discontinuous segments including residues on the N-terminal linker to β-strand

A (301-307), the BC loop region (327-332) and FG loop region (383-384) that in its entirety constitute an antigenic site located on the upper surface of the lateral side of EDIII. This finding corresponds to the results of studies of other flaviviruses like WNV (Nybakken, et al., 2005) and JEV (K. P. Wu et al., 2003) in which DIII of the E protein had been identified as the major antigenic site.

One of the greatest challenges in the current dengue field is to extrapolate the results from the studies conducted with murine anti-DENV mAbs to the human anti-DENV response because they might not recognize similar epitope(s). This is particularly important since this knowledge is critical for the design of effective vaccines to stimulate neutralizing antibody responses in immunized individuals since titers of neutralizing antibodies is widely believed to correlate with protective efficacy of vaccines. The human antibody response to DENV infection is complex since it encompasses a polyclonal response to primary, secondary or even subsequent infections to potentially up to 4 different DENV serotypes. Early studies conducted by Sabin in the 1950s demonstrated that protection against a homologous serotype was long-lasting, whereas that against a heterologous serotype was merely transient (Sabin, 1952). A large percentage of the human antibody response to DENV is cross-reactive with all four DENV serotypes, and even other related flaviviruses. These antibodies are also weakly neutralizing. In a recent study, it was found that early convalescent human sera consist of high levels of weakly neutralizing cross-reactive antibodies that can form large virus-antibody complex aggregates that bind the inhibitory FcγRIIB receptors on monocytes for its subsequent clearance (K. R. Chan et al., 2011). Since levels of such cross-reactive antibodies decrease with disease progression, protection from such cross-reactive antibodies is transient. Conversely, they found that strongly neutralizing, type-specific antibodies did not have to form aggregates to effectively neutralize DENV (K. R. Chan, et al., 2011).

Human antibodies that are type specific and strongly neutralizing constitute only a small percentage of the total anti-DENV antibody response (de Alwis et al., 2011). Most of the antibodies engendered in a natural dengue infection are cross reactive with all DENV serotypes and weakly neutralizing (Beltramello et al., 2010; de Alwis, et al., 2011; Dejnirattisai, et al., 2010). Using blocking experiments, Lai and

colleagues demonstrated that more than 90% of anti-E antibodies engendered in a primary DENV infection were cross-reactive and non-neutralizing against heterologous serotypes and these cross-reactive anti-E antibodies recognized the fusion loop of EDII (C. Y. Lai et al., 2008). When the sera of individuals infected with DENV were analyzed, antibodies against both structural proteins (E, prM and C) and non-structural proteins (NS1, NS3 and NS5) were identified (Abubakar, Azila, et al., 2002; Churdboonchart et al., 1991; Lazaro-Olan et al., 2008; Valdes et al., 2000).

Since E protein is the main antigen exposed on DENV surface and the target of neutralizing antibodies, it is the main focus of the majority of studies of DENV antibodies. Building on the work performed on murine antibodies against DENV, several groups have investigated if EDIII was the main neutralizing epitope targeted by human antibodies as well. It was found that although anti-EDIII antibodies are rare in the human antibody repertoire, constituting only 5 to 15% of the total serotype-specific neutralizing antibody titer (Midgley et al., 2011; Wahala, et al., 2009), they correlated significantly with protective neutralizing titers, a result consistent with that observed in murine studies (Crill et al., 2009). This hypothesis is supported by several studies. Wahala and colleagues (Wahala, et al., 2009) found that when human immune sera were depleted of anti-EDIII antibodies, there was no appreciable decrease in neutralization potency, suggesting that antibodies targeted against EDIII play a minor role in DENV neutralization. They proposed that antibodies that target other epitopes on DENV are major subsets of antibodies of the human repertoire that which is suggested to be mainly orchestrated by antibodies that target other epitopes on DENV (Wahala, et al., 2009). Recombinant viruses containing mutations in the lateral ridge and A strand epitopes were also strongly neutralized by human DENV immune sera, suggesting that these epitopes which are targeted by strongly neutralising murine antibodies are not the main targets of human neutralising antibodies (Wahala, et al., 2012). Human DENV-immune serum depleted of anti-EDIII antibodies were also found to protect mice infected with a homologous DENV serotype (Williams et al., 2012). Similarly, antibodies mapping to the lateral ridge of EDIII of WNV do not constitute the bulk of neutralizing activity in immune serum of naturally infected human patients and horses (Oliphant et al., 2007; M. D. Sanchez et al., 2007). This suggests that anti-EDIII antibodies are probably not the major subset

of antibodies that contribute to the strong serotype-specific neutralising antibody response generated in humans following a natural DENV infection.

The two human mAbs generated in this study are serotype-specific. 14C10 binds and neutralizes only DENV1 while 10.15 binds and neutralizes only DENV2. Both antibodies were isolated from individuals who have recovered from DENV infection. By scanning the immune repertoire of such individuals, it would theoretically be possible to isolate type-specific potently neutralizing antibodies for eventual use as a potential therapeutic to treat DENV infections.

The generation and initial characterization of 14C10 has been previously described (Teoh, et al., 2012). Briefly, 14C10 was generated from screening two thousand B cell clones immortalized by EBV derived from a convalescent DENV1 patient. Supernatants were screened for their binding activity and subsequently neutralizing activity using a standard PRNT. The clone 14C10 was identified based on its $PRNT_{100}$ at a dilution factor of 1:1028 of the B cell line supernatant. Total RNA was extracted from the 14C10 B cell line clone and the heavy and light chain templates cloned into the pTT5 vector for expression of IgG1. 12 recombinant IgG1 mAbs were cloned and expressed from the 14C10 EBV B cell line by from the random combinations of the several heavy and light chain templates extracted. Only one recombinant mAb template exhibited binding activity to DENV1 on a sandwhich ELISA, named mAb 14C10. PRNT activity of 14C10 was tested against DENV1, 2, 3 and 4. 14C10 neutralized only DENV1. It also bound to only DENV1 and not the other DENV serotypes on ELISA.

As part of this study, the binding activity of 14C10 to all 5 genotypes of DENV1 was compared with hu4G2. Hu4G2 is the humanized form of the mouse monoclonal flavivirus group-reactive antibody 4G2 that recognizes the fusion loop residues on flavivirus E protein. Hu4G2 was found to bind more strongly than 14C10 to all the genotypes tested except to 3146SL (genotype 5) where 14C10 exhibited slightly better binding. This observation that 14C10 exhibits weaker binding to DENV1 compared to hu4G2 is interesting since hu4G2 is weakly neutralizing and 14C10 is a strong neutralizer. This suggests that strongly neutralizing antibodies may not bind strongly to the virus. This has implications for the screening of mAbs for strong

neutralizers – a functional screen for neutralizing activity rather than binding activity only would be necessary since the strong neutralizers might be overlooked.

Within each DENV serotype are various genotypes that can differ at their nucleotide and amino acid levels by about 6% and 3% respectively (Rico-Hesse, 1990). Genotypic differences in the *in vitro* and *in vivo* neutralizing activity and epitope binding specificities of large panels of anti-DENV mAbs against DENV1, DENV2 and DENV3 have been addressed in several studies (Brien et al., 2010; Shrestha et al., 2010; Sukupolvi-Petty, et al., 2010). In one study, a panel of 79 murine anti-DENV1 antibodies raised against a strain of DENV1 belonging to genotype 2 exhibited lowered 6 to 9000 fold reduction in $PRNT_{50}$ values against DENV1 strains from heterologous genotypes, indicating that the limited amino acid differences within each DENV serotype can lead to variable antibody neutralization (Shrestha, et al., 2010). Only 2 of these mAbs retained strong binding and neutralizing activity against DENV1 from all 5 genotypes. Similarly, 4 out of 18 strongly neutralizing DENV2 mAbs could not neutralize DENV2 of a heterologous genotype (Sukupolvi-Petty, et al., 2010). Several DENV3 mAbs found to have highly protective *in vivo* effects in mice had no effect on a heterologous strain of DENV3 (Brien, et al., 2010). These observations suggest that it might be important to assess the ability of an antibody to neutralize more than one genotype of DENV1. The neutralizing activity of 14C10 was tested against all 5 genotypes of DENV1. 14C10 neutralized all 5 genotypes of DENV1 albeit to various extents, with slightly better neutralizing activity for genotypes 1, 3 and 5 as compared to genotypes 2 and 4. This is consistent with the observation that the epitope residues across all genotypes are conserved. The difference in neutralization activity across genotypes could be then due to differential exposure of epitope residues for binding.

Although antibodies specific for viruses are generally considered to play important roles for the control of viral infections via various means, there are some instances whereby the presence of virus-specific antibodies that benefit the virus instead and are detrimental to the host. This phenomenon is known as antibody dependent enhancement (ADE) of viral infections. ADE involves the enhancement of entry and/or replication of the virus into monocytes, macrophages and granulocytic cells via

engagement of Fc and/or complement receptors (Cardosa et al., 1983; Halstead, 1982; Mady et al., 1991; Porterfield, 1986). ADE has been reported for viruses from numerous families and orders including YFV (Schlesinger et al., 1981), HIV-1 (Robinson et al., 1988), RSV (Gimenez et al., 1989), hantavirus (Yao et al., 1992), Ebola virus (Takada et al., 2001), WNV (S. Nelson et al., 2008), JEV (Kimura-Kuroda et al., 1988), influenza virus (Ochiai et al., 1992). These viruses share several similarities such as their ability to replicate partly or exclusively in macrophages (Halstead, 1989; Stueckemann et al., 1982; Theofilopoulos et al., 1976), their ability to induce antibodies that have poor neutralizing activity even against homologous virus and antigenic diversity among isolates which can render isolates partly resistant to antibodies raised against heterologous isolates (Halstead, 1982; Porterfield, 1986). ADE activity in DENV infections has been associated with severe dengue such as DHF and DSS, which occurs DENV forms complexes with antibodies at subneutralizing concentrations, which bind to cells that express Fc receptors. This results in increased uptake of virus and secretion of pro-inflammatory cytokines and chemokines (Halstead, 2003). Unsurprisingly, 14C10 causes homotypic enhancement of DENV1 when it is titrated to sub-neutralizing concentrations but not heterotypic enhancement of DENV2, 3 and 4 to which it does not bind (Teoh, et al., 2012). ADE activities of antibodies have been shown in several viruses including flaviviruses and EV71 to be dependent on IgG subclass (Mehlhop, et al., 2007) (Cao et al., 2013). When we compared the ADE activity of 14C10 IgG1, 14C10 IgG2, 14C10 IgG3 and 14C10 IgG4 in an *in vitro* K562 model of ADE, we found that it correlated partially with reported binding activities for FcγRIIA expressed on K562 cells, with IgG3 > IgG1 > IgG2 > IgG4. We can conclude that ADE activity of 14C10 is dependent on Fcγ receptor binding although the K562 ADE model used in our study failed to address the contributions of FcγRI and complement component. Particularly, complement component C1q has been shown to restrict ADE by anti-flavivirus IgG antibodies in a subclass-dependent fashion both *in vitro* and in mice – IgG subclasses that bind C1q strongly induced minimal ADE when C1q was present (Mehlhop, et al., 2007).

Since ADE has been postulated to cause increased disease severity by increasing the viral load and/or number of infected cells, the fact that 14C10 causes ADE at subneutralizing levels might be a valid concern when evaluating 14C10 for use as a

potential therapeutic. We demonstrated that the Fab fragment of 14C10 that eliminates Fc receptor binding due to its lack of its Fc portion and the N297Q variant of 14C10 which has diminished Fc receptor binding capabilities, both demonstrated reduced homotypic ADE with DENV1. This suggests that these variants could be better candidates for administration as a therapeutic since it would eliminate the risk of disease enhancement.

The humoral immunity is a crucial component of host defense against flavivirus infection. While the main target or neutralizing antibodies is the E protein on the surface of flaviviruses, antibodies against prM and NS proteins have also been found. To understand the mechanism of how antibodies neutralize viruses, an important step would be to identify the target epitopes of neutralizing antibodies that is made possible with epitope mapping studies. Numerous sites on the E protein of flaviviruses have been identified as targets of neutralizing antibodies (Pierson et al., 2008). Some of these include the lateral ridge of EDIII of WNV (Nybakken, et al., 2005) and JEV (K. P. Wu, et al., 2003), A-strand of EDIII of DENV (Cockburn, Navarro Sanchez, Fretes, et al., 2012) (Lok, et al., 2008), CC' loop of EDIII of DENV1 (Austin et al., 2012), fusion loop of EDII of WNV and DENV (Cherrier, et al., 2009), EDI of DENV4 (Cockburn, Navarro Sanchez, Goncalvez, et al., 2012) and a complex structural epitope at the hinge of EDI and EDII of WNV (Kaufmann et al., 2010) and DENV (de Alwis, et al., 2012) and a composite epitope comprising of regions on the lateral ridge and A-strand of EDIII of DENV1 (Edeling et al., 2014).

In this study, the epitope of 14C10 has been solved using 7Å resolution cryoEM map of 14C10 Fab complexed with DENV1 (Teoh, et al., 2012). The densities connecting the E protein of DENV1 and 14C10 Fab can be directly observed from this map hence the epitope can be inferred directly from it. The epitope of this antibody was found to span across 2 adjacent E proteins, connecting EDIII of one E protein and EDI and the hinge region of EDI-EDII on the neighboring E protein.

This is consistent with observations made by de Alwis and colleagues, who proposed that the majority of neutralizing antibodies in human dengue immune sera bound to a complex quaternary structural epitope that is preserved only on the intact virion but not on recombinant E protein. They determined that this epitope bridges neighboring

E protein dimers and includes residues at the hinge region between EDI and EDII (de Alwis, et al., 2012). This hinge region between EDI and EDII has been reported to be recognized by neutralizing antibodies against other flaviviruses like WNV, JEV (Goncalvez et al., 2008; Kaufmann, et al., 2010; Oliphant, et al., 2006).

The observation that human antibodies isolated from immune sera of subjects who have recovered from viral infections such as WNV, DENV, ebolaviruses and HIV bind to complex quaternary structural epitopes suggest a generalized structural strategy by which naturally engendered antibodies inhibit viral infections (Dias et al., 2011; Kaufmann, et al., 2010; Spurrier et al., 2011). In this study, the epitope recognized by 14C10 bridges adjacent E proteins, making it a complex epitope that is preserved only when surface E proteins are properly arranged in their symmetrical structures on the intact virion surface. The E proteins of most flaviviruses share a high degree of homology in their quaternary arrangement based on the cryoEM structures of WNV (Mukhopadhyay et al., 2003) and DENV (W. Zhang, et al., 2003). The epitope recognized by 14C10 is very similar to that recognized by human mAb CR4354 against WNV.

The anti-WNV mAb CR4354 was generated from a phage displayed antibody library constructed from B cells of convalescent patients who were infected with WNV (Vogt et al., 2009). The cryoEM map of WNV complexed with the Fab of CR4354 has been solved to a resolution of 14Å. The pseudo-atomic resolution structure generated from the fitting of Fab CR4354 crystal structure allowed the interacting residues to be identified. CR4354 binds a disjointed epitope made up of protein segments from 2 adjacent E proteins, occurring at two independent positions in the same icosahedral asymmetric unit to yield 120 binding sites on the surface of the virus (Kaufmann, et al., 2010). The epitope recognized by 14C10 is also dependent on the quaternary structure of DENV, with 2 molecules of 14C10 Fab binding to 3 molecules of DENV E protein in an asymmetric unit. At full occupancy, 120 copies of Fab 14C10 bind to all 180 available copies of E protein on the virion surface.

Upon comparison of the epitope of CR4354 on WNV and the epitope of 14C10 on DENV1, it is clear that CR4354 has a larger portion of interacting resides on EDIII whilst most of that of 14C10 lies on EDI. When the sequences of epitope residues of

these 2 antibodies are compared, only 20% of residues overlap, and most of these overlapping residues are non-conserved (Teoh, et al., 2012).

Another recently described anti-DENV1 human mAb 1F4 that was shown to be highly neutralizing both *in vitro* and *in vivo* binds to EDI and the EDI-EDII hinge region on one E protein monomer but not across 2 neighboring E proteins (Fibriansah et al., 2014). 1F4 is proposed to recognize a conformational epitope centered on the hinge region since it binds only to intact DENV1 that has a conserved DI-DII hinge angle but not recombinant E protein with highly variable hinge angle. Both 1F4 and 14C10 bind to DENV1 at this EDI-EDII hinge region, suggesting that this region may be one of the key determinants of eliciting serotype-specific neutralizing antibodies in humans.

The importance of the role of the EDI-EDII hinge region as a major target of human anti-DENV antibodies is further illustrated by the chimpanzee mAb 5H2. 5H2 is specific for DENV4 and has both *in vitro* and *in vivo* neutralizing activity (C. J. Lai et al., 2007). The epitope of 5H2 was found to be on EDI (Cockburn, Navarro Sanchez, Goncalvez, et al., 2012). The epitope of 5H2 was found to partially overlap with the epitopes of 14C10 and 1F4 on the β-strands F_0 and G_0 and the loop F_0G_0, but 5H2 did not bind to the EDI-EDII hinge region, as observed for 14C10 and 1F4. However it is unclear if the epitope of 5H2 spans 2 neighboring E proteins since the Fab 5H2 complex structure is a crystal structure (Fibriansah, et al., 2014).

More recently, a potently neutralizing antibody against DENV1 E106 was found to bivalently bind across 2 adjacent EDIII subunits on a single virion and if this bivalent engagement was not present, as with the use of E106 Fab, the neutralization potency of the antibody reduced about 20,000 fold (Edeling, et al., 2014). Although rare, it suggests that antibodies against flaviviruses may be dependent on the unique icosahedral arrangement of E proteins on DENV surface.

There are several proposed models by which antibodies neutralize the infectivity of viruses, of which two are favored. “Single-hit” models describe neutralization with the binding of a virion with one antibody, based on the observation made by Dulbecco and colleagues in 1956 on the neutralization of two animal viruses that proceeded

with time as a first order reaction (Dulbecco et al., 1956). This suggests that the binding of virions at "critical sites" is adequate to trigger irrevocable conformational changes in the virion causing it to be non-infectious. On the other hand, "multi-hit" model of neutralization proposes that virus neutralization is related to the number of antibodies bound to the virion (Della-Porta et al., 1978). In this model, neutralization happens when a single virion binds with a stoichiometry that exceeds a certain threshold and neutralization is reversible. This threshold of number of antibodies required for neutralization differs significantly across various classes of viruses. The factors that contribute to the stoichiometric threshold of a given virus remains unclear, although Burton and colleagues have proposed that neutralization requirement is related to the number of antibodies needed to "coat" the virion surface (Burton et al., 2001). Antibody neutralization of flaviviruses is thought to be a multi-hit phenomenon, requiring multiple antibodies to bind a single virion until a neutralization threshold is achieved. This is backed by several evidences including (1) the affinity of antibodies for their respective virions is linked to their *in vitro* neutralization activity (2) the observation that not every antibody can bind to the virion with a stoichiometry required to neutralize it (3) the capability of complement to augment neutralization and (4) the capability of antibodies to cause ADE in FcγR bearing cells (Pierson, et al., 2008).

Although the binding activity of 14C10 to whole DENV1 on a sandwhich ELISA is lower than that of hu4G2 that binds to a flavivirus group specific determinant, 14C10 exhibits potent neutralization activity. This may suggest that 14C10 binds to a less easily accessible epitope but a small number of Ab molecules bound to the virion is sufficient to inhibit infectivity. This is consistent with the observation from studies of EDIII lateral ridge antibodies that neutralization necessitates only the engagement of a modest percentage of epitopes accessible to antibodies on the virion surface, exemplified by the WNV EDII lateral-ridge specific mAb E16 that neutralizes WNV when about 25% of the 120 accessible epitopes are bound by E16 (Pierson et al., 2007). Like E16, the potent neutralization activity of 14C10 could be attributed to their high affinity to the virion and a relatively small percentage of the epitope required to be bound for neutralization to occur.

The neutralization of viruses can be described as the abolishment of virus infectivity when antibodies bind to the virion. This coating of virions by antibodies is typically necessary and sufficient for neutralization to occur and such coating very often leads to a blockage of viral attachment to target cells. There are a variety of ways by which this can occur (Klasse et al., 2002). Firstly, antibodies can block attachment of the virion to target cells by means of receptors or other ancillary molecules. This is an essential step in virus replication since if its complete abrogation effectively prevents infection. In the event that attachment is simply reduced, repeated encounters with cells that are susceptible to infection can still lead to effective infection. Neutralization can even be brought about by aggregation since it lowers the cell number that virions can potentially attach to, although there is the possibility of delivery of a higher dose of virus when these aggregates attach to target cells compared to single virions. Secondly, antibodies can impede with post-attachment interaction of virions with receptors and co-receptors. Thirdly, antibodies can reduce internalization of virions by endocytosis if this is a necessary replicative step. However if endocytosis is not necessary for productive infection, rerouting of virions into this pathway though antibody-Fc receptor interactions could lead to neutralization. Fourthly, antibodies could interfere with necessary fusogenic protein-protein interactions or with conformational changes essential to the fusion process of virions with cell surface or within endosomal compartments of enveloped viruses. A fifth stage that can be possibly blocked by antibodies is the uncoating or correct intracellular localization of core of capsid proteins of viruses. The sixth potential step is the inhibition of initial metabolic events catalyzed by viral enzymes such as transcription, that can inhibit replication and hence infectivity of viruses.

The entry pathway of DENV is similar to that of other flaviviruses and there are numerous steps in the entry pathway that can be potentially blocked by the binding of antibodies to neutralize the infectivity of flaviviruses. There are several steps in the entry pathway of DENV and other flaviviruses that can be potentially blocked by the binding of antibodies. These include the (1) attachment of the virion to target cells, (2) interaction of virions with host cell factors that mediate internalization and (3) conformational changes of the E protein that facilitate membrane fusion.

Although the exact entry pathway of flaviviruses is still not well defined, the initial step is generally believed to involve interaction of the virions with one or more cellular factors on the surface of target cells. Despite extensive efforts, the exact cellular receptors that function as the essential flavivirus receptor has yet to be defined, although there have been numerous candidate receptors that have been proposed. Of which, DC-SIGN is the most well characterized receptor thought to mediate internalization of DENV into human dendritic cells (Tassaneetrithep, et al., 2003) (Navarro-Sanchez, et al., 2003). 14C10 has been found to be able to inhibit DENV1 infection at both a pre-attachment and post-attachment step, although a much higher concentration of 14C10 was required for the inhibition at a post-attachment step compared to that required for pre-attachment inhibition. This suggests that 14C10 works primarily by preventing viral attachment. Viral attachment is essential for infection and hindering viral attachment inhibits viral entry.

Neutralization of viruses by the inhibition of viral attachment has been described for antibodies against numerous viruses including the extracellular enveloped form of vaccinia virus (Law et al., 2001), rhinovirus (Colonno et al., 1989) and coronaviruses (Reguera et al., 2012). The most well-characterized attachment inhibition antibodies are those that block the binding of HIV-1 envelope protein to CD4 receptor or CCR5 co-receptor on T cells (Pantophlet et al., 2006).

It has been shown that mouse mAb 3H5 and human immune sera block DENV2 attachment to Vero cells and that there is a good correlation between neutralizing activity and the ability to block virus-cell attachment, suggesting that antibody-mediated neutralization of DENV occurs principally at a pre-attachment step (He et al., 1995). 3H5 targets the lateral ridge of EDIII and several indirect lines of evidences suggest that EDIII plays a crucial role in viral attachment. Firstly, EDIII extends furthest from the virion surface. Secondly, mutations that impact virulence of TBE, a member of the flavivirus family, mapped to EDIII (Mandl, et al., 2000) (Holzmann et al., 1990). Thirdly, soluble forms of recombinant EDIII of WNV was able to block entry of WNV and DENV2 into C6/36 cells, indicating that EDIII is responsible for binding to receptors on host cell surfaces (J. J. Chu et al., 2005). Part of the binding footprint of 14C10 lies on EDIII hence it is possible that the binding of 14C10 to DENV1 sterically hinders the attachment of DENV1 to receptors on

permissive cells, as seen in other EDIII-specific mAbs. The blockade of attachment presents as an attractive model for accounting for the neutralizing mechanism of EDIII-specific mAbs.

To further elucidate the mechanism of action of 14C10, we visualized a live infection of BHK cells with DENV1, in the presence of 14C10 and compared it to an isotype control antibody and hu4G2, a flavivirus group-specific antibody that binds targets the fusion loop of the flavivirus E protein. This was achieved using DENV1 fluorescently labeled with AF647 (red) and antibodies tagged to AF488 (green). The live infection visualized with high-resolution time-lapse confocal microscopy enabled us to decipher the mechanism of action of 14C10 real time. We observe that binding of 14C10 to DENV1, visualized as by yellow-colored antibody-virus complexes, failed to bind to the target BHK cells. Instead, these complexes were observed to 'bounce' off the surface of target cells. This further substantiates our hypothesis that 14C10 is an attachment and/or entry inhibitor. This is in contrast to the poorly neutralizing hu4G2, which promotes viral aggregation but failed to inhibit internalization of these large complexes. An interesting and important observation is that DENV1 internalization into BHK is a rapid event, with virus visibly internalized just 18 minutes post-infection. To our knowledge, this is the first direct evidence that demonstrate the mechanism of a neutralizing antibody in preventing attachment to target cells, as opposed to previous studies which employed indirect pre- versus post-attachment inhibition cell culture assays to derive the mechanism of action of mAbs (Vogt, et al., 2009) (Deng et al., 2011). This prevention of attachment and/or subsequent entry of DENV1 by 14C10 into BHKs was quantified by the relative intensity of fluorescence derived from internalized virus in the presence of 14C10 compared to hu4G2 or isotype control antibody. Unsurprisingly, there was significantly less internalized virus in the presence of neutralizing amounts of 14C10, confirming that 14C10 reduces the infection of BHKs, by preventing the attachment and/or entry of DENV1.

14C10 was also found to be able to inhibit at a post-attachment step, albeit at higher concentrations. Flavivirus-specific mAbs can possibly neutralize viral infectivity after viral attachment, conceivably by obstructing conformational changes in E protein that facilitates membrane fusion. This was first demonstrated using electron microscopy

studies in 1986 whereby WNV complexed with neutralizing amounts of antibody retained its ability to enter target cells, suggesting that neutralization was engendered by a post-attachment mechanism (Gollins et al., 1986). The WNV mAb E16 that targets EDIII also occurs after viral attachment (Nybakken, et al., 2005). Structural studies with E16 proposed that the binding of E16 to WNV sterically inhibits rearrangement of E proteins that drive fusion under acidic conditions (Nybakken, et al., 2006) (Kaufmann et al., 2006).

For the fusion of virus to endosomal membranes to occur after the virus has successfully entered the host cell via endocytosis, E proteins have to first expose the fusion loop and reorganize the arrangement of E proteins from dimeric to trimeric forms before achieving the proper post-fusion structure (Modis, et al., 2004). The hinge region between EDI and EDII has been shown to be highly flexible and it may be critical in facilitating this structural change essential for successful viral infection (Y. Zhang et al., 2004). Since part of the binding footprint of 14C10 lies on the EDI-EDII region, part of its neutralizing action may be to prevent this structural change of E protein dimers to trimers for fusion to occur. Neutralization escape mutant analysis on 14C10 demonstrated that a single amino acid substitution from threonine to lysine at position 51 (T51K) located in the EDI/EDII hinge region obliterated DENV1-14C10 interaction (Zou et al., 2012). Moreover, recombinant DENV1 with this T51K mutation could not be inhibited by 14C10 both *in vitro* and *in vivo*, demonstrating that binding of 14C10 to this region is critical for its neutralizing activity. T51 is positioned within the flexible EDI-EDII hinge region. This hinge region is made up of four peptide strands that link EDI and EDII, designated H-1 to H-4. These four strands provide the flexibility necessary for conformational changes of the E protein for fusion to occur in the endosome when triggered by acidic conditions (Hurrelbrink et al., 2001) (Rey, et al., 1995). The hinge region between EDI and EDII swivels by a 27° angle when the virus switches from the immature to mature state after prM is cleaved by furin (Y. Zhang, et al., 2004) and swivels back 30° as the virus transits from its pre- to post-fusion arrangement (Bressanelli et al., 2004) (Modis, et al., 2004). Hence a likely mechanism of action of 14C10 to inhibit DENV1 infection at a post-attachment step could be by preventing this movement. Additionally, the T51 residue was found by sequence analysis to be present in 887 out of 896 DENV isolates which could contribute to the ability of 14C10 to neutralize all genotypes of

DENV1 tested, since it was shown that the single T51K mutation confers resistance in DENV1 (Zou, et al., 2012).

The proteins located on the surface of DENV have been proposed to experience constant physiological changes – a phenomenon termed "breathing" (Lok, et al., 2008). This dynamic structural change has been observed not only in flaviviruses where 3 E protein molecules are arranged in 60 icosahedral asymmetric units on the viral surface, but also other icosahedral viruses such as rhinovirus (J. K. Lewis et al., 1998) and nodavirus (Bothner et al., 1998). Moreover, DENV surface structure was also shown by cryoelectron microscopy to undergo a conformational change from smooth to bumpy as temperature switches from 25°C in the mosquito host to physiological temperatures of 37°C in humans, where its infectivity was found to be about 1.6 fold higher than the smooth form of DENV (X. Zhang et al., 2013) (Fibriansah, et al., 2013). Once again this requires rearrangement of surface E proteins. "Breathing" of DENV possibly contributes to the facilitation of attachment of DENV to host cells. Since 14C10 cross-links surface E proteins by binding across 2 adjacent E proteins, it could lock surface proteins and prevent them from undergoing structural changes involved in "breathing". This is similar to what have been observed in rhinoviruses where binding of an antiviral agent stabilized the viral capsid and reduced the amount of "breathing" which would expose internal regions of rhinovirus proteins for proteolytic cleavage (J. K. Lewis, et al., 1998).

Hence we conclude that there is probably more than one way by which 14C10 neutralizes DENV1. However we propose that the major mode of action is via blocking attachment to target cells by inhibiting viral surface proteins from "breathing" that facilitates attachment or by sterically hindering EDIII, which has been shown to be critical for host cell attachment. This is similar to what is seen in anti-WNV mAb CR4354, which targets a similar conformational epitope as 14C10. When CR4353 was complexed with WNV, it efficiently neutralized WNV infection. It was also able to inhibit WNV infection after virus had been adsorped onto the cell surface, signifying that its neutralizing action was at least partly occurring at a post-attachment stage of the viral replication cycle (Vogt, et al., 2009).

The final mechanism whereby antibodies can hinder flavivirus infection is via the activation of Fc dependent effector functions, including activating complement components. Mice lacking the third component of complement (C3) or complement receptor 1 (CR1) and CR2 were found to be more vulnerable to WNV infection with increased central nervous system virus burdens. These mice also developed significantly lower levels of specific anti-WNV IgM and IgG, suggesting that complement activation controls WNV infection, in part via inducing a protective antibody response (Mehlhop et al., 2005). In another study, mice deficient for the classical and lectin pathways demonstrated diminished B and T cell responses to WNV challenge (Mehlhop et al., 2006). In other viral systems, complement components were found to enhance neutralization potency of antibodies directly by increasing the avidity of the antibody or the steric effects of when the antibody is bound to achieve better inhibition of virus attachment or fusion (Meyer et al., 2002) (Feng et al., 2002). Neutralization potency of neutralizing antibodies against YFV have also been found to be dependent on Fcγ receptor dependent clearance pathways and was related to variable interaction of IgG subclasses with complement and/or Fcγ receptors instead of merely dependent on epitope specificity of these antibodies (Schlesinger et al., 1995) (Schlesinger et al., 1993). The contribution of complement components on the neutralization action of 14C10 has not been addressed in this study and we do not rule out the possibility of 14C10 augmenting complement-mediated or Fc-mediated clearance of DENV1.

Knowledge of the mechanism of neutralization of an antibody would help infer its probability of promoting ADE at a certain concentration. Neutralizing antibodies generally can promote ADE *in vitro* when present at concentrations below their neutralizing thresholds (Morens, 1994). The ability to promote ADE at sub-neutralizing concentrations *in vitro* has been observed for numerous neutralizing antibodies and it could be generalized for all neutralizing antibodies (Morens, 1994; Morens et al., 1990; Morens et al., 1987). Stoichiometric analysis of anti-WNV antibodies demonstrated that there exist a threshold occupancy requisite for neutralization to occur – potently neutralizing mAbs inhibit infection at concentrations that yield low occupancy of accessible sites, as little as 30 of 180 E proteins, while poorly neutralizing mAbs require almost complete occupancy to inhibit infection (Pierson, et al., 2007). This implies that mAb concentrations that are

below or not yet at this threshold even at maximal binding will cause ADE *in vitro* in Fcγ receptor bearing cells. The neutralization mechanism of mAbs could govern this occupancy requirement for neutralization and consequently impact upon the range of concentration that cause ADE. Antibodies that inhibit attachment via steric effects by blocking receptor binding might need higher concentrations for neutralization and hence sustain ADE over a larger range of concentrations, as observed with EDII-specific anti-WNV mAbs (Oliphant, et al., 2006). However it was proposed that the neutralizing mAb1A1D-2 that bind a cryptic epitope exposed at 37°C and bound to two of three E proteins in each of the 60 icosahedral asymmetric units on DENV preventing E protein arrangement, had a lower occupancy requirement for neutralization and consequently lower possibility of causing ADE over a larger range of antibody concentration (Lok, et al., 2008). Since 14C10 binds across 2 adjacent E proteins it inhibits surface E protein movements as well in addition to theoretically have a low occupancy requirement. Consequently like 1A1D-2, 14C10 should have the lower propensity to cause ADE over a wider range of antibody concentration. This is a particularly useful attribute as a potential therapeutic candidate.

Passive transfer of neutralizing mAbs or immune sera have been shown to confer protection against DENV (Wan et al., 2014) (Kaufman et al., 1989) (C. J. Lai, et al., 2007) (Kyle, et al., 2008) (C. H. Tan et al., 1990) and other flaviviruses (Schlesinger et al., 1985) (Engle et al., 2003) (Throsby, et al., 2006) (Oliphant et al., 2005) in mice. Moreover, the *in vivo* protective potential of antibodies generally correlated with their *in vitro* neutralizing activity (Roehrig et al., 2001) (Oliphant, et al., 2005) (Brandriss et al., 1986) (Gould et al., 1986). The most direct way of assessing the therapeutic potential of candidate mAb 14C10 is to investigate its neutralizing activity *in vivo*. The AG129 mouse that is deficient for both type I and type II interferon receptors is a commonly used small animal model for DENV infections. Small animal models are commonly used as the first step to assess the safety and efficacy of and potential therapeutic drugs before moving to the more cost prohibitive higher primate model. Due to the lack of a strain of DENV1 that causes lethal disease in AG129 mice, we were unable to assess the protective effect of 14C10 in a lethal challenge. However, we were able to assess the effect of 14C10 in AG129 mice infected with two strains of DENV1 belonging to two different genotypes and via two different routes of infection. These mice develop a transient viremia but display no overt signs of

disease. In the first model, mice were infected s.c. with $1x10^6$ PFU per mouse of EHI.D1 DENV1 belonging to genotype 1. 4μg/mouse of 14C10 administered (i.p.) prophylactically 24 hours pre-infection was able to reduce plasma viremia to undetectable levels 4 days post-infection in a standard plaque assay. When administered 48 hours post infection as a therapeutic dose, 2μg per mouse of 14C10 was able to significantly lower plasma viremia whilst 16μg per mouse of 14C10 lowered plasma viremia to undetectable levels. In the second model, mice were infected i.p. with $1.25x10^7$ PFU per mouse of Westpac74 DENV1 belonging to genotype 4. 15μg per mouse of 14C10 administered prophylactically 24 hours pre-infection was able to reduce plasma viremia to undetectable levels 3 days post-infection in a standard plaque assay. When administered therapeutically 48 hours post infection, 0.6μg per mouse of 14C10 significantly lowers plasma viremia while 6μg per mouse of 14C10 reduces viremia to undetectable levels 3 days post-infection. This demonstrates the potent *in vivo* neutralizing effect of 14C10 since the lowest possible dose of 14C10 to lower viremia significantly was at 0.6μg per mouse.

The anti-WNV CR4354 that targets a similar conformational epitope as 14C10 also exhibits potent neutralizing activity *in vivo*. 0.4μg per mouse of CR4354 administered as a prophylaxis conferred complete protection against lethal WNV encephalitis in wild-type C57BL/6 mice while the pre-treatment of the highly susceptible NIH Swiss mice with 50μg per mouse of CR4354 protected 94% of mice from lethal WNV challenge (Vogt, et al., 2009). However, post-exposure protection efficacy of CR4354 was not evaluated and the survival from lethal infection was the sole readout from this study. This makes it difficult to compare the *in vivo* efficacy of 14C10 and CR4354 since the parameters of *in vivo* protection evaluated are different.

Since part of binding of both CR4354 and 14C10 map onto the hinge region of EDI and EDII, this region might be a major neutralization determinant of flavivirus antibodies. Indeed there have been other anti-flavivirus mAbs with neutralizing activity both *in vitro* and *in vivo* that have mapped onto this region, including mAbs against WNV, JEV (Goncalvez, et al., 2008) and MVEV (McMinn et al., 1995). However a study comparing the *in vivo* neutralizing activity of WNV antibodies found that although mAbs that target EDI-EDII protect mice against WNV infection, they had a lower efficiency compared to EDIII-specific neutralizing mAbs (Oliphant,

et al., 2006). Also, the protective efficacy of EDI-EDII specific mAbs reduced when administered post infection compared to preceding infection. Comparatively, neutralizing anti-EDIII mAbs protected mice and hamsters 5 days after WNV infection (Morrey et al., 2006) (Oliphant, et al., 2005). Notably, an anti-WNV humanized monoclonal antibody E16 was the first humanized mAb demonstrated to be successful as a post-exposure therapy against a viral disease, reinforcing our hypothesis that antibody-based therapeutics is viable for treating viral diseases (Oliphant, et al., 2005). Passive transfer of the serotype-specific humanized anti-DENV4 chimpanzee mAb 5H2 that recognized an epitope on EDI was found to be protective *in vivo* - 20μg per mouse of 5H2 provided 50% protection against 25 50% lethal dose (LD_{50}) of mouse-neurovirulent DENV4 strain H241 while 2mg/kg of 5H2 conferred complete protection against 100 50% monkey infectious dose (MID_{50}) as shown by the absence of viremia and lack of seroconversion (C. J. Lai, et al., 2007). This is the first study to evaluate the protection of primates against DENV infection by means of passive antibody transfer.

There have been other attempts at generating anti-DENV1 antibodies as potential therapeutics. The Diamond group generated a panel of murine DENV1 mAbs against a strain of DENV1 belonging to genotype 2, of which the two most potently neutralizing mAbs E105 and E106 were found to target EDIII via epitope mapping and X-ray crystallographic analyses (Shrestha, et al., 2010). These antibodies were tested for their prophylactic and therapeutic protective efficacy in a lethal DENV1 infection model. AG129 mice were infected i.p. with 10^6 PFU per mouse of Westpac74 DENV belonging to a heterologous genotype of DENV1. 20μg or 100μg per mouse of E105 administered one day before DENV1 challenge had a 50% protective efficacy while 20μg per mouse of E106 conferred 100% protection. When administered as a post-exposure treatment, a single 500μg per mouse dose of E105 or E106 administered 2 days post infection protected 75% and 82% of AG129 mice respectively. Percentage protection dropped to 20% and 40% for mice treated with 500μg E105 and E106 respectively when treatment was administered four days post-infection. Compared to 14C10, the protective efficacy of E105 and E106 are significantly poorer. However it should be noted that while both groups used the same strain of mice (AG129) and DENV1 (Westpac74), the amount of Westpac74 inoculated was different. The Diamond group infected their mice with 10^6 PFU per

mouse of Westpac74 DENV1 i.p. while we infected our mice with $1.25x10^7$ PFU per mouse via the same route. In our model, the mice developed a transient viremia and our readout was the reduction in viremia upon administration of mAbs. However the mice utilized by the Diamond group succumbed to infection and their readout was survival of the mice with mAb treatment. It remains unclear why there is a discrepancy in the 2 animal models since the same strain of mice and virus were used. Also it is still not known if there is a direct correlation between the reduction in viremia and survival of AG129 mice hence it remains to be seen if treatment with 6μg per mouse of 14C10 that lowers viremia to undetectable levels would confer protection in a lethal challenge model.

We explored alternative approaches for generating subsequent anti-DENV human mAbs. It was hoped that with alternative approaches we could address some of the problems we encountered with the method of generating mAbs using EBV-immortalized human B cells. One of the most significant problems we have encountered is the lost of neutralizing activity of B cell clones. B cell clones that have been identified to produce antibodies neutralizing for DENV have been found to lose the ability to produce neutralizing antibodies after some time in culture. This could possibly be due to the action of the enzyme activation–induced deaminase (AID) that is required for the process of somatic hypermutation (SHM) (Muramatsu et al., 2000). SHM generates diversity in B cells by introducing random nucleotide substitutions in the variable regions of heavy and light chain Ig genes (Neuberger, 2008). From a study that demonstrated that EBNA2 and LMP regulate AID expression, it is evident that AID is expressed in EBV-immortalized B cells (Tobollik et al., 2006). There is a possibility that AID induced Ig gene mutation in EBV-immortalized B cells can abolish their ability to produce functional antibodies or cause them to produce antibodies with altered specificities. This implies that we would have to identify neutralizing B cell clones before they have accumulated enough point mutations that can result in the production of non-neutralizing antibodies. This is a major limitation that we have encountered with this methodology of producing human mAbs. The strategy we used to generate 14C10 was to clone out the heavy and light chains of the IgG of a neutralizing B cell clone we identified in the shortest amount of time for expression as a recombinant antibody.

To circumvent this problem we looked into other strategies that would allow us to survey the immune repertoire of dengue convalescent patients. We chose to construct a human immune library from the B cell repertoire of an individual who has recovered from DENV2 infection that led to the generation of anti-DENV2 mAb 10.15. Since this methodology involved the extraction of total RNA from memory B cells sorted from PBMCs of immune donors, it eliminates the need to immortalize these B cells with EBV and stimulate their proliferation to sufficient numbers for subsequent screening. After the heavy and light chains of Ig genes are amplified from cDNA converted from the extracted total RNA, they are randomly combined before they were cloned to encode combinatorial library consisting of Fab fragments displayed on individual phages. A major limitation of this methodology is that although the entire B cell Ig repertoire is preserved, the heavy and light chains are separately amplified and subsequently randomly paired again. This results in numerous artificial pairings between heavy and light chains to result in multiple fold more combinations of resulting Fab fragments that may not have existed originally.

Another feature of the phage displayed immune library is the screening methodology involved to identify phages displaying Fab fragments of interest. This involves multiple rounds of panning cycles whereby phages can be positively selected by allowing it to bind to the antigen of interest and eluting only the bound phages. Multiple rounds of positive selection will enrich the final pool of phages with a greater percentage of which is specific for the desired antigen. Negative selection could be carried out prior to positive selection, to rid phages expressing Fab fragments with undesired specificity – in our case, cross reactive Fab fragments which bind to the other DENV serotypes. Hence the panning strategy for anti-DENV mAbs involved a depletion step for DENV4 before selecting the remaining phages for binding for DENV2. An additional step introduced into the panning cycle was the usage of the Fab fragment of an anti-prM antibody D29 to pre-block DENV2 used for positive selection. Similarly, the phage library was also pre-blocked with D29 before positive selection with DENV2 was carried out. This would prevent Fab fragments specific for prM from being selected. This is important since prM protein is expressed on the viral surface and is highly conserved between DENV serotypes, implying that antibodies that recognize prM are usually serotype cross-reactive (Dejnirattisai, et al., 2010).

This panning strategy for selecting phages expressing Fab fragments of interest would work very well for identifying antibodies with high binding affinity to the antigen the library is screened against. It also implies that this methodology is most useful for identifying antibodies that has the major functionality as a good binder. Unfortunately, high binding affinity does not necessarily equate to high neutralizing potency for anti-DENV antibodies, although the ability to bind to DENV is a pre-requisite for neutralizing DENV antibodies. Hence by utilizing this methodology, we select for Fab fragments with high binding affinity for DENV, which may not necessarily translate to the desired functionality of neutralizing activity against DENV. Comparatively, the initial screen for 14C10 was a micro-neutralization assay that allows for the direct selection of clones that demonstrate neutralizing activity for DENV.

10.15 was selected out of a DENV2 immune phage library from PBMCs obtained from convalescent DENV2 patient 2 months after infection. 105 monoclonal phages specific for DENV2 only were obtained after enrichment of the phage library using several panning cycles. There were 27 unique pairings of heavy and light chain templates obtained from these monoclonal phages, of which 26 were successfully expressed as full IgG antibodies. 17 of these antibodies were cross-reactive, binding to two or more DENV serotypes on ELISA, while 9 antibodies bound to DENV2 only. Three antibodies, named 10.15, 12.17 and 14.19, displayed strong neutralizing activity on a PRNT and were selected for further *in vitro* characterization.

As compared to the positive control hu4G2, the three DENV2 mAbs demonstrated comparable or stronger binding activity on a sandwhich ELISA across all strains of DENV2 tested (Figure 26). However, 14C10 demonstrated lower binding activity to the five genotypes of DENV1 tested as compared to hu4G2 (Figure 4). This indicates that the three DENV2 mAbs bind to DENV with a higher affinity than 14C10, which could be due to their selection from the phage library on the basis of their binding affinity to DENV2.

Of the three DENV2 mAbs, 10.15 exhibited the strongest neutralizing activity *in vitro* (Figure 27). The $PRNT_{50}$ values of 10.15 against various strains of DENV2 tested range from 0.00782μg/ml to 1.09μg/ml (Figure 28) whilst the $PRNT_{50}$ values of

14C10 against various strains of DENV1 range from 0.0381μg/ml to 1.05μg/ml (Figure 5). Hence 10.15 was found to be more neutralizing than 14C10 on a PRNT. However, this *in vitro* neutralizing potency of 10.15 did not translate to strong *in vivo* neutralizing activity. Whilst 6μg per mouse of 14C10 was sufficient to lower plasma viremia in Westpac74 DENV1 infected AG129 mice to undetectable levels using plaque assay (Figure 21), 300μg per mouse of 10.15 was required to do so in D2Y98P-PP1 or MT5 DENV2 infected AG129 mice (Figure 37). Also, this complete abrogation of plasma viremia could be observed 1 day after treatment with 14C10 (Figure 21) but there was no significant reduction in 10.15-treated mice 2 days post-treatment (Figure 37). A significant reduction in plasma viremia was observed could be observed 4 days after treatment and complete reduction observed 5 days post-treatment (Figure 37). The lowest dosage of 10.15 required for a significant reduction in plasma viremia was found to be 75μg per mouse that results in a 1.5 log reduction in viremia 5 days post treatment (Figure 43). While we could not assess the protective effect of 14C10 in a lethal challenge model, it was possible with 10.15 since D2Y98P-PP1 DENV2 was lethal to infected mice. We found that administration of 300μg of 10.15 prolonged the survival of infected mice as compared to controls, with 70% survival by the time point at which all control mice succumbed to infection (Figure 36). It was also possible to assess viremia in various organs of mice infected with D2Y98P-PP1 DENV2 since infectious viral particles were reported to be present (G. K. Tan, et al., 2011). Treatment with 300μg of 10.15 significantly lowered infectious viral particles detected in the liver, intestine, spleen, kidney and spinal cord of infected mice (Figure 38). One of the most well characterized anti-DENV2 mAb is 3H5, which targets the lateral ridge of EDIII. We compared the neutralizing activity of 10.15 and hu3H5 *in vivo* and found that 10.15 exhibited better neutralizing activity compared to hu3H5 (Figure 42). This result is significant since 3H5 is the most neutralizing DENV2 specific lateral ridge antibody.

10.15 was able to increase the survival of mice infected with a lethal dose of D2Y98P

Although we have yet to pin-point the exact epitope of 10.15, we have determined that it binds to E protein from an immunoprecipitation assay (Figure 31) and more specifically, it binds to EDIII as evident from the dot blot (Figure 33) and a direct binding ELISA (Figure 34). We also have some suggestion that 10.15 binds to a

conformation specific epitope since it binds very weakly to reduced purified DENV2 on a reducing SDS-PAGE, as compared to hu3H5 which exhibited strong binding (Figure 32). Preliminary cryo-EM data from our collaborators suggest that 10.15 binds a cryptic epitope that is more exposed at 37°C compared to at RT. When the neutralizing activity of 10.15 was compared between these two temperature, we found that the $PRNT_{50}$ value was approximately 4-fold lower at 37°C, indicating that the epitopes recognized by 10.15 might be preferentially exposed at 37°C (Figure 29). This suggests to us that although 10.15 also binds to EDIII, its epitope might be distinct from 3H5. We do not rule out the possibility that the binding epitope of 10.15 includes a site distinct from EDIII and that this binding epitope may be conformation specific. Further work is required to pinpoint the exact epitope of 10.15.

Although 10.15 shows approximately 50-fold lower neutralization potency *in vivo* compared to 14C10 with a dose of 300μg per mouse of 10.15 required to reduce plasma viremia levels to undetectable levels on a plaque assay whilst only 6μg per mouse of 14C10 is required to do so, it is still within a dosage range translatable for use in humans. The differences we observed in these antibodies – 10.15 being a stronger binder and neutralizer *in vitro* but a weaker neutralizer *in vivo*, could be attributed to the difference by which these antibodies were generated. The EBV-immortalization strategy of generating human antibodies was probably more effective in preserving the original specificity of human B cells engendered as part of a natural infection, as opposed to amplifying the heavy and light chains of these B cells separately before they are randomly combined again to form full IgGs with the phage displayed immune library.

More importantly we have identified two potential therapeutic candidates that could be used for the treatment of DENV1 and 2 infections. We suggest that generating similar human mAbs against DENV3 and DENV4 and combining all 4 mAbs into a cocktail could be a possible novel prophylactic and/or therapeutic option for the future treatment of DENV infections.

7 Appendix

7.1 Construction of Human Immune Library from purified B cells

Materials Required:

- Purified B cells by MACs separation
- Trizol and Purelink RNA mini kit with DNAase (Invitrogen)
- Superscript III 1st strand cDNA synthesis kit (Invitrogen)
- DEPC treated water & RNAse away (Invitrogen)
- AmpliTaq Gold Polymerase for 1st round PCR
- Q5 Polymerase for 2nd round PCR
- Human Library Primers
- pCES1X- MCS HC/LC Pst library cloning vectors

Protocol:

Extraction and purification of RNA

Expected yields from MACS purification
B cells ≈3% total PBMCs (by CD19 MACs)
IgM^+ cells ≈ 1.7% total PBMCs (by IgM MACs)
$CD27^+$ IgM^+ cells ≈ 0.06% total PBMCs (by depletion followed by IgM selection- Note: highly inefficient)

1. Resuspend harvested cells in Trizol at ratio of 1ml Trizol per 1 million cells
2. Process as per protocol in Purelink RNA mini kit, briefly:
    a. Add 200ul Chloroform/1ml Trizol (for smaller amounts adjust volume at same ratio) in 1.7 ml eppendorf
    b. Shake vigorously by hand 15s and sit 3min at room temperature
    c. Spin 12000g/15min/4°C
    d. Transfer 500-550ul upper aqueous layer to equal volume of 70% Ethanol in 1.7ml eppendorf
    e. Vortex to mix then spin down briefly to collect liquid
    f. Pass sample through 1 spin cartridge, adding 700ul of sample and spinning 12000g/30s/RT each time
    g. Wash column 350ul of Wash Buffer I
    h. Prepare DNAse (8ul 10x buffer, 10ul DNAse and 62ul DEPC water)
    i. Spin column 12000g/30s/RT, change collection tube and add DNAse for 15min/RT on-column to remove genomic DNA
    j. Add 350ul Wash Buffer I, spin column 12000g/30s/RT
    k. Add 500ul Wash Buffer II, spin column 12000g/30s/RT and then repeat wash
    l. Give final spin 12000g/1min/RT to remove remaining wash
    m. Elute with 2x30ul 10mM Tris pH7.4, incubate 1min then spin 16000g/2min/RT to elute
    n. Store RNA at -80°C

Expected yield: 420ng from $5x10^4$ B cells or 660ng from $5x10^5$ B cells (probably inaccurate)

1st strand cDNA synthesis (refer to 1st strand cDNA sheet in Excel document)

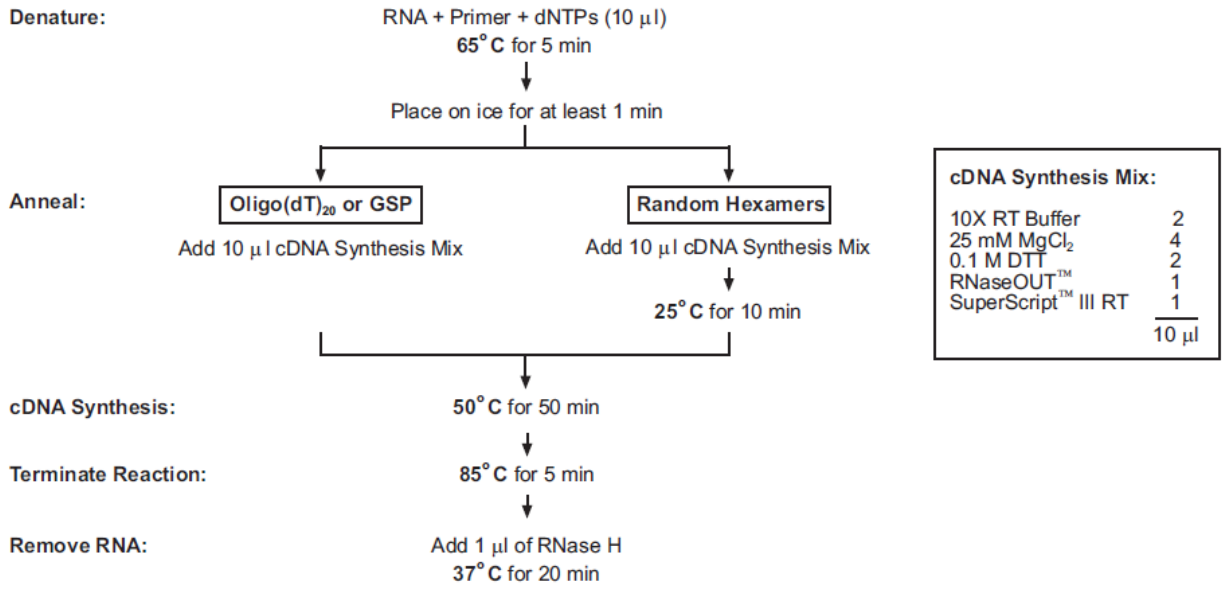
1. Following protocol as in SSIII 1st strand cDNA synthesis kit: Use 1x vol of RNA for synthesis via random hexamer (for heavy chain PCR) and 2x vol of RNA for synthesis via oligodT (for light chain PCR)
    a. e.g. for 30ul of RNA, use 8ul RNA with 1ul each of random hexamer primer and dNTP and 16ul RNA with 2ul each oligodT and dNTP
2. Requires minimum 20ul of random hexamer cDNA and 40ul of oligodT cDNA using formula below.

Heavy chain: reaction includes 1ul of RNA, 1ul of **random hexamer primers**, 1ul of dNTPs

Light chain: reaction includes 1ul of RNA, 1ul of **oligo dT primers**, 1ul of dNTPs

cDNA synthesis mix is prepared separately and added after 65°C 5min, 4°C 1min step.

Don't forget to add 1ul of RNase H per 10ul of reaction at the end.



PCR (refer to 1st PCR round sheet in Excel document)

1st round PCR

- Light Chain Forward – HuVκ1B-6/Vλ1A-9-For (6 kappa & 11 lambda primers) w/o ApaL1
- Light Chain Reverse – HuCκ-Asc (1single primer) or HuCλ2/7-Asc (2 primer mix)
- Heavy Chain Forward- HuVH1B/7A to HuVH6A-For-Sfi (9 forward primers)
- Heavy Chain Reverse- HuIgG or HuIgM-Rev (1 single primer)
- Use AmpliTaq as Q5 gives no yield due to proofreading activity being incompatible with degenerate primers

Assembly (9 heavy, 6 kappa, 11 lambda)			**Cycling**
10mM dNTP	1ul	95°C	5min
10uM forward primer	1ul	95°C	30s

10uM reverse primer mix	1ul	55°C	30s	35x
AmpliTaq Gold polymerase	0.25ul	72°C	1min 30s	
cDNA	2ul	72°C	15min	
25mM $MgCl_2$	3ul	4°C	∞	
AmpliTaq no Mg^{2+} 10x Buffer	5ul			
MQ water	36.75ul			
Total	**50ul**			

1. Run gels on 1% gel agarose gel, 22-well, thick comb, 1 reaction per well
2. Correct band size should be approximately 700bp for both heavy and light chain.
3. Extract gel with gel cutter and use Qiagen gel extraction kit to purify DNA
4. Wash with QG buffer (500ul) to ensure a clean fragment
5. **Elute with 30ul EB heated to 50°C to ensure better yield**

2nd round PCR

Light Chain Forward – HuVκ1B-6/Vλ1A-9-For-IF (6 kappa & 11 lambda primers)

- Light Chain Reverse – HuCκ-IF (1single primer) or HuCλ2/7-IF (2 primer mix)
- Heavy Chain Forward- HuVH-ForIF (1 single primers)
- Heavy Chain Reverse- HuJH1-6-Rev-Xho (5 primer mix)
- Use Q5 which gives much better yield then AmpliTaq
- For each gene family use same forward primers, do a 100ul reaction for light chains and a 2x100ul reaction for heavy

Assembly (9 heavy, 6 kappa, 11 lambda)		**Cycling**		
1st round PCR product	50ng			
10mM dNTP	1ul	95°C	5min	
10uM forward primer	2.5ul	95°C	30s	30x
10uM reverse primer mix	2.5ul	55°C	30s	
Q5 hot start polymerase	0.5ul	72°C	1min 30s	
5X Q5 Reaction Buffer	10ul	72°C	15min	
5X Q5 High GC Enhancer	10ul	4°C	∞	
MQ water to **Total of**	**50ul**			

1. Run gels on 1.5% agarose gel, 22-well, thick comb, light chain reactions split into 2 wells, heavy chain reactions split into 3 wells
2. Correct band size should be approximately 400p for heavy chain and 700bp for light chain.
3. Extract gel with gel cutter and combine 2/3 fragments into one Qiagen gel extraction kit column to purify DNA
4. Wash with QG buffer (500ul) to ensure a clean fragment
5. **Elute with 30ul EB heated to 50°C to ensure better yield**

Digestion and cloning in of PCR products (refer to LC/HC digestion sheets in Excel document)

Digestion and extraction

PCR product

1. Digest a total of 8ug of heavy chain PCR product (about 888.9ng each gene family) as follows: (Note: order of digest can be reversed)
    a. Add **18ul NEB SfiI**, **4ul 100x BSA**, **40ul 10x NEB Buffer 4** and MQ water to total volume of 400ul
    b. Split into 4x100ul in PCR tubes and digest 50°C/7.5hrs
    c. Add **4.5ul NEB XhoI** to each tube and digest 37°C/15-16hrs (overnight)
2. Digest a total of 4ug each lambda and kappa light chain PCR product (about 666.7ng each kappa and 363.6ng each lambda gene family)
    a. Add **16ul high concentration NEB ApaL1** and **20ul NEB AscI**, together with **4ul 100x BSA**, **40ul 10x NEB Buffer 4** and MQ water to total volume of 400ul
    b. Split into 4x100ul in PCR tubes and digest 37°C/15-6hrs (overnight)
3. Run on 1.5% gel, 6 wells for each set of digests. Heating PCR products 85°C/30s and chilling on ice just prior to running on gel can remove odd sized bands and improve cloning efficiency

Vector **(refer to Vector digestion sheet in Excel document)**

4. Digest a total of 20ug pCES-1 MCS HC Pst plasmid vector as per step 1 for heavy chain PCR product
5. Digest a total of 20ug pCES-1 MCS LC Pst plasmid vector as per step 2 for light chain PCR product
6. Mix 400ul digest with 2000ul PB and split into 3 QiaQuick columns. Elute each column with 2x30ul of heated EB buffer
7. Combine with **10ul of NEB PstI**, **3-4ul 100x BSA**, **30-40ul 10x NEB Buffer 3** and MQ water to total volume of 300-400ul and digest 37°C/7hrs (can also be overnight)
8. Run on 1.0% gel, 6 wells for each set of digest.

Gel extraction

1. Slice gel out with gel cutter and split gel slices into 2 freeze squeeze tubes, leave to freeze at -20°C for at least 10-20min
2. Spin 16100g/3min
3. Repeat 3 times cutting up gel remnants further to help extracted additional liquid
4. Ethanol ppt DNA from extracted liquid:
    a. top up to 300-400ul and add 750-1ml ice-cold 100% EtOH and 105-140ul 3M sodium acetate and split into 2 x 1.7ml eppendorf tubes
    b. Incubate 4hrs to overnight at -20°C
    c. Spin down 16100g/30min in cold room and remove supernatant
    d. Add 400ul 70% EtOH to each eppendorf and spin down again 16100g/5min in cold room
    e. Remove supernatant and dry pellet at 50°C in thermoblock
    f. Resuspend DNA in **20ul of 50°C heated sterile EB** and combine for total of 40ul
5. Nanodrop DNA to determine yield

Ligation and Electroporation

1. Assemble ligation as follows:

1ug	cut vector
700ng or 420ng	cut LC/HC PCR product respectively
4ul	10x T4 ligation buffer
2ul	NEB T4 ligase (high concentration)
MQ water to 40ul	

Incubate 16hrs@16°C (overnight) then **heat inactivate 65°C for 10min**

2. Top up mix to 100ul and then add 250ul ice-cold 100% EtOH and 35ul 3M sodium acetate and incubate at least 1hr at -20°C
3. Spin down 16100g/30min in cold room and then take off supernatant
4. Wash DNA pellet w/ 100ul 70% EtOH and spin down 16100g/5min in cold room
5. Remove supernatant and **repeat wash steps 4-5 two more times to wash DNA**
6. Dry pellet and re-suspend in 8ul of 50°C heated MQ water.
7. **Chill electroporation cuvets but do NOT Freeze them.**
8. Thaw one or two tubes of electrocompetent **XL1 blue or E10 E. coli** on ice and split into 2x50ul aliquots in chilled 1.7ml eppendorf tubes. XL1 blue or E10 E. coli do not have endonuclease activity and are more suitable for long term plasmid storage.
9. Take 4ul of ligation mix and add to one tube of TG1, then take second tube of TG1 and transfer TG1 to remaining 4ul of ligation mix to ensure no ligation mix is lost.
10. Incubate on ice approximately 5min
11. Electroporate on Eppendorf electroporator
    a. Transfer bacteria/ligation mix (approx 60ul) to pre-chilled@-20°C electroporation cuvette
    b. Place in electroporator and electroporate at **1700V for XL1 blue or 2350V for E10**
    c. Add 225ul SOC/2% glucose media and transfer to 14ml snap cap tube
    d. Add additional 225ul SOC/2% glucose to wash out remaining bacteria and transfer to 14ml snap cap tube
    e. Rinse with MQ water, remove water with 200ul fine tipped pipette
    f. Repeat electroporation (steps a-d) with second aliquot of bacteria/ligation mix, transferring mix to 14ml snap cap tube
    g. Final volume is 1ml of culture
12. Shake snap cap tube for 1hr@37°C, plate out onto 2 2TYC (Carbenicillin) G(2% glucose) agar 15cm plates keeping 5ul to titre
13. Grow overnight at **37°C (33°C is only for TG1)** and then scrape bacterial lawn with 5ml 2TY per plate, rinse plates with 5ml 2TY then spin down scraped culture at 4000g/20min and midiprep pellet

Checking electroporation efficiency

1. Take 5ul of SOC media and do 1:10 dilutions from 10^{-2} to 10^{-4} by serial dilutions in 450ul of 2TY (first dilution 1:100, 5ul into 495ul)
2. Plate out 100ul on 2TYCG 90mm plates
3. Count colonies to determine electroporation efficiency

4. Pick 24 colonies each for heavy chain and light chain electroporation and grow up in 5ml 2TYCG media and miniprep

Digest 4ul of miniprep with 0.5ul each NEB SfiI/XhoI (digest 2hrs@37°C then 2hrs@50°C) or 0.5ul each NEB AscI/ApaL1 (digest 4hrs@37°C) in a 20ul volume for heavy and light chain respectively **(refer to Digestion test sheet in Excel document)**

After HC into LC Use 0.5ul each ApaL1/XhoI in CutSmart, 4hrs 37°C. Should be about 1.2kb insert. Sequence 24 positives HX01-F/HX01-R.

5. Sequence with HX01F/pCES-LR for light chain and pCES-HF/HX01R for heavy chain to check for stop codons and frame shifts

Cloning heavy chain into light chain library plasmid

N.B. Both heavy and light chains (HC and LC) are digested with Sfi1/Xho1

Note: **Heavy chain is cloned into light chain vector** as a significant percentage of heavy chain sequences contain ApaL1 restriction sites and so makes cloning light chain into the heavy chain vector unsuitable

1. Cut 40ug of heavy chain library plasmid and 20ug of light chain as described in pro **"HC into LC sheet in Excel document"**
2.
    a. Add **18ul NEB SfiI**, **4ul 100x BSA**, **40ul 10x NEB Buffer 4** and MQ water to total volume of 400ul
    b. Split into 4x100ul in PCR tubes and digest 50°C/7.5hrs
    c. Add **4.5ul NEB XhoI** to each tube and digest 37°C/15-16hrs (overnight)
    d. Run on 1.5% gel, 6 wells for each set of digests. Heating PCR products 85°C/30s and chilling on ice just prior to running on gel can remove odd sized bands and improve cloning efficiency
3. Gel extraction

Slice gel out with gel cutter and split gel slices into 2 freeze squeeze tubes, leave to freeze at -20°C for at least 10-20min

Spin 16100g/3min

Repeat 3 times cutting up gel remnants further to help extracted additional liquid

Ethanol ppt DNA from extracted liquid:

a. top up to 300-400ul and add 750-1ml ice-cold 100% EtOH and 105-140ul 3M sodium acetate and split into 2 x 1.7ml eppendorf tubes
b. Incubate 4hrs to overnight at -20°C
c. Spin down 16100g/30min in cold room and remove supernatant
d. Add 400ul 70% EtOH to each eppendorf and spin down again 16100g/5min in cold room
e. Remove supernatant and dry pellet at 50°C in thermoblock
f. Resuspend DNA in **20ul of 50°C water** (less if you have more than 1 tube, total volume should be 20ul)
g. Nanodrop DNA to determine yield

4. **Assemble duplicate ligation as follows:**

2000ng cut vector (**light chain**)
700ng cut insert respectively (**heavy chain**)
4/6ul 10x T4 ligation buffer
2/3ul NEB T4 ligase (high concentration)
MQ water to 40/60ul
(Use 40 or 60ul depending on minimum volume of DNA)

Incubate 16hrs@16°C (overnight) then **heat inactivate 65°C for 10min**

5. Combine ligation mix and top up to 200ul with water and ethanol precipitate with 500ul 100% ethanol and 70ul 3M sodium acetate, incubate for at least 1hr at -20°C. **Chill electroporation cuvets but do NOT Freeze them.**
6. Spin down 16100g/30min in cold room and then take off supernatant
7. Wash DNA pellet w/ 180ul 70% EtOH and spin down 16100g/5min in cold room
8. Remove supernatant and **repeat wash steps 4-5 two more times to wash DNA**
9. Dry pellet and re-suspend in 16ul of 50°C heated MQ water.
10. Take 4ul of ligation mix and add to one tube of TG1, then take second tube of TG1 and transfer TG1 to remaining 4ul of ligation mix to ensure no ligation mix is lost.
11. Incubate on ice approximately 5min
12. Thaw 2 tubes of electrocompetent TG1 E. coli on ice and split into 4x50ul aliquots in chilled 1.7ml eppendorf tubes.
13. Electroporate on Eppendorf electroporator
    a. Transfer bacteria/ligation mix (approx 55ul) to pre-chilled@-20°C electroporation cuvette
    b. Place in electroporator and electroporate at 1700V
    c. Add 475ul SOC/2% glucose media and transfer to 14ml snap cap tube
    d. Add additional 475ul SOC/2% glucose to wash out remaining bacteria and transfer to 14ml snap cap tube
    e. Rinse with MQ water, remove water with 200ul fine tipped pipette
    f. Repeat electroporation (steps a-d) with next aliquot of bacteria/ligation mix, transferring mix to 14ml snap cap tube (Use cuvette twice before switching to new cuvette)
    g. Final volume is 4ml of culture
14. Shake snap cap tube for 1hr@37°C, plate out onto 12 2TYC (Carbenicillin) G(2% glucose) agar 15cm plates keeping 5ul to titre (approx 333ul per plate)
15. Grow overnight at **33°C (TG1 grows at 33°C)** and then scrape bacterial lawn with 5ml 2TY per plate, rinse plates with 5ml 2TY
16. Take 1/10 and 1/20 dilutions and measure OD
17. Spin down pellet and resuspend in 2TY/20% glycerol to OD50-OD80
18. Aliquot into 1ml aliquots and store

QC for HC-into_LC ligation/electroporation

1. Pick up 48 colonies from control plates.
2. Culture them over-nigh in 2TY medium, 2% glucose with carbeniciline (37°C on shaker). Culture is 2-5ml.
3. Make 48 minipreps.
4. Digest plasmids using Apal1 and Xho1 digestion as described in protocol **Digestion test** in Excel sheet (37°C for at least 2h).
5. Run 1% agarose gel at 160V.
6. Digestion with Apal1/Xho1 gives bend of 1.2kb.
7. Samples that give 1.2kb bend send for sequencing. Use Hxo1 Forward and Hxo1 Reverse primers. If you can send **36 samples** (72 reactions, forward/reverse primer sequencing).

Phage expression

1. Determine actual cfu titre of glycerol stock by plating out 100ul of 10^{-5}, 10^{-6}, 10^{-7}, 10^{-8} dilutions of glycerol stock on 90mm 2TYC (Carbenicillin) G(2% glucose) - 2TYCG plates. Make dilutions starting from 1/10 (450ml of 2TY medium + 50µl of transformed TG1 from glycerol stock). Incubate overnight at 37°C.
2. Add enough glycerol stock for at least 100-fold excess over number of individual clones in volume sufficient for at least 2 doublings into 400ml 2TYCG (should be about 0.5ml)
3. Plate out 100ul of 10^{-4}, 10^{-5}, 10^{-6} dilutions of freshly inoculated culture on 90mm 2TYCG plates to confirm actual cfu titre in culture
4. Grow up library culture at 220rpm/37°C, monitor OD closely and proceed once **OD600=0.5±0.05** (if exceed then dilute culture 1:4 with 2TYCG and grow up again)
5. Take 10ml for –ve control culture in 50ml Falcon, DO NOT ADD HELPER PHAGE
6. Add 1:40 MOI KM13 helper phage to remainder of culture
7. Incubate both cultures for 30min at 37°C in incubator WITHOUT shaking. Then **shake both cultures for a further 30min at 37°C/100rpm.**
8. Process –ve control culture as follows:
    a. Spin down at 4000g/10min and discard supernatant
    b. Resuspend pellet with 200ul 2TYCG media
    c. Plate out 200ul neat culture on a 150mm 2TYCK plate to check for background infection
    d. Take 5ul of culture to do dilutions and plate out 100ul of 10^{-8}, 10^{-9}, 10^{-10} dilutions on 90mm 2TYCG plates
9. Process helper phage infected culture as follows:
    a. Spin down at 8000g/15min and discard supernatant
    b. Resuspend infected culture in 5ml 2TYCG media
    c. Take 5ul of culture to do dilutions and plate out 100ul of 10^{-8}, 10^{-9}, 10^{-10} dilutions each on 90mm 2TYCG and 2TYCKG plates
    d. Plate out remainder of culture on 150mm 2TYCK plates, 200ul per plate (should required between 26-30 plates).
10. Incubate all plates at 30°C/15-16hrs

Phage Recovery

1. Add 5ml TBS (20mM Tris pH7.5, 150mM NaCl) to each 2TYCK plate and incubate 15min at 4°C
2. Process plates in groups of 5 at intervals of 7-8 minutes
3. Scrape each plate and transfer scraped culture to a 250ml centrifuge tube with a plastic Pasteur pipette, keep 250ml centrifuge tube on ice
4. Wash all 5 plates with another 5ml TBS, transferring the wash from one plate to another and then finally into the 250ml centrifuge tube with a Pasteur pipette
5. After all plates are processed, spin down culture at 10000g/20min and recover phage in supernatant
6. Split supernatant into 4x37ml centrifuge tubes and add 1/5 volume 20% PEG/2.5M NaCl

and incubate 1½-2hrs on ice

7. Spin down 10000g/15min/4°C and discard supernatant
8. Re-suspend phage pellets in total of 8mls of 1xPBS and split into 8-9 2ml eppendorf tubes with 1ml of phage suspension in each
9. Spin down 161000g/2min at RT
10. Transfer 1ml supernatant from each tube to another 2ml eppendorf tube with 200ul of 20% PEG/2.5M NaCl, mix thoroughly and incubate 20min on ice
11. Spin down 16100g/5min at RT
12. Remove supernatant with micropipette and add 750ul PBS/20%gly to each tube and resuspend phage pellet again
13. Spin down 16100g/2min at RT
14. Combine supernatant into one 15ml Falcon, mix phage thoroughly and then aliquot 800ul into 1.7ml eppendorf tubes and freeze at -80°C

7.2 Time-Lapse Confocal Microscopy Video Clips

7.2.1 Live infection of BHK cells with DENV1 in the presence of an isotype control antibody

7.2.2 Live infection of BHK cells with DENV1 in the presence of an hu4G2

7.2.3 Live infection of BHK cells with DENV1 in the presence of 14C10

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