Defining the role of Antibody FcR regions in Dengue virus neutralization, clearance and post infection immunity.

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Lim SY, Chan CE, Lisowska MM, Hanson BJ, MacAry PA. The Molecular Engineering of an Anti-Idiotypic Antibody for Pharmacokinetic Analysis of a Fully Human Anti-Infective. PLoS One. 2015 Dec 23;10(12):e0145381. doi: 10.1371/journal.pone.0145381

List of Abbreviations

Ab   antibody

ADCC   antibody dependent cell-mediated cytotoxicity

ADE   antibody dependent enhancement

ALT   alanine transaminase

BHK-21   baby hamster kidney

bNAb   broadly neutralizing antibody

C   capsid (protein)

CCL   chemokine (C-C motif) ligand

CLEC5A   C-type lectin domain family 5 member A

CDR   complementarity determining region

CMC   carboxymethyl cellulose

DENV   Dengue virus

DENV1-4   Dengue virus serotype 1-4

DF   Dengue Fever

DHF   Dengue Hemorrhagic Fever

DSS   Dengue Shock Syndrome

E   envelope (protein)

EDI   Envelope protein domain I

EDII   Envelope protein domain II

EDIII   Envelope protein domain III

EHI	Environmental Health Institute
ER	Endoplasmic reticulum
Fab	fragment antigen binding
FBS	fetal bovine serum
Fc	fragment crystallizable
FcRN	neonatal receptor for Fc
FL	fusion loop
g	gram
Hu	humanized
i.p.	Intraperitoneal
i.v.	intravenous
IACUC	Institutional Animal Care and Use Committee
Ig	Immunoglobulin
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
JEV	Japanese encephalitis virus
LC	light chain
LAV	Live-attenuated vaccines
M	membrane (protein)
mAb	monoclonal antibody
MIF	migration inhibitory factor
mu	murine

NK	natural killer
NS	non-structural
OD	optical density
ORF	open reading frame
PBST	PBS containing 0.01% Tween-20
PCR	Polymerase Chain Reaction
PEI	polyethyleneimine
PFA	paraformaldehyde
PFU	Plaque forming units
RIG-I	helicase
RNA	Ribonucleic Acid
RT	room temperature
SAE	severe adverse events
s.c.	subcutaneous
SD	standard deviateon
STAT	signal transducer and activator of transcription
TBEV	tick-borne encephalitis virus
TLR	toll-like receptor
TMB	tetramethylbenzidine
VH	Variable heavy
VL	Variable light
WHO	World Health Organization

WNV   West Nile virus

YFV   Yellow fever virus

Summary

Dengue disease is the most common arboviral (viral-arthropod borne) disease in humans, with over 40% of the human population in 100 countries at risk of infection. Dengue virus causes an estimated 50–100 million infections each year. It has been observed endemically in tropical and subtropical regions and recently the records of dengue cases have extended geographically to Europe and the United States of America. The latest World Health Organization reports show the increasing numbers of dengue cases globally; and it is suggested that due to limited access to Dengue diagnostics, the actual infection number is more than three times the dengue burden estimate of the WHO (World Health Organization) (Nature 2013; 496: 504-7). The spectrum of clinical symptoms ranges from an acute debilitating, self-limited febrile illness called Dengue Fever (DF) to a life-threatening vascular leakage syndrome, referred to as Dengue Hemorrhagic Fever/Shock Syndrome (DHF/DSS) (Guzman, Halstead et al. 2010). An increased risk of severe disease manifestation has been associated with sequential infection by different viral serotypes and this is hypothesized to be an effect of antibody dependent enhancement (ADE) (Sabin 1952, Halstead, Nimmannitya et al. 1967, Balsitis, Williams et al. 2010). To date, there is no virus-specific treatment available and the economic costs of dengue disease are estimated to be very high. Previously our laboratory has generated a fully human antibody based on the natural immune response of dengue patients termed 14C10. The 14C10 antibody has remarkable protective activity at low concentrations *in vitro* and *in vivo*. Thus, it is a good therapeutic candidate for dengue virus serotype 1 (DENV1) infection (Teoh, Kukkaro et al.

2012). ADE in dengue infection is proposed to occur when antibodies at low concentrations create immune complexes with the virus and facilitate virus entry into Fc (Fragment crystallizable) receptor bearing cells. In my project, I employ molecular engineering methodologies to dissect the role of Fc-receptor binding through the Fc-region of an anti-Dengue antibody in DENV neutralization and clearance. Specifically we use modifications to the genetic template of the 14C10 antibody to investigate how changes in subclass, glycosylation and Fc-receptor binding activity impacts *in vitro* and *in vivo* on neutralizing activity. We also aim to establish the role of the Fc domain of the 14C10 antibody in DENV1 infectivity versus neutralization. Furthermore, we test human and mouse-human variants of the 14C10 and their different subclasses on their ability to trigger heterotypic and homotypic ADE. Finally, my project aims to investigate the impact of 14C10-based antibody therapy upon the ability of the treated hosts to generate their own protective immune response where the virus has been rapidly neutralized and cleared from circulation. The data presented herein represents a thorough mechanistic dissection of a therapeutic candidate antibody for DENV1 with important implications for how this may be employed in a clinical context.

1 Introduction

1.1 Dengue disease

1.1.1 Epidemiology

Dengue is the most common arboviral disease in humans, with over 3 billion people in 100 countries at risk of infection and with an estimated 50–100 million infections each year. Dengue disease had been observed mainly in tropical and subtropical regions but recent outbreaks in other parts of the world, including Europe and the United Stated of America have been reported. It is believed that it is an enzootic disease, which originates from non-human primates and is transmitted between them and humans by mosquitoes. Once infected, a mosquito remains a vector for the rest of its life (usually a few weeks). Moreover, it may also the transmit virus transovarially to its progeny (Murphy and Whitehead 2011). The main vectors of the Dengue virus (DENV) are two types of mosquito: *Aedes aegypti* (Yellow fever mosquito/jungle mosquito) and *Aedes albopictus* (Asian tiger), which are highly adapted to human habitats. *A. aegypti* is responsible for most dengue infections in South-East Asia, India, Africa, and South and Central America. Due to the decline of vector control actions in the Americas, the number of cases transmitted by *A. aegypti* has increased significantly in that part of the world over the past three decades. *A. albopictus* is more resistant to lower temperatures and within the last 10 years, has spread across southern European countries. Overall, the last 50 years saw a 30-fold increase in the incidence of dengue globally (Pierro, Varani et al. 2011, Caminade, Medlock et al. 2012, Wan, Lin et al. 2013).
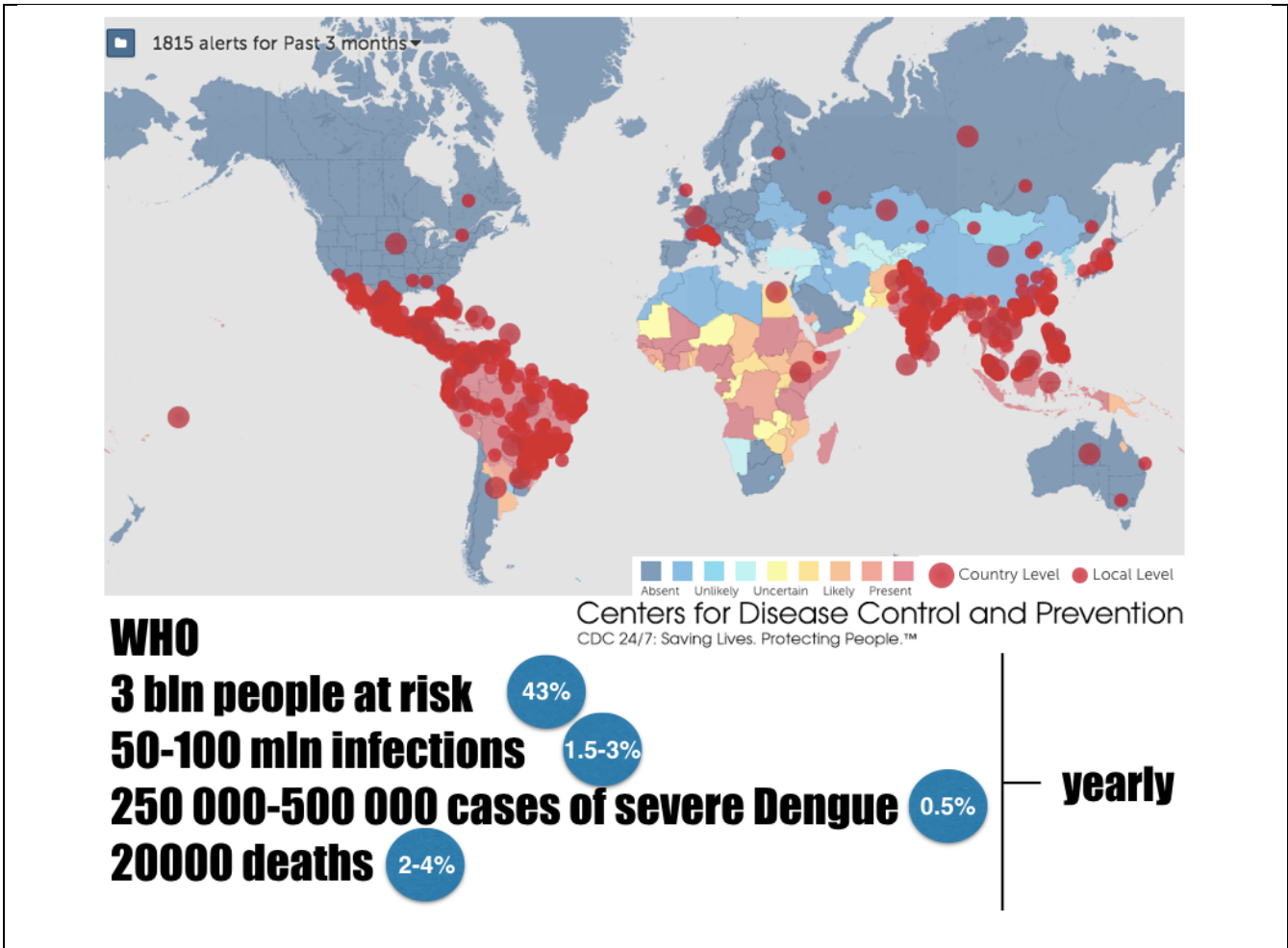
Figure 1. The map representing the Dengue endemic areas as well as the cases outside these regions over January-March 2016 (WHO 2016)

43% of all human population is at risk of Dengue infection
1.5-3% of the people at risk develop Dengue infection
0.5% of infected people develop life threatening severe form of Dengue disease
2-4% of the patients who encounter severe Dengue disease never recover

A comparison to the influenza annual epidemiological situation (WHO 2016):

5-15% of the population is affected with influenza (0.3-1 bln infections)
0.3-0.5% of the infected people develop severe influenza illness (3-5 mln severe influenza infections)
8-16% of the severe influenza patients die (250 000 - 500 000 deaths, mostly among the elderly over 65 years of age)

1.1.2 Clinical disease

DENV causes a spectrum of clinical symptoms and signs ranging from an acute debilitating, self-limited febrile illness termed Dengue Fever (DF) to a life-threatening vascular leakage syndrome, referred to as Dengue Hemorrhagic Fever/Dengue Shock Syndrome (DHF/DSS). Around 75% of DENV infections in humans are asymptomatic (Duong, Lambrechts et al. 2015). The symptomatic infections vary in different individuals and the symptoms are classified by WHO in the 'Dengue Guidelines for Diagnosis, Treatment, Prevention and Control' to help health practitioners in the fast diagnosis and in the avoidance of misdiagnosis (Figure 2). Most of the DENV- induced disease symptoms are not specific; these include fever, headache, abdominal pain, nausea, vomiting, muscle pain, bone and joint pain, maculopapular or macular rash and mucosal bleeding. More characteristic is a low platelet count and in the most serious cases, severe bleeding linked to increased vascular permeability and shock. There are no pathognomonic, i.e. distinctively characteristic, symptoms of dengue disease. Depending on the medical observations, the disease is classified as Dengue Without Warning Signs/Undifferentiated Dengue, Dengue With Warning Signs/Dengue Fever and Severe Dengue/Dengue Hemorrhagic Fever. Further, DHF can be classified into four severity grades. Grade I (Dengue Without Warning Signs) and II (With Warning Signs) are classified as Non-severe Dengue. Grade III and IV are defined as Dengue Shock Syndrome (DSS) (WHO 2009, Narvaez, Gutierrez et al. 2011). Several hypotheses for why most dengue cases are asymptomatic while others lead to non-severe Dengue Disease or Dengue Shock Syndrome and death-these will be discussed in more detail in this thesis.

Dengue Virus Infection		
Non-severe Dengue		**Severe Dengue**
without Warning Signs	with Warning Signs	**Dengue With Warning Signs + 1 of:**
Fever + 2 of the following: nausea/vomiting rash (maculopapular) myalgia arthralgia leukopenia positive tourniquet sign	**Dengue Without Warning Signs + 1 of the following:** abdominal pain/tenderness persistent vomiting clinical fluid accumulation mucosal bleeding lethargy liver enlargement lab: ↑HCT concurrent with rapid ↓platelet count	1.**sever plasma leakage** leading to: *shock (DSS) *fluid accumulation with respiratory distress 2.**severe bleeding** 3.**severe organ involvement**: liver AST of AST>1000 CNS: impaired consciousness failure of heart and other organs

Figure 2. WHO classification of the dengue disease distinguishes 3 forms of dengue disease.

According to WHO classification of Dengue Disease, Dengue Virus Infection is observed as Non-severe or Severe Dengue. Non-severe Dengue Without Warning Signs includes all the febrile cases with any two out of gastrointestinal symptoms, skin conditions, changes in blood morphology, capillary fragility (measured with Tourniquet test) or muscles/joints pain. Dengue With Warning Signs is diagnosed when one additional abnormality is observed on top of the Non-sever Dengue case. To diagnose Severe Dengue one additional serious symptom must be observed: severe plasma leakage, severe bleeding or sever organ involvement (WHO 2009).

1.1.3 Transmission of DENV infection

Dengue disease is transmitted by mosquitos susceptible to DENV infection (Chapter 1.1.1.).

Mosquitos breed in stagnant water and as little as a few millimeters of water is enough for

the new insects to go through all the developmental stages to form adult mosquitos.

Mosquitoes lay 70-100 eggs in three to four batches. The larvae hatched from eggs moult

four times, and between moults they grow rapidly. In two to three days they become pupas,

which then metamorphose into adult mosquitoes. Hence, a single female mosquito, which

has access to water might give rise to up to hundred daughter mosquitos in a few days.

When an adult mosquito bites a dengue-infected host, it draws blood containing DENV into

its stomach. Seven to twelve days later virus reaches the salivary glands of the mosquito.

When biting another person, an insect releases saliva together with DENV into the skin

transmitting the virus and spreading the disease (Salazar, Richardson et al. 2007).

Once in the human body, the virus infects immune cells in the skin tissue such as

dendritic cells and the skin-resident macrophages (St John, Abraham et al. 2013). Infected

immature dendritic cells and macrophages of the skin enter the lymphatic system and reach

the lymph nodes where monocytes become infected. Humoral and cellular immune

responses to viral infection trigger strong inflammatory reaction (Chapter 1.2). During the

replication period, the virus first replicates locally and after three to six days of incubation

spreads to the bloodstream (Marchette 1973). The reproduction of virus in the bloodstream

is termed viremia. Around six hours after the onset of viremia, the first symptoms of the

dengue disease can be noted. Within next six to eight hours the host develops fever. For

two to ten days victims can potentially transmit the infection to mosquitoes that bite them (Halstead 2007).

1.2 Immunopathogenesis of DENV response

The pathogenesis of dengue disease is an effect of interplay between multiple risk factors, which can be classified either as viral or host in origin (Clyde, Kyle et al. 2006, Srikiatkhachorn 2009, Guzman, Halstead et al. 2010). Thus, the development and severity of dengue disease depends on many conditions such as the specific strain of infecting DENV, the time interval between infections, previous exposure to heterologous DENV serotype, and the form and functionality of the patients' immune system. The age, ethnicity, genetics, and presence of additional disorders change the probability of severe forms of the disease. DHF is diagnosed more frequently in children, elderly and people with weakened immune systems (Costa, Fagundes et al. 2013, Acosta and Bartenschlager 2016).

1.2.1 Innate Immunity

Pattern Recognition Receptors (PRRs)

The initial targets for DENV at the site of mosquito bite are Langerhans cells, dermal dendritic cells, and interstitial dendritic cells (Navarro-Sanchez, Despres et al. 2005). Viral recognition activates interferon (IFN) pathways and pro-inflammatory transcription factors such as NF-kB. IFN-α/β and inflammatory cytokines trigger an antiviral response in dendritic cells (Severa and Fitzgerald 2007). Other cells, that detect Pathogen Associated Molecular

Patterns (PAMPs) and viral antigens include lymphocytes, monocytes, macrophages, Kupffer cells (liver macrophages), and endothelial cells. Host pattern recognition receptors (PRRs) in these cells are responsible for sensing viral molecules (Jessie, Fong et al. 2004).

Families of sensors for viral nucleic acids detection by mammalian cells like the cytoplasmic receptors of DExD/H box RNA helicases such as retinoic acid inducible gene I (RIG-I) and melanoma differentiation - associated gene 5 (MDA5), together with the endosomal Toll-like receptors (TLRs) are the most important PRRs engaged in DENV recognition and anti-DENV innate immune response. Interaction of DENV with receptors of the DExD/H box RNA helicases activates a macromolecular signaling complex that stimulates IFN regulatory factor 3 (IRF3) and NF-kB, which in turn induces IFN-β promoter (Loo, Fornek et al. 2008). During antibody-enhanced DENV infection, it was also observed that mast cells activated via RIG-I trigger massive chemokine CCL4, CCL5, and CXCL10 production (Brown, McAlpine et al. 2012). Overall, RIG-I, MDA5, and TLR3 act as synergistic sensors inducing IFN-β production and inhibiting replication of DENV (Nasirudeen, Wong et al. 2011). Additionally, it was shown that both STAT1-dependent and STAT1-independent pathways play a critical role in the anti DENV response in mice, induced by type I IFNs (Shresta, Sharar et al. 2005). However, DENV was presented to subvert the IFN induced antiviral response by blocking JAK/STAT signaling (Munoz-Jordan, Sanchez-Burgos et al. 2003).

Except for DENV-sensing receptors, there are also other molecular mechanisms responsible for the induction of potentially overwhelming anti DENV-inflammatory responses. For example, C-type lectin domain family 5 member A (CLEC5A) was

suggested to interact directly with DENV, stimulating the release of proinflammatory cytokines *in vitro* and *in vivo*. Anti-CLEC5A monoclonal antibodies suppressed plasma leakage and internal bleeding in STAT1-deficient DENV infected mice, reducing mortality by around 50% (Chen, Lin et al. 2008, Huang, Chen et al. 2016). However, human clinical data in the context of CLEC5A activation is required to confirm the importance of this receptor in DENV pathogenesis.

Cytokine storm

The production of cytokines and chemokines in the DENV host response may have dual protective and pathologic roles. 'Cytokine storm' is a term describing elevated levels of proinflammatory cytokines and chemokines. It can be seen in patients with DF, however it is much higher in severe dengue cases suggesting its contribution to the pathogenesis of DHF/DSS (Srikiatkhachorn 2009, Kim 2011). Massive production of the following cytokines was reported in patients with severe dengue disease: TNF-α, IL-6, IL-8, IL-10, chemokine ligand (CCL)-2, CCL-3, CXCL-8, CXCL-10, and IFN-γ (Chaturvedi and Elbishbishi 2000, Navarro-Sanchez, Despres et al. 2005, Tolfvenstam, Lindblom et al. 2011, Costa, Fagundes et al. 2013, Singla, Kar et al. 2016). Some of the inflammatory cytokines represent crucial protective molecules in DENV infection. For example IFN-γ as well as IL-12 and IL-18, which precede production of IFN-γ, what was demonstrated in murine immunocompetent models, play essential protective roles during DENV infection (Fagundes, Costa et al. 2011).

Nevertheless, other proinflammatory cytokines target endothelium triggering endothelial cell dysfunction and a transient increase in vascular permeability, hemorrhagic

occurrence, hemoconcentration, and shock, which can be fatal (Basu and Chaturvedi 2008, Rothman 2011). Moreover, decrease in the levels of some of the cytokines, for example IL-8 and IL-10 was described as the most significant marker of recovery from severe dengue (Singla, Kar et al. 2016). Although the patho-mechanism behind the induction and control of 'cytokine storm' is not fully understood, a number of studies have reported a relationship between increased levels of particular cytokines (e.g. TNF- α or migration inhibitory factor (MIF)) and more severe dengue cases (Chaturvedi and Elbishbishi 2000, Assuncao-Miranda, Amaral et al. 2010, Costa, Fagundes et al. 2013). Similarly, the chemokine production system seems to have both protective and pathological roles during DENV. The activation of CXCR3 and CXCL10 was shown to improve host anti DENV resistance during infection (Chen, Lu et al. 2006). On the contrary, increased levels of CCL2, CCL3, CCL4 and CCL5 have been associated with severe disease outcome and symptoms like hypotension, thrombocytopenia, hemorrhagic shock (CCL2-4), and hepatic dysfunction (CCL5) (Bozza, Cruz et al. 2008, Conceicao, El-Bacha et al. 2010).

1.2.2 Adaptive immunity

Humoral Immunity and Antibody Dependent Enhancement (ADE)

There are four antigenically-distinct serotypes of Dengue virus (DENV 1-4). Antibodies against one serotype confer life-long protection against that specific serotype but only temporary protection against the other serotypes. It has been suggested in several previous studies that secondary (2°) infection with a new serotype of DENV leads to more severe

dengue disease and that this is due to antibody dependent enhancement (ADE). In the proposed mechanism of ADE, heterotypic antibodies bind to the new DENV serotypes but do not neutralize them, instead they facilitate the DENV entry into FcR bearing target cells (Halstead 1979). As a result, they enhance DENV replication during 2° infection and trigger higher viral load inside the cells compared to the primary disease.

It has been reported that the complexes of anti-DENV antibodies and FcR down-regulate TLR expression and up-regulate negative regulators of NF-kB, suppressing innate immune response in peripheral blood mononuclear cells of 2° severe dengue patients but not in DF patients. The results of the treatment with anti-FcϒRI or anti-FcϒRIIa antibodies confirmed reduced viral loads, up-regulated IFN-β synthesis, and increased gene expression in the TLR-dependent signaling pathway (Modhiran, Kalayanarooj et al. 2010).

The correlation of homotypic and heterotypic neutralizing antibodies (NAbs) titers and the disease outcome is still not understood. Conversely, it was presented that the NAbs after subsequent infection with another serotype become broadly neutralizing and might limit the severity of dengue disease (Olkowski, Forshey et al. 2013). Similarly, new studies carried out in a longitudinal cohort by Eva Harris' group prove that the pre-infection presence of cross-reactive NAbs correlates with long-term protection against DF and severe forms of DENV infection (Katzelnick, Montoya et al. 2016).

In infants who receive the anti-DENV IgG by transplacental transfer from the DENV-immune mothers, the antibodies were hypothesized to cause ADE (Chau, Quyen et al. 2008). However, according to other studies infants with higher NAbs titers at birth usually

experience symptomatic disease later than those with lower titers (Libraty, Acosta et al. 2009).

Among all those theories there is a consensus that an exacerbated host response, uncontrolled immune cell activation and excessive inflammation is directly related to the pathogenesis of severe dengue disease (Basu and Chaturvedi 2008, Rothman 2011).

1.3 Dengue virus (DENV)

1.3.1 Classification of the virus

The Dengue virus (DENV) belongs to the family Flaviviridae, genus Flavivirus next to many other globally significant viral pathogens such as Japanese encephalitis virus (JEV), Yellow fever virus (YFV), tick-borne encephalitis virus (TBEV) and West Nile virus (WNV) (Mackenzie, Gubler et al. 2004, Westway EG, Tidona et al. 2011). Recently a number of new Flaviviruses were discovered enlarging the group of flaviviruses to 74-these members are classified into groups based on serological and phylogenetic parameters (Blitvich and Firth 2015, Colmant, Bielefeldt-Ohmann et al. 2016). The first classification of the group into eight serocomplexes was based on a positive hemagglutination inhibition (HI) assay with polyclonal sera (Calisher CH, Karabatsos N et al. 1989).

Later, in 1997, a comprehensive phylogenetic study was undertaken to establish the relationship among the flaviviruses (Kuno J, Gwong-Jen JC et al. 1998). The analysis of the genes encoding the non-structural 5 protein (NS5) and the structural envelope protein (E) led to the classification of the Flaviviruses into clusters reflecting their arthropod association, clades correlated with existing antigenic complexes and species. The study showed that from the putative ancestor, two clusters of the primary non-vector and secondary vector-borne viruses evolved. The non-vector flaviviruses were defined as transmitted between rodents or bats with no known arthropod vectors. The vector-borne cluster was further separated into mosquito (50% of identified flaviviruses) and tick-borne (28% of identified flaviviruses) virus clusters (Kuno J, Gwong-Jen JC et al. 1998). Phylogenetic analysis of

nucleotide sequence of complete ORF of flaviviruses produced refined and more detailed

classification of the genus Flavivirus and it is presented in Figure 3 (Blitvich and Firth 2015).

Insect-Specific Flaviviruses (ISF) are divided into two phylogenetic groups, first, of which is

classical ISF (cISF) comprising 12 viruses. The second, polyphyletic group consists of 9

viruses.
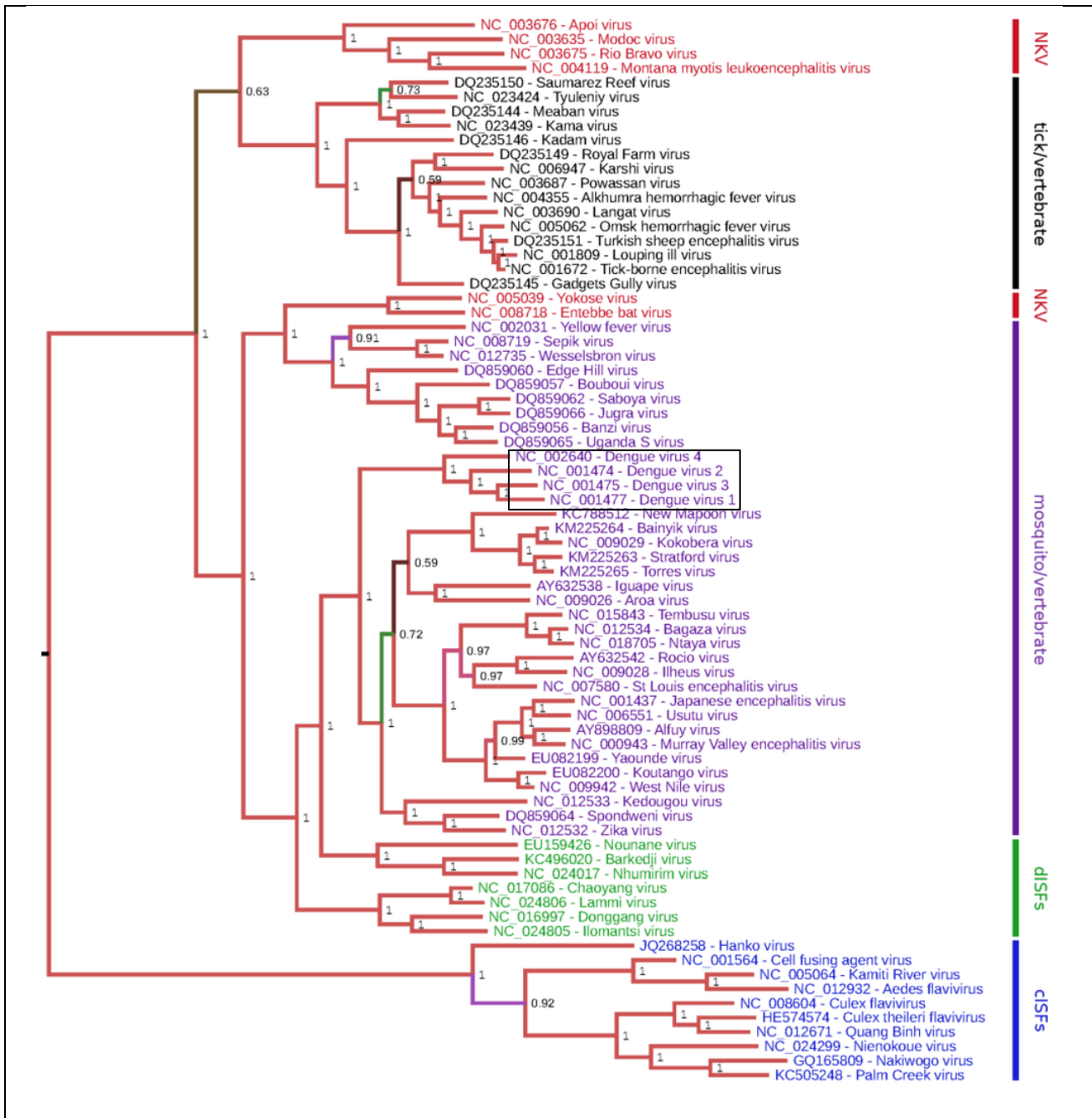
Figure 3. Phylogenetic tree for genus Flavivirus (Blitvich and Firth 2015).

The classification is based on the whole open reading frame (ORF). The tree is midpoint-rooted, nodes are labeled with posterior probability values and branches are highlighted with alternative colors. The clade in blue, at the highlighted node, represents the YFV subgroup. Species names are color - coded as follows: blue - classical Insect-Specific Flaviviruses (cISFs), discovered first; green - dual-host affiliated ISFs (dISFs); red – No Known Vector (NKV) Flaviviruses; purple - mosquito/vertebrate Flaviviruses; black - tick/vertebrate Flaviviruses. Bar 0.2 - substitutions per nucleotide position (Blitvich and Firth 2015).

1.3.2 Structure of DENV

Dengue virus (DENV) is approximately ~50nm in diameter with a smooth outer surface covered with envelope proteins surrounding a lipid bilayer derived from the host cell (Kuhn, Zhang et al. 2002, Acosta and Bartenschlager 2016). Inside the envelope is a capsid shell that contains the viruses' genetic material. DENV like all the Flaviviruses possesses a single-stranded, plus-sense RNA genome which is around 11kb long (Lindenbach, Thiel et al. 2007). The genome encodes a single open reading frame (ORF) flanked by 5' and 3' untranslated regions (UTRs) of ~100 and ~400–700 nt, respectively (Markoff L 2003). ORF is expressed as a large polyprotein, cleaved by viral and cellular proteases into ten mature proteins. Three structural proteins include capsid (C), pre-membrane (prM)/membrane (M), envelope (E) proteins and seven non-structural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5 (Murphy and Whitehead 2011), (Zou, Kukkaro et al. 2012). The genome encodes proteins in the following order 5'–C–prM(M)–E–NS1–NS2A–NS2B–NS3–NS4A–2K–NS4B–NS5-3' (Rice, Lenches et al. 1985, Lindenbach, Thiel et al. 2007). The E, C and prM proteins are built of 495, 120 and 165 amino acids respectively (Mukhopadhyay, Kuhn et al. 2005). The prM protein, the E protein and NS1 are the principal target of an antibody response of infected individual. The E protein of the Dengue virus (DENV) carries the main antigenic determinants of the DENV and it is a major target of neutralizing antibodies. Around 65% of the amino acid sequence of the E protein is identical in all DENV serotypes, and it contains two glycosylation sites at positions N67 and N153 (Johnson, Guirakhoo et al. 1994). In mature viral particles, 180 envelope proteins lie flat as 90 head to tail homodimers with a T=3 quasi-icosahedral symmetry (Kuhn, Zhang et al. 2002). The residues employed

in the formation of the antiparallel dimer and the highly conserved fusion loop (FL) required for the low-pH-driven membrane fusion are incorporated into E-DII. The single subunit of each dimer consists of three domains: E-DI, E-DII and E-DIII (Figure 4). Elongated E-DII and immunoglobulin-like folded E-DIII are connected through lateral hinges of E-DI, a central β-barrel structure of each E protein (Modis, Ogata et al. 2003, Rey 2003).
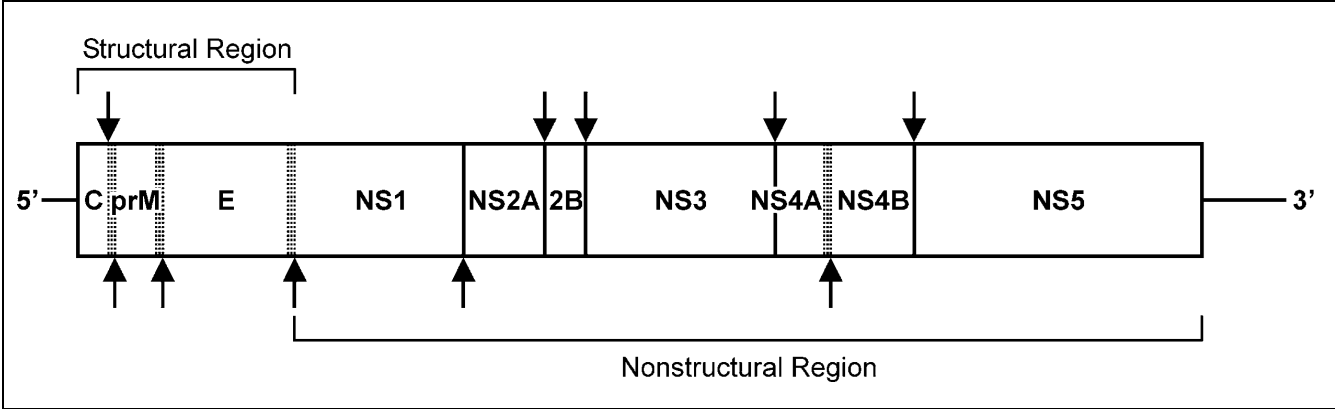
Figure 4. Genome organization of the Flavivirus

In the polyprotein, upper arrows indicate cleavage sites in the cytosol by the viral serine protease NS3 and cofactor NS2B, and lower arrows cleavages in the lumen of the endoplasmic reticulum; signal sequences are shown as shaded bars. (Coia G 1988, Speight G 1989, Westway EG, Tidona et al. 2011)

1.3.3 Life cycle of DENV

Immune cells of the host are targeted by DENV. The process starts with recognition and interaction between DENV and cellular receptors through heparan sulfate proteoglycans. The internalization of the virus into the cell occurs through receptor mediated endosytosis - Figure 5A (Acosta and Bartenschlager 2016). There are two cell receptor molecules pivotal in dengue infection, the cognate receptor involved in normal infection and Fc receptors (FcRs) believed to play a role in antibody dependent enhancement (ADE). The E glycoproteins in addition to being the major target of neutralizing antibodies (Abs) (and thus vaccine development) are responsible for the main steps involved in the viral entry process through cognate receptor recognition and cell and viral membrane fusion. The interaction with cellular receptors to initiate viral entry into the host cell is believed to be mediated by the E-DIII domain of E protein (Bhardwaj, Holbrook et al. 2001, Chu, Rajamanonmani et al. 2005, Chin, Chu et al. 2007, Watterson, Kobe et al. 2012). After the virus is internalized into the host cells endocytic pathway, acidic conditions cause irreversible conformational changes in E proteins which form spike-like trimeric structures aimed outward (Modis, Ogata et al. 2015, Zhang, Sheng et al. 2015). The tips of the spikes are hydrophobic what allows them to penetrate the membrane of the host endosome. The spikes bend bringing the endosomes' and viruses' membranes in close proximity leading to their fusion and the release of the viral nucleocapsid into the cytoplasmic space - Figure 5B (Modis, Ogata et al. 2015, Zhang, Sheng et al. 2015).

Next, the capsid disintegrates and the viral RNA is released into the cytoplasm where direct translation of the virus RNA genome takes place. The whole viral genome is translated into a single polyprotein chain (Acosta, Kumar et al. 2014).

The capsid protein is on the cytoplasmic side of the ER while the E protein and the pre-membrane (prM) protein are in the lumen where they are activated by host peptidases. In the cytoplasm the viral proteases in collaboration with cellular proteins, promote major rearrangements of the ER microenvironment and activate proteins in the polyprotein chain to form the RNA replication complex (Chatel-Chaix and Bartenschlager 2014, Junjhon, Pennington et al. 2014). Some of large number of synthetized copies of RNA are translated to make viral proteins used to assemble new viral particles - Figure 5 (Welsch, Miller et al. 2009).

The E proteins aggregate in the lumen of the ER, multiple copies of C proteins associated with the viral RNA, aggregate on the cytoplasmic side and form the new virus particle as it detaches into the lumen of the ER. In the still immature virus, 180 prM proteins cover the tips of 180 E proteins preventing the premature fusion inside the host cell. Those prM-E heterodimers form 60 spikes on the surface of immature virus. Thus, the surface of the immature particles, unlike mature virus, is rough and the diameter is around 10nm longer (Zhang, Corver et al. 2003). The virus is believed to be released from ER via the conventional secretory pathway. It travels through the trans-Golgi network, where it is exposed to acidic conditions, towards the cell surface. Before reaching the surface the prM proteins are processed into M proteins as the dissociation of prM-E heterodimers takes place, the E proteins are reorganized to form homodimers and prM is exposed for the Golgi-

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resident serine protease furin (Yu, Zhang et al. 2008, Zhang, Hunke et al. 2012). After the cleavage of the pr (after exposure to the neutral pH of the extracellular space) peptide viral particles gain infectivity and can be released from the cell to infect other cells (Zybert, van der Ende-Metselaar et al. 2008).
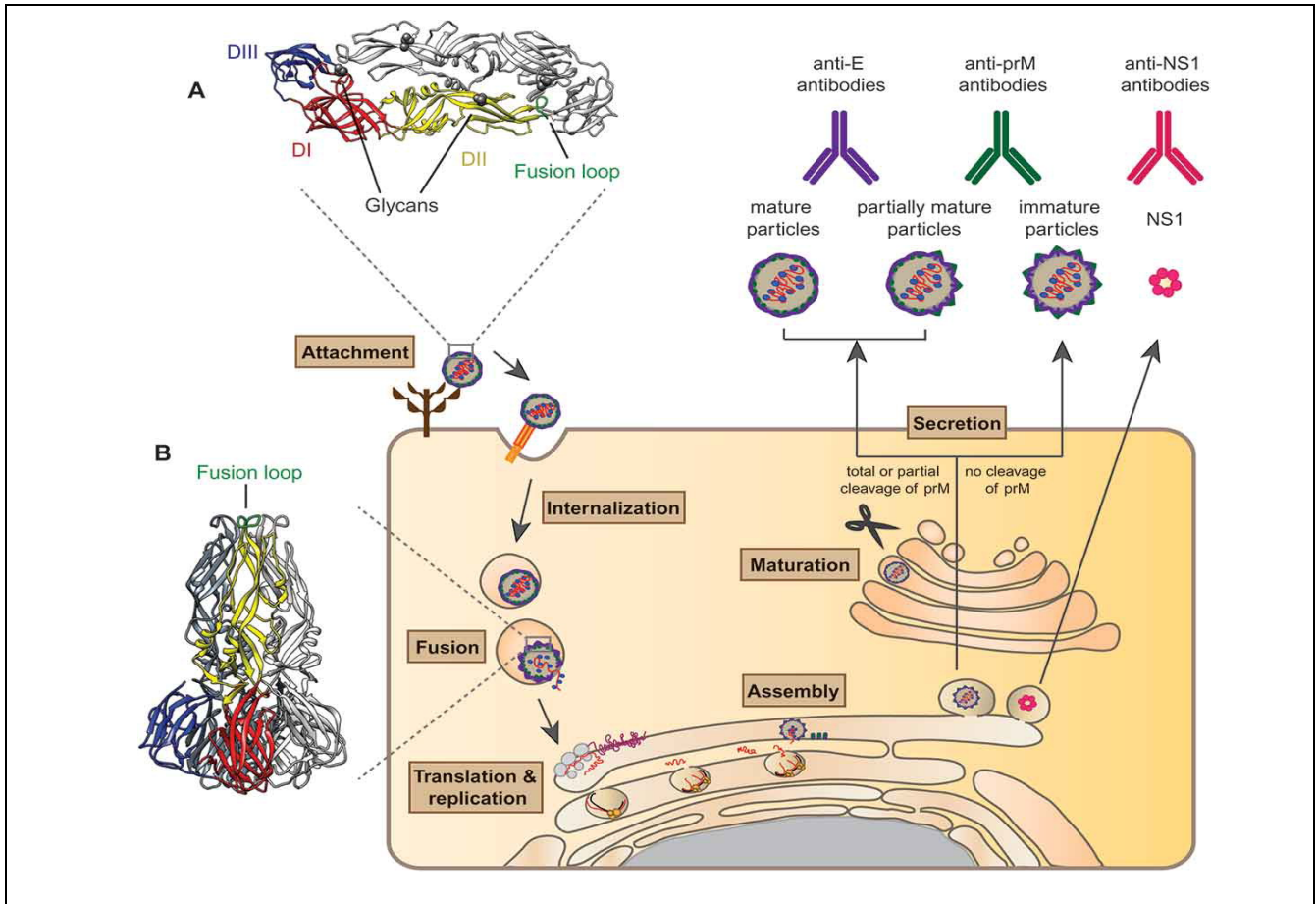
Figure 5. DENV life cycle and antibody responses to DENV infection.

The attachment of the DENV to the cells is followed by receptor-mediated endocytosis. The conformational changes of the E proteins triggered by acidic pH of the endosome exposes the fusion loop (green). This allows the membrane of the endosome and the virus to fuse and the viral genome is released into the cytoplasm. Now, the virus can replicate and RNA is translated making immature virus particles. Fully mature virus develops in the trans-Golgi network, where the prM proteins are cleaved by furin. Both premature and mature DENV particles are released from the cell

and induce antibody responses to E protein and prM protein respectively. Since NS1 is secreted out of the cell anti-NS1 antibodies are also produced. (Acosta and Bartenschlager 2016)

1.3.4 Phylogeny of DENV

As mentioned earlier, there are four confirmed DENV serotypes DENV1, DENV2, DENV3, and DENV4. All the serotypes are capable of triggering the clinical disease in all its forms (Murphy and Whitehead 2011). Although all four serotypes are endemic in Singapore, the majority of dengue patients in this country are infected with dengue serotype 1 and 2. Interestingly, periodic shifts between infections with the two most common dengue serotypes are observed. For instance in 2010, 70% of dengue patients in Singapore were infected with serotype 2 (Lee, Lai et al. 2010). One of the complexities of DENV is the presence of its multiple genotypes within each serotype. Genotype was defined by Rico-Hesse as a genetically distinct group within each serotype, characterized by genome sequencing (Rico-Hesse 2003). The genotypes of DENV1-4 are homologous in their nucleotide sequence up to 94% and their amino acid composition may differ by up to 3% within the E/NS1 junction (Teoh, Kukkaro et al. 2012, Zou, Kukkaro et al. 2012). For example there are five distinct genotypes within DENV serotype 1. Genotype I was found in Southeast Asia, China and East Africa, genotype II in Thailand, genotype III in Malaysia, genotype IV in West Pacific islands and Australia, and genotype V in North and South America, West Africa and Asia. The sequence of epitope residues of all DENV1 genotypes is conserved, what is consistent with an observation that 14C10 binds to all of them. The reason of differences in neutralization activities could be different expostion of target residues. (Teoh, Kukkaro et al. 2012).

The phylogeny of the virus is listed as one of risk factors for the development of symptomatic and severe form of dengue (Chapter 1.2). For instance, specific strains of

DENV3, genotype III were associated with an increase in severe dengue cases in South Asia, East Africa and Latin America (Messer, Gubler et al. 2003, Diaz, Black et al. 2006). Till date, the mechanisms explaining the correlation between mentioned DENV strains and the severity of the DENV-induced disease remain obscure (Costa, Fagundes et al. 2013).

An online news report published in October 2013 suggested the existence of a DENV serotype 5, identified in Sarawak, Malaysia in 2007 (Normille D. 2013). To date, there is no confirmation that an additional serotype of DENV exists as the 'new' serotype is closely related to DENV4 and might be a variant or genotype of this existing serotype (da Silva Voorham 2014).

1.4 Vaccine development

The development of highly effective dengue vaccine has been a challenge. One of the many reasons is that DENV has four serotypes and the recovery from an infection caused by one of the serotypes results in a lifelong immunity against only that particular serotype. Even though historically there have been only a few places where more than one serotype of DENV circulated simultaneously, global travel and urbanization increase the chances of the presence of two and more dengue serotypes at the same time and place. Thus, the vaccine needs to be tetravalent, triggering the immune protection against all described DENV serotypes. Moreover, the adaptive immune response to DENV, which is crucial for the antiviral response and the protection from the reinfection, was also reported to escalate the chances of an enhancement of disease severity. Nevertheless, since the neutralization of

DENV is assumed to be the main mechanism by which protection is acquired, elicitation of neutralizing antibodies at protective levels is the capital goal of immunization. Importantly, an immunization against DENV, needs to address protective immunity and hypothesized enhancing role of antibodies (Murphy and Whitehead 2011). The lack of an appropriate animal model is another difficulty for dengue vaccine development.

1.4.1 Live-attenuated vaccines (LAV)

Live-attenuated Dengue virus vaccine candidates were obtained by tissue culture passage, chimerisation, or an introduction of attenuating mutations (Murphy and Whitehead 2011). Recently a **c**himeric **y**ellow fever virus–**D**ENV **t**etravalent **d**engue **v**accine (CYD-TDV) known as Dengvaxia® or ChimeriVax (before only ChimeriVax) by Sanofi Pasteur was endorsed by WHO and has already been licensed in Mexico, Brazil, El Salvador and the Philippines. It is based on ChimeriVax technology, where certain genes (in Dengvaxia DENV prM and E structural genes) are inserted into Yellow Fever (YF) 17D vaccine backbone (Guy, Barrere et al. 2011). The choice of YF 17D backbone is justified as it was confirmed to be safe over 60 years use in other vaccines such as LAV against JEV, WNV, and Modoc virus (Govindarajan, Guan et al. 2016). Phase 3 studies involving 31,000 children between age 2 and 14 were carried out in Latin America and Asia. Clinical trials included 10 275 children aged 2–14 years children (CYD14) in Southeast Asia (Capeding, Tran et al. 2014) and 20 869 children, aged 9–16 years (CYD15) Latin America (Villar, Dayan et al. 2015) and revealed this vaccine to be 70 percent effective for those with pre-

exposure to DENV. Based on the results, schedule of three injections over one year is advisable in children more likely to be seropositive that is above nine yesr old, living in endemic areas, and previously exposed to DENV. Age and location restrictions as well as requirement for pre-existing immunity triggered with previous infection are significant limitations for dengue-control with Dengvaxia (Guy and Jackson 2016). The vaccine was shown before to be safe, not to trigger disease enhancement or vaccine-related severe adverse events (SAE) and resulted in relatively low viremia detected in vaccinated individuals (Morrison, Minnick et al. 2010, Capeding, Luna et al. 2011). However, the results of a phase IIb clinical study carried out in Thailand in children aged 4 to 11 years showed disappointing efficacy at 30.2% (Sabchareon, Wallace et al. 2012). The reason for such a low protective efficacy was that the incidence of mild disease caused by DENV2 endemic in the region at the time of the trial infection was the same in controls and in partially or fully vaccinated children (Halstead 2012). Surprisingly, the finding that immuogenicity against all four DENV serotypes did not assure the protection for DENV2 infection, is against the general consensus that the protective efficacy of anti DENV vaccine is a result of production of protective tetravalent neutralizing antibodies.

A non-chimeric version of DENV was also utilized as a LAV formulation. Each serotype of the virus was weakened by multiple passages in Vero cells and then four serotypes were combined into a tetravalent formula (Butrapet, Huang et al. 2000). This formulation entered phase II of clinical trials, but in some of the patients the vaccine candidate was not immunogenic against all four DENV serotypes resulting in unbalanced immunity (Sabchareon, Lang et al. 2004). A similar vaccination strategy was employed by the Walter

Reed Army Research Institute (WRAIR) and it was also found to stimulate immunity to only some of the serotypes (Sun, Edelman et al. 2003). Improved versions of these formulations were found to be safe and yielded tetravalent response rates 60% in subjects seropositive to at least one dengue virus (DENV) type and 66.7% in unprimed subjects (Thomas, Eckels et al. 2013).

Another way to attenuate the DENV for vaccine formulation is site directed mutagenesis of the viral genome. Delta30 vaccine contatins a non-chimeric mutated virus. 30-nucleotide deletion (Δ30) in the 3'-UTR was effective in attenuating DENV1 and DENV4 and did not affect the immunogenicity of these serotypes (Men, Bray et al. 1996, Whitehead, Falgout et al. 2003, Blaney, Matro et al. 2005). To address the lack of the immunogenicity of DENV2 and DENV3, the modification with genetic backbone of DEN4Δ30 was utilized (DEN2/4Δ30 and DEN3/4Δ30) (Blaney, Durbin et al. 2010). After initial *in vivo* tests in animal models, the mixtures of monovalent forms started being tested in human trials (Durbin, Kirkpatrick et al. 2013).

Another recombinant tetravalent, chimeric, dengue vaccine named DENVax was developed by Division of Vector-Borne Infectious Diseases of the Centers for Disease Control and Prevention and is in phase III clinical trials (CDC).

On many occasions, the first clinical phase of testing of these attenuated viruses, generated excellent immunological results and this lead to false degree of optimism for the vaccine's efficacy (Halstead 2012).

1.4.2 Subunit vaccines

Another vaccine strategy used for Dengue is a recombinant subunit vaccine, which utilizes only fragments of the virus. This method is considered safe, as it includes protein subunits instead of full infectious agent. Moreover it can stimulate balanced immune response to four DENV serotypes with minimal risk of ADE when employed in an accelerated immunization schedule. The disadvantage is that to achieve the immunity of live attenuated vaccines, the subunit vaccines must be administered with an adjuvant and need to administered as repeated doses multiple times. The subunit used for anti DENV production is the full or fragment of E protein, which is the target of neutralizing antibodies (Clements, Coller et al. 2010, Coller, Clements et al. 2011).

1.5 Antibodies against DENV

It has been suggested that antibodies against DENV with the highest capacity of neutralization and clearance are serotype-specific (Sabin 1952). Although dengue patients produce a broad repertoire of dengue binding antibodies, most of them are postulated to be immunologically redundant, e.g. by targeting poorly protective epitopes like prM, or by being cross-reactive or having overall weak neutralizing activity (Teoh, Kukkaro et al. 2012). Only a tiny fraction of all antibodies are serotype specific, targeting E protein and assuring the efficient neutralization of the virus (Teoh, Kukkaro et al. 2012). They are the key antibodies, which mediate long-term protection and resolve dengue infection (Teoh, Kukkaro et al. 2012). It is believed that once infected and recovered a person acquires lifelong serotype-

specific immunity due to the production of neutralizing homotypic IgG. Nonetheless, neutralizing and partially or non-neutralizing heterotypic IgG constitute the biggest fraction of all antibodies produced in DENV. Neutralizing heterotypic (cross-protective) IgG are responsible for cross-protective immunity, which typically persists for a period of several months to a few years (Goncalvez, Engle et al. 2007). Over longer periods, heterotypic IgG antibody titers, decrease due to the preferential selection of long-lived memory B cells producing homotypic antibodies (Chau, Quyen et al. 2008).

As mentioned earlier, it is hypothesized that secondary infection with DENV may lead to severe disease due to the presence of sub-neutralizing concentrations of antibodies produced during the initial infection (Guzman, Alvarez et al. 2007). This phenomenon is called antibody dependent enhancement (ADE) and it includes the mother-offspring ADE effect and ADE as a result of acquired immunity. Maternally derived anti DENV antibodies increase the risk of DHF in the infant infected with DENV for the first time (Green, Beatty et al. 2014). The proposed mechanism is that cross-reactive but non-neutralizing antibodies bind to, but do not neutralize the virus. It was shown that the cross-reactive antibodies might facilitate DENV infection of myeloid cells *in vitro* by promoting virus entry through FcγR. The antibodies would form complexes with DENV and bind to Fc receptor-bearing cells. As a result, the virus uptake increases and signaling cascades initiated via the pattern recognition receptors TLR-3 and MDA5/RIG-I are down regulated, reducing the antiviral response of the cell (Guzman, Alvarez et al. 2007, Rodenhuis-Zybert, Wilschut et al. 2010).

At the same time, the inflammatory immune response and the production of cytokines and chemokines are unusually high. In such cases, the viremia level is much

higher and the course of the disease more severe (Balsitis, Williams et al. 2010, Sasaki, Setthapramote et al. 2013). The Harris group has previously intimated that FcγR interactions play a critical role in ADE *in vivo* in both pre and post-exposure treatment strategies. Still, the exact mechanism of ADE remains unclear. There are many factors, which might influence an ADE effect *in vitro* and *in vivo.* In the presence of laboratory-adapted virus strains, high levels of ADE, but no neutralization activity were observed in serum taken from patients during the acute phase of a secondary infection. In contrast, the same serum assayed with the autologous virus showed high levels of neutralization activity and no ADE (Chaichana, Okabayashi et al. 2014). Even though, the ADE *in vitro* assay in K562 cells is considered the current gold standard, an *in vivo* ADE assay in an animal model might be of more significance (Chan, Watanabe et al. 2015, Milligan, Sarathy et al. 2015).

1.6 Human IgG1 14C10

A fully human, serotype specific human monoclonal antibody termed 14C10 was derived from a dengue resolved patient and is under pharmaceutical development for employment in clinical trials. Time-lapse confocal microscopy was employed to determine the mechanism of action of 14C10. This study demonstrated that 14C10 functions as a receptor antagonist. It impacts on the attachment of the DENV1 to the target cell surface and on a post-attachment step to stop the virus from cell entry (Zou, Kukkaro et al. 2012). *In vitro* characterization of 14C10, using PRNT method results in $PRNT_{50}$ = 0.33 μg and a novel quantitative flow assay provides $PRNT_{50}$ = 4 ng. Importantly, 14C10 has strong neutralizing activity for all known genotypes of the serotype 1 of DENV, isolated from the blood of patients globally (e.g. Cuba, Hawaii, Vietnam, Singapore). The binding footprint (Figure 6) from the binding Fab domain of 14C10 on the virus shows that 120 Fab domains of the antibodies bind to 180 E-proteins present on the capsid of the virus.
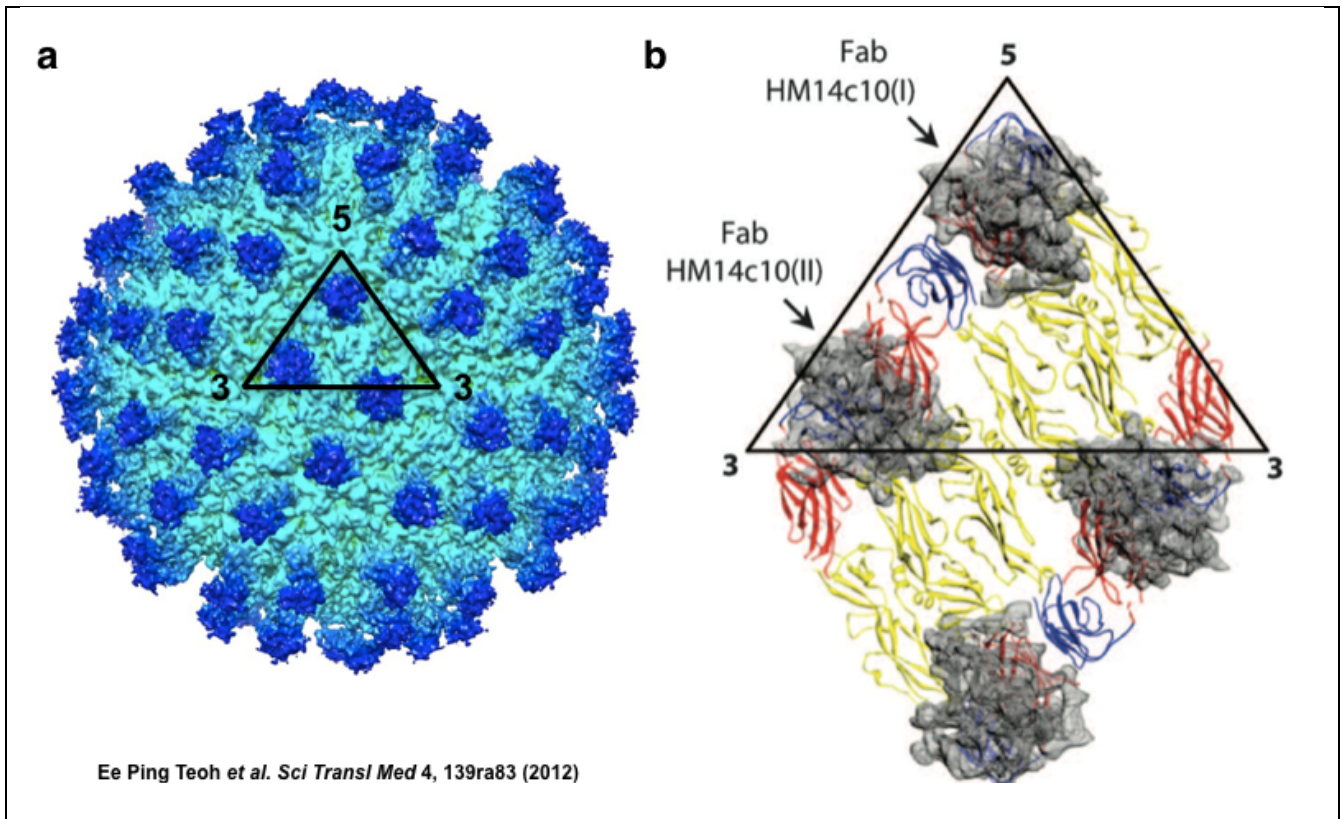
Figure 6. 14C10 binds a virus quaternary structure–dependent epitope

(**a**) CryoEM map of a Fab HM14C10-DENV1 complex showing 120 Fabs (blue) binding to 180 envelope proteins on the virus surface (cyan). Black triangle represents an asymmetric unit. (**b**) View of connecting densities of a Fab 14C10 (I) to envelope protein epitope (purple spheres). The domains of an envelope protein: E-DI, E-DII, and E-DIII are colored in red, yellow, and blue, respectively. (Teoh, Kukkaro et al. 2012)

The 14C10 antibody does not bind to DENV2, DENV3, and DENV4 and, thus it does not cause heterotypic enhancement at a sub-neutralizing concentrations. On the other hand, it binds and neutralizes the five known genotypes of DENV1 and exhibits some homotypic enhancement of DENV1 infection at low sub-concentrations (Teoh, Kukkaro et al. 2012). Homotypic ADE was seen in all the subclasses of the human antibody and it was ranked as follows IgG3 > IgG1 > IgG2 > IgG4, which may be related to the binding affininty to FcγRIIA expressed by K562 cells used in the assay (Chiofalo, Teti et al. 1988, Teoh, Kukkaro et al. 2012). The exact role of the Fc domain in 14C10 function is unknown. To further determine the interactions between 14C10 Fc and the FcγRs, we have designed a mutation of the human antibody and a point mutation of mouse 14C10 that also abolishes FcR binding. We produced 14C10-hG1LALA, which abrogates binding to human FcγRs and mouse antibody hCH1mG1HCH2-3-D265A, which is null for FcγR binding. Moreover, to evaluate an effect of antibody subclass on virus neutralization and associated heterotypic and homotypic antibody dependent enhancement (ADE), we have generated two different subclasses of a mouse variant of HM14C10.

1.7 Fc effector domain of IgG and FcγRs

An antibody binds to an antigen on a virus through its Fab (fragment antigen-binding) portion, and the targeting of 14C10 Fab for DENV1 has been previously characterized (Teoh et al, 2012). Thus, my project is aimed at characterizing the other part of the antibody that encodes functionality-the fragment crystallizable (Fc) portion. Binding of antibody Fc-regions to Fc receptors (FcR) on cells can induce both pro and anti-inflammatory responses, called effector mechanisms (Nimmerjahn and Ravetch 2008). This may be important to neutralize the pathogen and eventually stop the inflammation that underlies dengue morbidity. FcRs are cell surface transmembrane dimeric receptors. Binding IgG through the Fc domain can cause phagocytosis of immune complexes, release of various cytokines by many different cells, the antibody-dependent cell-mediated cytotoxicity (ADCC) and the complement cascade. Fc domain also binds the neonatal receptor for Fc (FcRN), which is responsible for the maintenance of the antibodies serum half-life. One way to account for this diversity of biological responses for a single molecular entity is to invoke a number of different binding partners.

Antibody coated pathogens are seen by effector cells through Fc receptors and the main Fc effector property is an activation of different FcR-bearing cells. In response to FcR binding, macrophages, neutrophils, and dendritic cells phagocytoze virus-antibody complexes and clear them from circulation. Non-phagocytic cells of the immune system like natural killers cells (NK), eosinophils, basophils, and mast cells, are stimulated to secrete cytokines. Activated NK cells destroy antibody-coated targets in a process termed antibody-dependent cell-mediated cytotoxicity. Moreover, as the IgG is made and secreted into the

circulation, it stimulates feedback mechanisms on plasma cells and DC through their FcγRs that control their activation. Antibody binding to the virus can trigger some or all of these mechanisms, which result in the pathogen neutralization and clearance (Murphy, Travers et al. 2012).

A significant factor in the functionality of Fc receptors is the expression of both activatory and inhibitory forms on the same cell types. Though, the number and specificity of FcRs in humans and in mice differ, there is significant homology between them (Figure 7), which makes the mouse model suitable for this project (Nimmerjahn and Ravetch 2008, Bruhns 2012). Thus, this work aims to establish the role of FcγRs binding, clearance, protection and neutralization of a representative therapeutic anti-DENV1 14C10 antibody *in vitro* and *in vivo*.
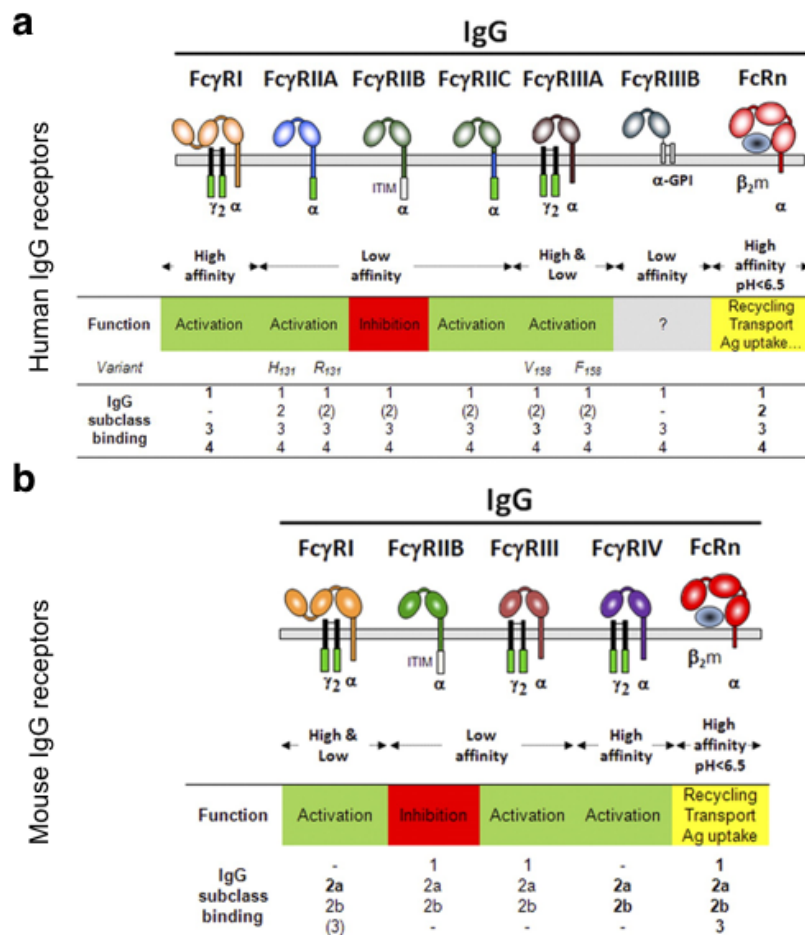
Figure 7. Human and mouse FcγRs

FcγRI is a receptor, which has high affinity for IgG1, IgG3 and IgG4 in human and IgG2a, IgG2b and IgG3 in mouse. Additionally we can distinguish 5 and 3 low to medium affinity receptors in the human and mouse respectively. The second classification of FcγRs into activators or inhibitors is based on the signaling pathways they induce. In both human and mouse there is one inhibitory FcγR, FcγRIIB, containing immunoreceptor tyrosine-based inhibitory motif (ITIM in its cytoplasmic domain. All the activatory receptors of mouse and FcγRI anf FcγRIII (A/B) consist of a ligand-binding α-chain and immunoreceptor tyrosine based activating motifs (ITAMs) in signal-transducing γ-chain dimer. In human all IgG subclasses bind to all FcγRs except for FcγRIIIB, which does not bind IgG2. (Nimmerjahn and Ravetch 2008, Bruhns 2012).

1.8 IgG isotypes

The properties and functions of diverse IgG subclasses, which are already uncovered, give an insight into the complexity and importance of the Fc portion of an antibody not only between the main antibody isotypes (IgA, IgM, IgD, IgG) but also within the IgG group. In human we distinguish four IgG isotypes (IgG1 IgG2 IgG3 IgG4), also termed as IgG subclasses.

In my project we engineered two mouse-human chimeric IgG subclasses of 14C10 human IgG1 in order to study the effect of those modifications *in vivo.* We chose mouse as a good model for studying the roles of different IgG immunoglobulins as four existing IgG subclasses (IgG1, IgG2a, IgG2b, IgG3) in mouse are well characterized and to some extent represent the equivalents of human IgG isotypes. IgG1 antibody is the most prevalent antibody and it comprises 60% of all IgG isotypes, while IgG2 comprises 25%, IgG3 – 10% and IgG4 – 5% of all IgG isotypes in human (Salfeld 2007). Mouse IgG2a was proposed to be an equivalent of human IgG1, mouse IgG3 represents human IgG2, mouse IgG2b – human IgG3 and mouse IgG1 - human IgG4 (Nimmerjahn and Ravetch 2008).
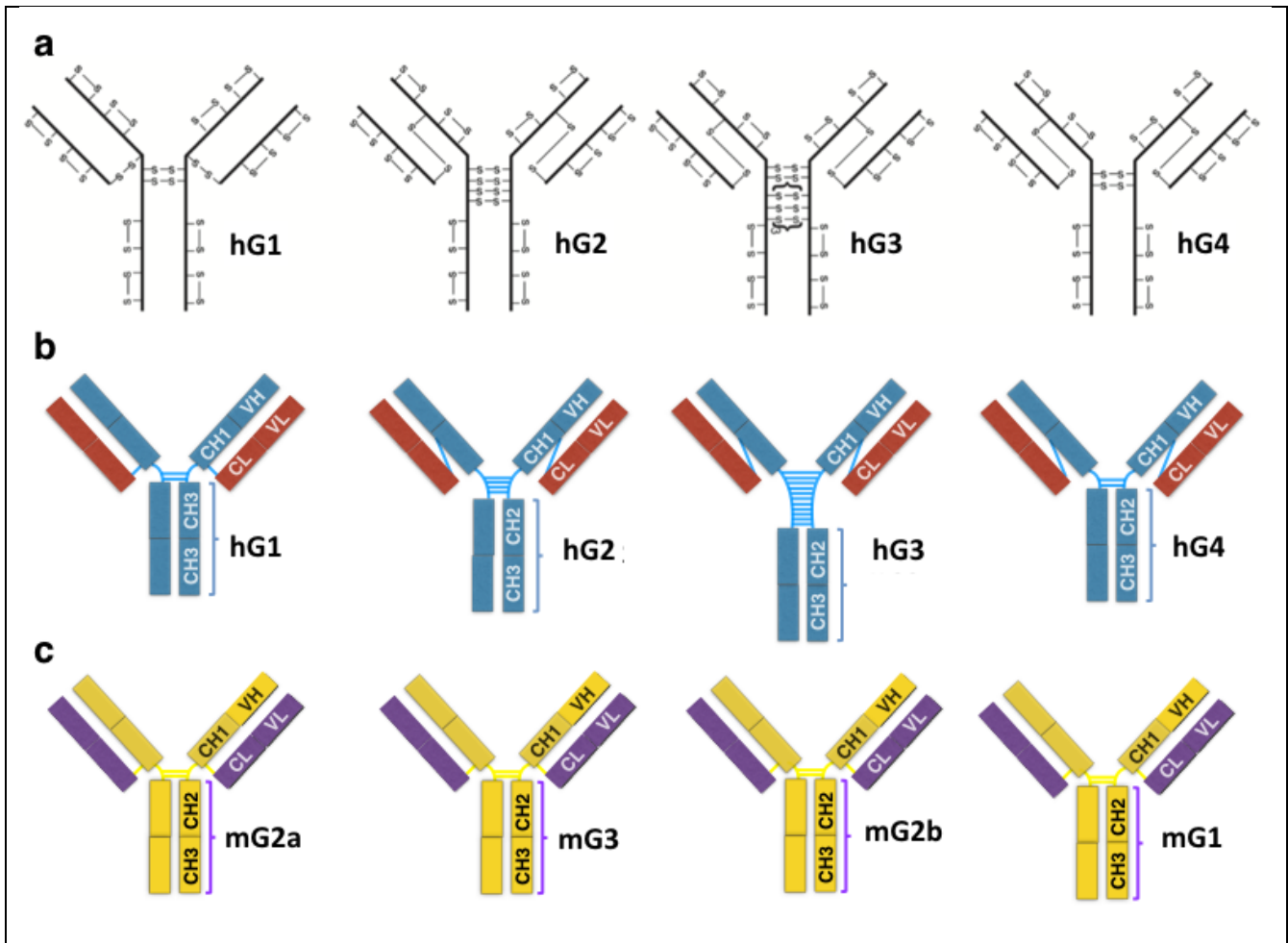
Figure 8. Subclasses of IgG antibodies in the human and mouse.

The figure presents subclasses of IgG antibodies in the human and mouse. All subclasses bind to FcRn, which is responsible for their recycling. (**a**) Disulfide bond structures in the subclasses of human IgG antibodies (Liu and May 2012). (**a, b**) **Human IgG subclasses.** In humans, IgG1 is the most prevalent subclass of IgG antibodies. IgG1, IgG3 and IgG4 cross the placenta. With respect to complement activation the effectiveness decreases IgG3 > IgG1> IgG2. IgG4 does not activate the complement system. IgG1 and IgG3 mediate opsonization by binding to Fc receptors on phagocytic cells. IgG4 has moderate affinity for Fc receptors while IgG2 has extremely low affinity. (**c**) **Mouse IgG subclasses.** Mouse equivalent of IgG1, IgG2, IgG3 and IgG4 are IgG2a, IgG2b, IgG1 and IgG3 respectively. IgG2a is the most prevalent mouse antibody. IgG2a and IgG2b bind to all FcγRs and are the most potently activating, while IgG3 binds only to FcγRI with low affinity, whereas IgG1 is the least activating and preferentially interact with inhibitory FcγRIIB (Hussain, Dawood et al. 1995).

1.9 Animal models

Currently, the AG129 mouse is the most widely available and best characterized animal model used in dengue studies (Johnson and Roehrig 1999). However, it should be noted that no animal exhibits the full disease spectrum found in humans naturally or experimentally. Thus, no ideal animal model for dengue infection is available. We can make the following conclusions about Dengue immunology based upon studies in AG129 and human cohorts. The first line of viral defense begins with an intracellular signaling cascade resulting in the production of interferon α/β (IFN-α/β), which helps initiate, the adaptive response during the course of DENV infection (Green, Beatty et al. 2014). IFN α/β is produced by virally infected leukocytes and fibroblasts respectively. After IFN α/β diffuses to adjoining cells, it activates gene products that interfere with viral replication, and stimulate the production of MHC I: thus enhancing the ability of virally infected cells to present peptides to T cells. It also activates NK cells, which recognize and kill host cells infected with viruses. Natural killer cells and T cells produce IFN γ, which activates function of macrophages. It was demonstrated that DENV subverts the IFN induced antiviral response, which highlights the importance of type I IFN system in the host response (Munoz-Jordan, Sanchez-Burgos et al. 2003, Costa, Fagundes et al. 2013). The AG129 mouse is genetically modified and defective in its IFN α/β and IFN γ responses, which allows effective replication of DENV, with some variability in susceptibility (Johson and Roehrig 1999, Tan, Ng et al. 2010).

2   Aims of the project

In summary the aims of my PhD are:

I. To employ advanced molecular engineering to develop murine and human scaffolds for 14C10 beyond the standard human IgG1 format (it's current formulation), including different human/mouse chimerics, IgG sub-classes plus defined mutations that altered antibody functionality through Fc receptor binding.

II. To utilize engineered antibodies to investigate the role of Fc receptor biology in antibody mediated neutralization and viral clearance *in vitro* and *in vivo* employing a number of defined molecular, cellular and virological assays.

III. To determine the impact of employing different 14C10 antibody formats in therapeutic and prophylactic contexts *in vivo* on the ability of dengue-infected AG129 mice to mount their own anti-dengue memory immune response.

3 Materials and methods

3.1 Cell lines

C6/36 *Aedes albopictus* cells (American Type Culture Collection ATCC®CRL-1660™), Baby Hamster Kidney-21 (BHK-21) [C-13] (ATCC® CCL-10™) and Human erythroleukemia K562 (ATCC® CCL-243™) cell lines were used for this study. C6/36 cells were cultured in Leibovitz's L-15 medium (Gibco, Invitrogen) with 10% of fetal bovine serum (FBS) at 28ºC with 5% CO_2. BHK-21 cell line was maintained in Roswell Park Memorial Institute 1640 (RPMI 1640 medium, from HyClone) with 2.05mM L-Glutamine supplemented with 10% of FBS at 37ºC in 5%CO_2. K562 cells were maintained in RPMI 1640 supplemented with 10% of FBS at 37ºC with 5%CO_2.

3.2 Dengue virus propagation

Serotype 1 and 2 of DENV were used in this study, DENV1 and DENV2. The two most distinct genotypes of DENV1, EHI (genotype 1) and WestPac74 (genotype 4) and genotype 1 EHI of DENV2 were used in the study. DENV1 EHI and DENV2 EHI strains were isolated from dengue patients hospitalized in Singapore by the Environmental Health Institute (EHI). The DENV1 EHI.D1, which is a laboratory-adapted strain from clinical isolate, was obtained for this study from Prof. Ng Mah Lee laboratory and DENV1 WestPac74 from Novartis Institute of Tropical Diseases in Singapore. DENV2 EHI was obtained from Prof. Alonso

Sylvie's lab. Each virus stock was propagated in the monolayer of C6/36 cells in a non-vented T-75 flask (Nunc) infected with 1 ml of a stock virus at 1×10^6-1×10^7 PFU/ml. C6/36 cells were first incubated with the virus at 28ºC with 5% CO_2 for 1 hour and the flasks were rocked every 15 minutes. Afterwards, 9 ml of L-15 medium with 2% of FBS was added and the cells were monitored every day for the cytopathic effect. Culture media was harvested after 5-6 days from infected C6/36 cells and centrifuged for 10 minutes at 10,000 rpm. The viral supernatant was aliquoted into the cryovials (Nunc) and stored at -80ºC as viral stocks. Virus titer was determined by plaque assay on BHK cells.

3.3 Virus Quantification – Plaque Assay

A standard plaque assay was performed to measure the concentration of the DENV. The concentration of the virus was assessed as the number of infectious particles in 1 ml volume of supernatant. BHK cells were seeded on 24-well (Nunc) plates to obtain 80% confluency 24 hours later. Six 10-fold serial dilutions of viral stock were prepared (10^{-1} to 10^{-6}) in RPMI 1640 media. Triplicates of 100µl of each dilution were added onto the BHK cells monolayer. The stock virus was used as a positive control and the RPMI 1640 media was used as a negative control. The BHK cells were incubated with 100µl of samples for 1 hour at 37ºC with 5% CO_2. The plates were rocked every 15 minutes to avoid drying of the cells. Thereafter, each well of the cells was covered with 1ml 1% (w/v) carboxymethyl cellulose (CMC) with 2% FBS to assure that the spread of the virus was restricted to the neighboring

cells. Cells were incubated for 5 days at 37°C with 5% CO_2, before the CMC was removed, and the cells were fixed and stained with 1% (w/v) crystal violet dissolved in (w/v) paraformaldehyde (PFA), and incubated on the rocker for 2 hours. The plaques were counted manually.
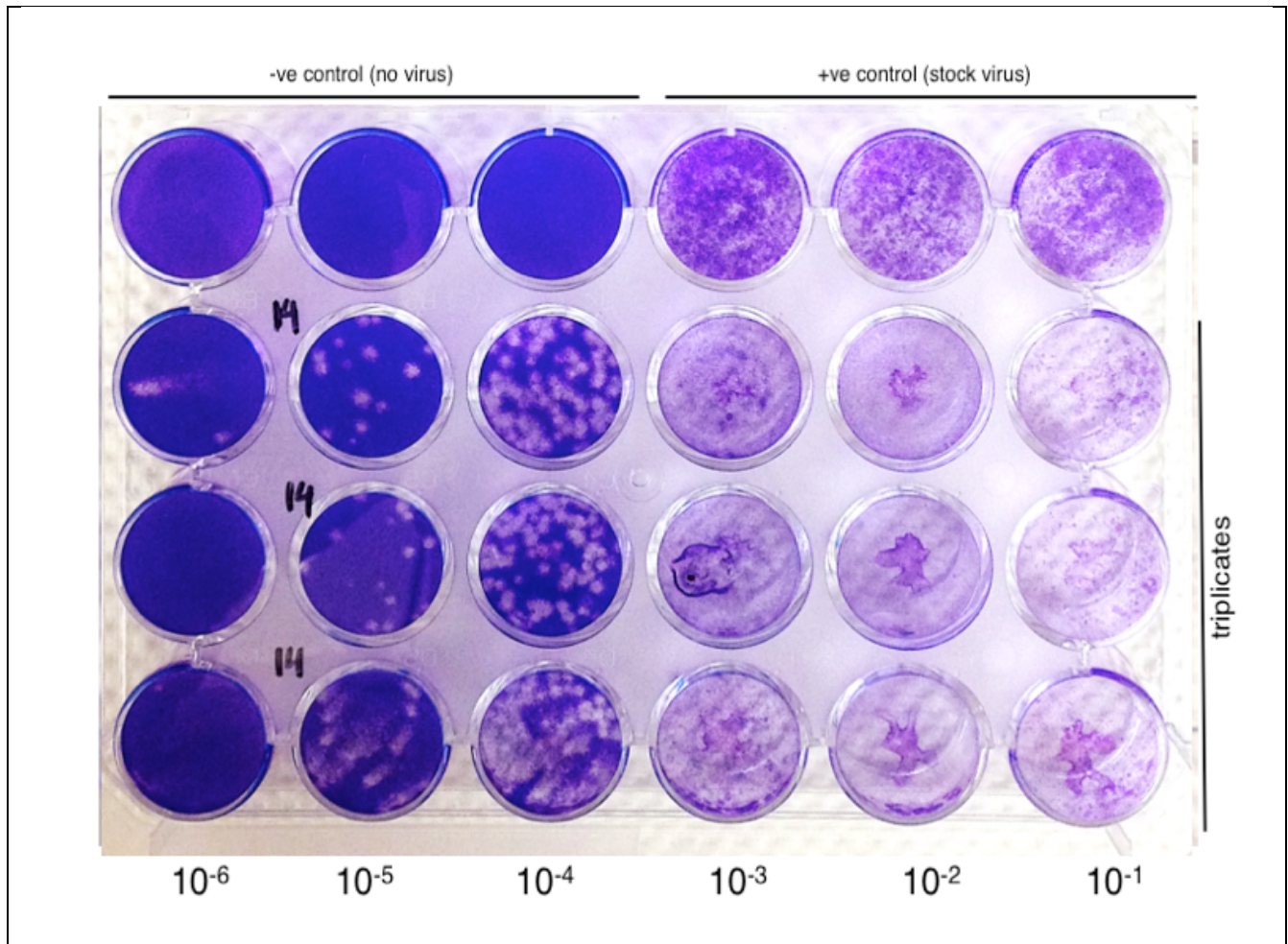
Figure 9. Plaque assay used for the assessment of the virus titer of the DENV1 EHI stock used in the *in vitro* and *in vivo* experiments.

Virus stock was serially diluted 10-fold in RPMI 1640 (10^{-1} to 10^{-6}) and incubated with the BHK-21 cells monolayer. The stock virus was used as a positive control and the RPMI 1640 media was used as a negative control. The plaques were counted manually at the concentration where 5-50 clear plaques were visible. The titer of this stock is 14 x 10^5 (dilution factor) x 10 (per ml)= 1.4x10^7 plaque forming units per ml (PFU/ml).

3.4 Cloning of 14C10 constructs

The sequence template of wild type 14C10 antibody, derived previously from the B cell of a human patient and immortalized via the EBV method (Teoh, Kukkaro et al. 2012), was used as a template for the expression of the four 14C10 constructs (LALA, mIgG2aEXT, mIgG1, mIgG1D265A).
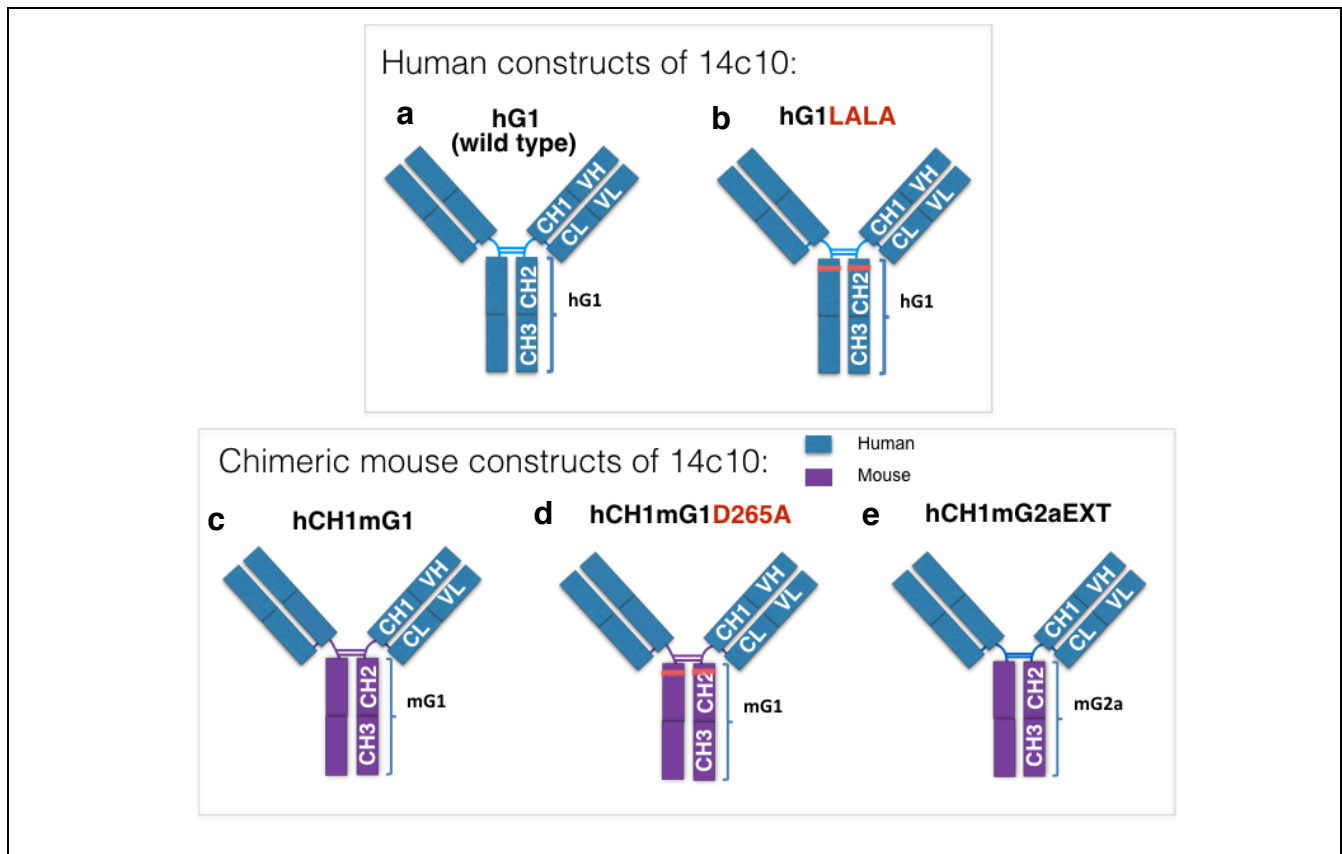
Figure 10. Schematics of the constructs engineered for the project.

(**a**) 14C10-hG1 (14C10 WT); (**b**) hCH1mG2aEXT-14C10 (mIgG2aEXT), human Fc domain substituted with mouse Fc domain; (**c**) hCH1mG1-14C10 (mIgG1), human Fc domain and human hinge replaced by mouse Fc domain and Fc hinge; (**d**) 14C10hG1LALA, two leucines L234, L235 are substituted with alanines in CH2 domain of hG1; (**e**) hCH1mG1HCH2-3-D265A (mIgG1D265A), construct is the same as (a) except point mutation at D265 in the CH2 domain of mIgG1.

3.4.1 Cloning of mG1, mG2a, mG2aEXT

To obtain mIgG2a (hCH1mG2a), mIgG2aEXT (hCH1mG2aEXT **-** mouse chimeric IgG2a with human IgG1 hinge) and mIgG1 (hCH1mG1-14C10 - mouse chimeric IgG1 with mouse IgG1 hinge) chimeric constructs of the 14C10 antibody, the following steps were carried out. The light chains of the 14C10 WT antibody were cloned from the library phagemid vector into the proprietary expression vector via ApaLI-PstI. The variable region of the heavy chain of 14C10 was cloned from the library phagemid vector into an in-house expression vectors via SfiI-BsmBI restriction sites for the engineering of hCH1mG2a and hCH1mG1. An internal ribosome entry site (IRES) insert allowed for expression of two genes from a single vector.

3.4.2 Cloning of hIgG1LALA, mG1D265A

The commercial QuikChange Mutagenesis kit (Stratagene®) was employed to generate two Fc mutants namely hG1LALA and mG1D265A (hCH1mG1D265A), according to the manufacturer's instructions. LALA mutation is localized in the CH2 domain of hG1. Construct hCH1mG1D265A is the 14C10hCH1mG1 construct with a point mutation at D265A in CH2 domain of mG1.
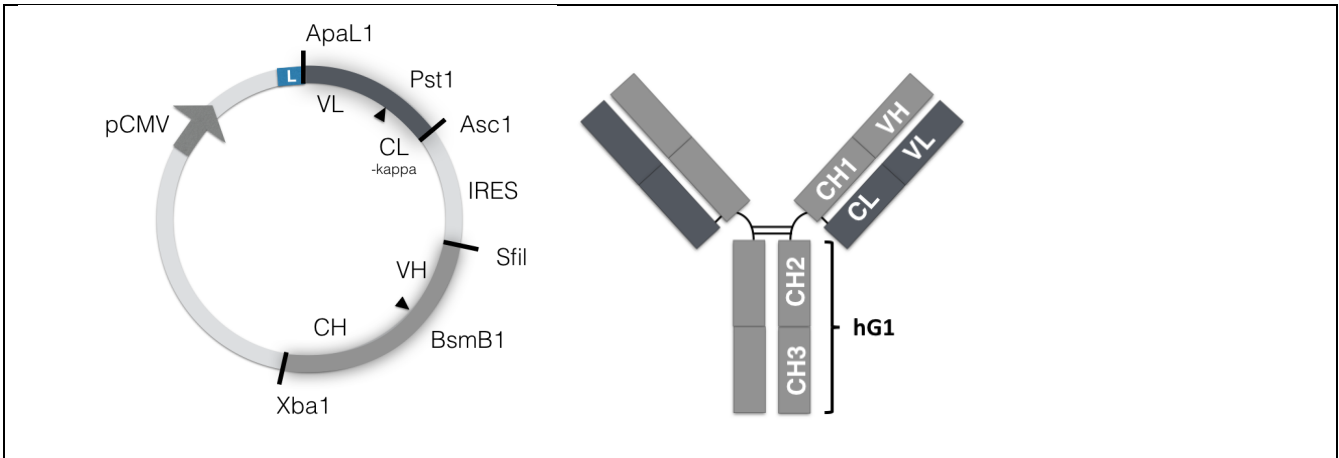
Figure 11. Schematic diagram of the in-house expression vector used to create human IgG.

The variable light chain and heavy chain of fully human 14C10 immunoglobulin were cloned separately via ApaLI-PstI and SfiI-BsmBI restriction sites into proprietary expression vector containing standard human constant regions of human G1 antibody and an IRES insert. (Dark grey=light chain; Light grey=heavy chain).
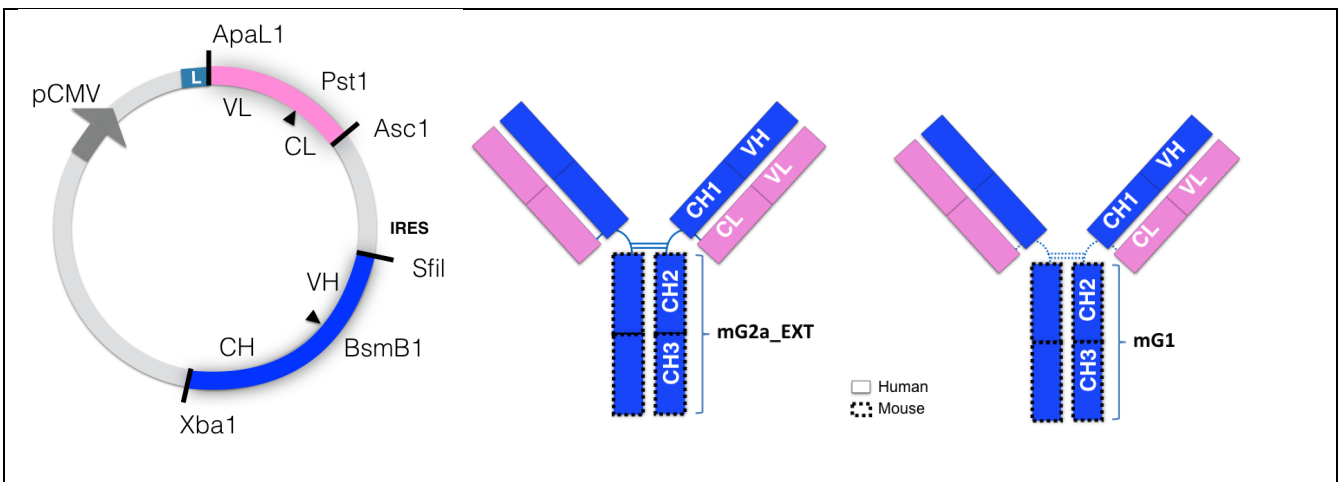
Figure 12. Schematic of the in-house expression vector used to create chimeric mouse-human IgG antibodies.

IRES sequence was inserted into vectors to allow for expression of heavy and light chains from a single vector. The variable light chain and heavy chains of fully human 14C10 immunoglobulin were cloned via ApaLI-PstI and SfiI-BsmBI restriction sites into proprietary expression vectors containing either mouse G2a or G1 constant regions (CH2 and CH3 domains). Mouse IgG2a consists of CH1 bound to CH2 through human hinge. (Pink=light chain; Blue=heavy chain).

3.5 Expression of the 14C10 constructs

Constructed plasmids were sent to the company 1st Base Asia for sequencing using our in-house designed primers. Once the sequences of the plasmids were verified, the light chain and the respective heavy chain were co-transfected into suspension HEK293-6E cells using branched polyethyleneimine (PEI) (Sigma-Aldrich, USA) and harvested 7 days later.
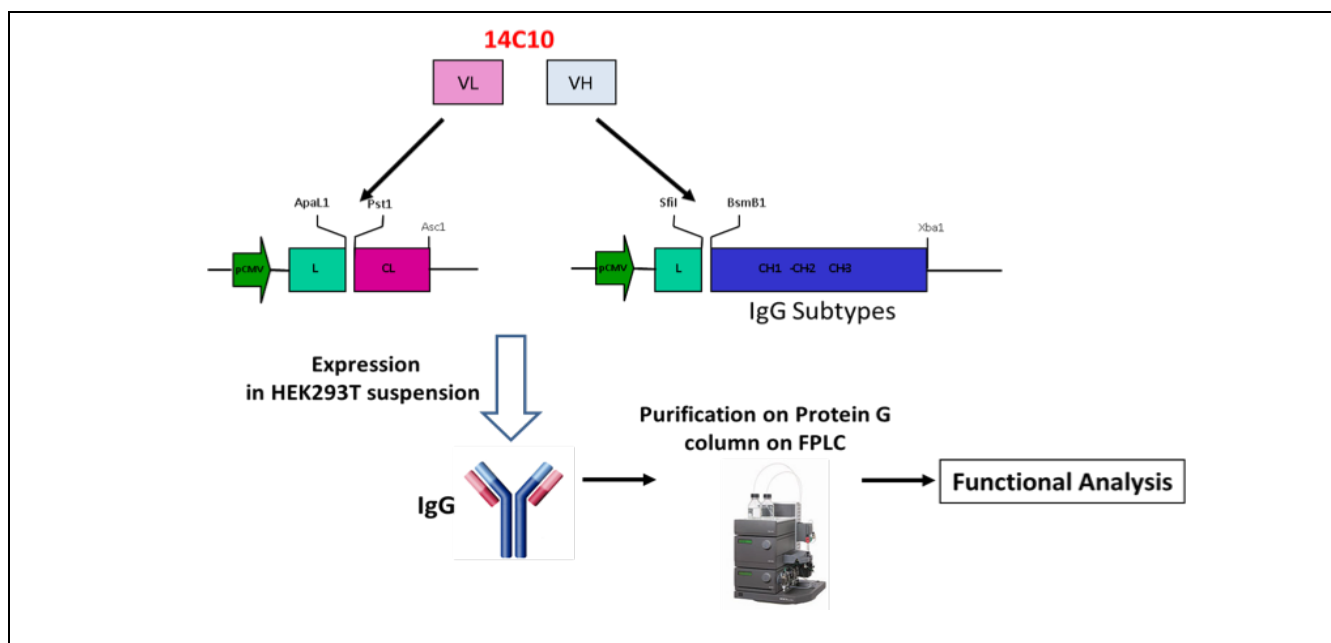
Figure 13. Cloning and expression of the 14C10 constructs.

The variable light chain and heavy chains of the various constructs of 14C10 immunoglobulins were cloned separately into proprietary expression vectors via ApaLI-PstI and SfiI-BsmBI restriction sites respectively. The commercial QuikChange Mutagenesis kit (Stratagene) was employed to generate the two Fc mutants hG1LALA and mG1D265, according to manufacturer's instructions. The constructed plasmids were sent to the company 1st Base Asia for sequencing using our in-house designed primers. Once the plasmids were sequence confirmed, the light chain and the respective heavy chain were co-transfected into suspension HEK293T cells via standard PEI method and harvested 7 days later. The culture supernatant was then purified on HiTrap Protein G column (GE Healthcare) on the FPLC. Purified antibodies were analysed by standard protein gel electrophoresis followed by their retention of binding specificity for Dengue1 via ELISA before conducting further functional studies.

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3.6 Protein G purification

The culture supernatant from transfected HEK293-6E was harvested and applied on 5 ml HiTrap rProtein G column (GE Healthcare, United Kingdom). The column was washed with five column volumes (CVs) of phosphate buffered saline (PBS) and eluted with IgG elution buffer (Thermo Scientific, USA). The eluted fractions were neutralised with 1 M Tris-HCl, pH 9.5. Protein G-purified antibodies were applied onto Centifugal Filter Concentrator with Ultracel® 30 Regenerated Cellulose Membrane and the buffer was exchanged to PBS. Purities of the protein G purified antibodies were analysed by standard 10% NuPAGE sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

3.7 Functional testing of the engineered antibodies (ELISA)

3.7.1 Testing of the binding specificity of the constructs

Retention of binding specificity for DENV1 was tested via Enzyme Linked Immunosorbent Assay (ELISA) before conducting further functional studies. Binding of hIgG1, hIgG1LALA, mIgG1, mIgG2aEXT and mIgG1D265A engineered antibodies versus 14C10 WT antibody were compared using the direct ELISA assay. Purified DENV1 EHI or DENV2 NGC was coated on the 96-well plates (Nunc MaxiSorp® flat-bottom) 50µl/well and incubated overnight at 4°C. Plates were then washed once with PBST 0.05% and twice with PBS and

blocked with 4% (w/v) skim milk (Sigma)/PBS at RT for 2 hours. After two rounds of washes with PBST 0.05%, purified antibody samples were added to the wells and incubated for 1 hour at RT. The plates were washed again, before the anti-mouse HRP antibody or anti-human HRP antibody was added at 1:5000 dilution in 2% (w/v) skim milk (Sigma)/PBS and incubated for 1 hour at RT. When hG1 was tested, goat anti-human IgG Fc cross-adsorbed (minimal cross-reactive with mouse serum proteins) secondary antibody, HRP-conjugate (Pierce), diluted 5000-fold in 2% (w/v) skim milk was utilized. When mIgG1, mIgG2aEXT and mIgG1D265A were tested, goat anti-mouse IgG (H+L) cross-adsorbed (minimal cross-reactive with human serum proteins) secondary antibody, HRP-conjugate (Pierce), 1:5000 in 2% (w/v) skim milk was used. Plates were washed as described above and tetramethylbenzidine (TMB) substrate (Pierce) was added. The reaction was stopped with 0.1 M sulfuric acid and the reading of absorbance was taken immediately at 450nm by spectrophotometer.

Additionally, sandwich ELISA was carried out to check the binding characteristics of mIgG1 and mIgG2aEXT. The primary antibody (4G2 - human IgG1 specific for Flavivirus group antigen) was coated at 5µg/ml, 50µl/well overnight at 4^oC onto Nunc MaxiSorp® flat-bottom 96-well plates. The wells were then washed once with PBST 0.05%, twice with PBS and blocked with 4% (w/v) skim milk (Sigma)/PBS at RT for 2 hours. Then non-purified DENV1 EHI or DENV2 NGC was added at 10^6 PFU/ml in 4% skimmed milk, 50µl/well. After an incubation of 1 hour at RT, the wells were washed and probed with the samples of purified antibodies. 3H5 is a murine monoclonal recombinant chimeric clone of the murine monoclonal IgG1 specific for DENV2 and it was used as a control. Goat anti-mouse IgG

(H+L) cross-adsorbed (minimal cross-reactive with human serum proteins) secondary antibody, HRP-conjugate (Pierce, 1:5000 in 2% (w/v) skim milk) was added 50µl/well and incubated for 1 hour. After the final triple wash, TMB substrate (Pierce) was added at 50µl/well and the reaction was stopped with 0.1 M sulfuric acid 50µl/well. Absorbance was read at 450nm by spectrophotometer.

3.7.2 *In vitro* neutralization efficacy of the 14C10 constructs

Plaque reduction neutralization test (PRNT) in BHK cells was performed to assess *in vitro* neutralization of two different genotypes of DENV1 EHI and WP74 by all four constructs of 14C10 versus WT HM14C10. BHK cells were seeded on 24-well plates (Nunc), 24 hours earlier, to obtain a monolayer of cells. Virus was diluted to around 400 PFU/ml to give around 40 plaques in 100µl. Serial dilutions of the antibodies were preincubated with the virus for 1 hour. Each sample of virus-antibody solution was added onto BHK cells in triplicates and incubated for 1 hour at 37°C. Each plate had a triplicate of the positive (with 40-100 viral plaques per well) and the negative (RPMI) controls. Carboxymethyl cellulose (CMC) 1% (w/v) was added to assure that the spread of the virus was restricted to the neighboring cells. After 6 days of the incubation, the BHK cells were fixed and stained with crystal violet in PFA. The level of neutralization was estimated by the number of plaques in BHK monolayer, in the plaque-forming units (PFU) per milliliter (ml).
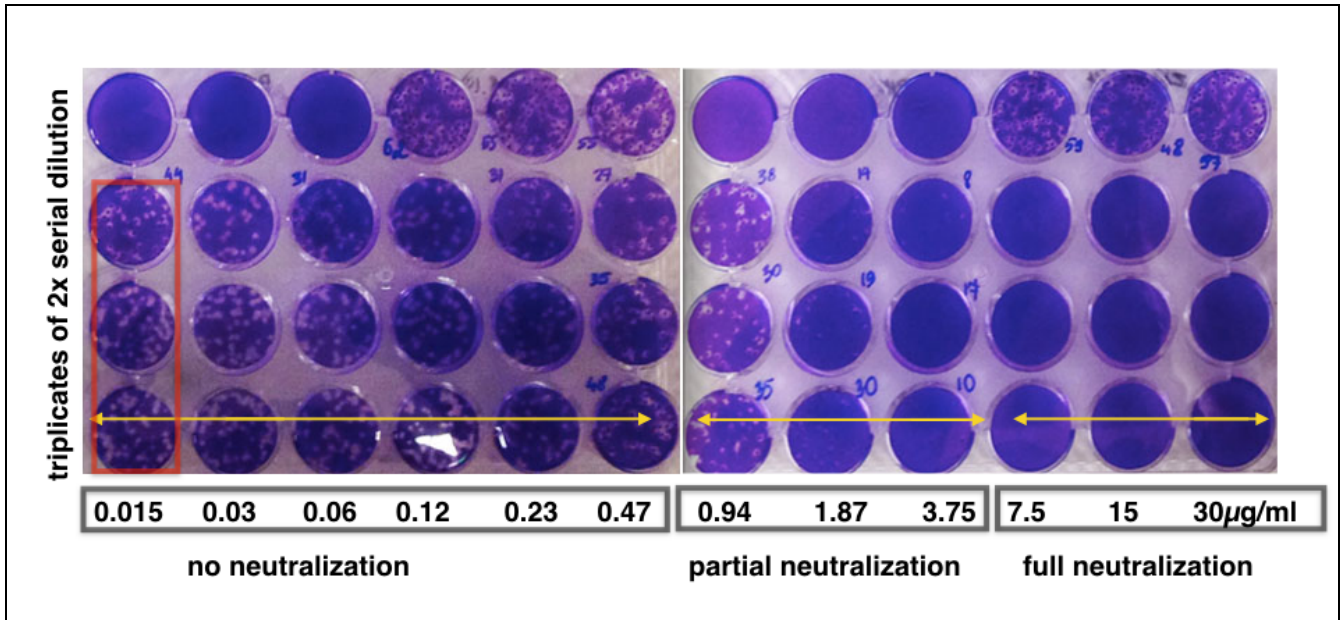
Figure 14. Plaque reduction neutralization test (PRNT).

In vitro neutralization activity of the constructs - sample setup used for testing neutralization characteristics of antibodies. The antibodies were serially diluted and pre-incubated with the virus for 1 hour. Each sample of virus - antibody mixture was added onto the BHK cells in triplicates and incubated for 1 hour at 37°C. Each plate had a triplicate of the positive (infected cells without the addition of antibodies) and the negative (neat RPMI) controls. Three highest concentrations of antibody resulted in the complete neutralization of the virus and no plaques were seen. The subsequent three dilutions of the antibody resulted in the partial neutralization with reduced number of plaques compared to the positive control. The antibodies at the concentrations 0.47 µg/ml and below do not neutralize the virus *in vitro.*

3.7.3 An influence of the molecular modifications of 14C10 on the ADE effect *in vitro*

The following procedure was carried out to check for the homotypic ADE of the engineered constructs in comparison to 14C10 WT. The K562 cell line (suspension cells cultured in RPMI supplemented with 10% FBS) was prepared beforehand. MOI of 0.1 was used (MOI= number of virus/number of cells = 0.1). The stock virus was thawed and diluted to the pre-determined amount. Virus suspension was distributed at 300μl per Eppendorf tube. Two positive (infected cells) and two negative (uninfected cells) controls (one for each 24-well plate - Nunc) were prepared. Twelve dilutions of an antibody (4-fold serial dilution, starting at 150μg/ml) were prepared. The serial dilution of an antibody was transferred into the tubes with the virus, and incubated at RT for 1 hour. Meantime the K562 cells were pelleted at 6000-8000 RPM for 5 minutes. The cells were re-suspended in media and counted. Cells were added to virus-antibody mixture and incubated for 1 hour at 37$^{\text{o}}$C. Afterwards the cells were pelleted down at 6000-8000 RPM for 5 minutes and the supernatant was discarded. Cells were washed twice with PBS, and re-suspended in 350μl RPMI supplemented with 2% FBS (for maintenance and expansion of cells) and transferred to 48-well plate and cultured for 48 and 72 hours. After the incubation period, cells were centrifuged at 10000 rpm for 5 minutes and the virus titre of the supernatant was quantified by plaque assay in BHK cells. The level of ADE was estimated by the number of infectious plaques in BHK monolayer, in the plaque-forming units (PFU) per milliliter (ml).

3.8 Setting up the *in vivo* model for defining the role of FcγRs in the Dengue virus neutralization and clearance.

The NUS Institutional Animal Care and Use Committee (IACUC) approved all the animal tests performed in this study. Mice were maintained in a pathogen-free environment in the ABSL-2 facility at the National University of Singapore. To minimize discomfort, stress and pain to an animal, all the *in vivo* procedures were performed under isoflurane anesthesia. The blood was collected from the jugular vein as the most recommended and the least invasive blood collection method (Hoff J 2000). Furthermore this method allowed us to withdraw blood as often as needed, according to the approved method and protocol. The isoflurane anesthesia allowed us to ensure the correct positioning of an animal. To minimize the changes within the blood vessels and the tissues surrounding them, the jugular veins at the alternate sides were used for the subsequent blood withdrawal. Each *in vivo* experiment was started with the blood withdrawal to carry out the control measurements of the viremia and the antibody levels.

3.8.1 Infection of the mice

To assess the most efficient route of an infection, the comparison of the viral titers after the subcutaneous and intra peritoneal injection was carried out. The subcutaneous infection with the same viral titer delivered higher viral blood loads than intra peritoneal infection, thus it was decided to utilize this route of infection in all the *in vivo* experiments of the project. The eight-to-nine week old AG129 mice were chosen as the most susceptible to DENV

infection and they were inoculated subcutaneously (s.c.) with 200µl of culture of a supernatant containing DENV1 EHI (genotype 1) at 10^6 PFU/ml or DENV1 WP74 (genotype 4) at 10^7 PFU/ml.

3.8.2 Treatment of mice

To decide about the route of an antibody treatment delivery, the following experiment was carried out. The mice infected s.c. with DENV1 WP74 (genotype 4) at 10^7 PFU/ml were treated with the 14C10 WT antibody s.c. or i.p. The results proved no significant difference in the viremia levels within the two groups, thus it was decided that the antibody would be delivered via intra peritoneal route as an experimentally easier procedure. The aspiration ensured that the antibody treatment was delivered into the right compartment of the mouse body, given only if no blood/urine or any other fluid was aspirated. The antibodies were tested at the following concentrations 30µg/mouse as the therapeutic/fully neutralizing concentration established by previous study (Teoh, Kukkaro et al. 2012), 10µg/mouse, 6µg/mouse and 1µg/mouse as the testing and sub-neutralizing concentrations in order to detect the differences between various constructs of the 14C10 antibody. The treatment schemes appropriate for our studies were established.

3.8.3 Establishing the therapeutic scheme for the *in vivo* model

Each group consisted of four to five AG129 mice. To control the initial levels of antibodies, the blood samples were taken before an infection. Afterwards, the mice were inoculated subcutaneously with 200µl of viral suspension containing 10^6 PFU/ml DENV1 EHI or 10^7 PFU/ml DENV WP74. Two days later, the mice were treated intraperitoneally with the 14C10 antibody. The PBS control group was injected with 200µl of sterile PBS and one group of mice was kept naïve. The blood was taken via the jugular bleeding at the following days: 3, 4, 5, 9, 15 and 29. A month after the initial infection, the mice were re-challenged with the same amount of DENV1 EHI or DENV WP74 as previously. At that time, the naïve control group was infected for the first time. Subsequently 3 and 5 days later, the blood was obtained from the jugular vein of the anesthetized mice.

3.8.4 Establishing the prophylaxis scheme for the *in vivo* studies

Each group consisted of five to six AG129 mice. To control the initial level of antibodies, the blood samples were first taken before the infection. The mice were first treated intraperitoneally with 14C10 antibody per mouse. The PBS control group was injected with 200µl of sterile PBS instead of an antibody. One day later, the mice were inoculated subcutaneously with 200µl of DENV1 EHI or DENV WP74 at 10^6 PFU/ml or 10^7 PFU/ml respectively. One group of mice was kept naïve (non-treated and non-infected). Next, the blood was taken on day 3, 4, 5, 9, 15 and 29. A month after the initial infection, all the mice were re-challenged with the same amount of DENV1 EHI or DENV WP74. The naïve control

group was infected for the first time at this point of time. Consequently, the blood was collected two and five days after re-challenge.

3.8.5 Virus quantification of plasma of infected mice

To assess the levels of the DENV1 and DENV2 viremia in the mouse serum 10, 20, 30 and 40-fold dilutions or four 10-fold dilutions respectively of the samples were prepared. 100µl aliquots were inoculated onto BHK cell monolayer seeded on 24-well plates (Nunc) 24 hours earlier. Each sample was added in quadruplicates and incubated for 1 hour at 37ºC before carboxymethyl cellulose (CMC) 1% (w/v) with 2% FBS was added. After 5-7 days of the incubation, BHK cells were fixed and stained with crystal violet in PFA and the plaques were counted. The titer of the virus was calculated in plaque-forming units (PFU) per ml.

3.8.6 Collection of the organs

After the *in vivo* experiment was completed, all the mice were sacrificed and the organs were collected for assessing the viremia in the organs. The tissue samples were snap-frost in the liquid nitrogen and stored at -80°C.

3.8.7 ELISA binding assays for the assessment of the antibody levels in the mouse serum

3.8.7.1 <u>Direct ELISA</u>

To measure levels of 14C10 in mouse serum, 96-well plates (Nunc, Maxisorp) were coated with novel anti-idiotype antibody engineered and generated in our lab, namely E1 (anti-HM14C10) at 5µg/ml, 50µl/well overnight. Plates were washed once with PBST 0.05% and twice with PBS and blocked with 4% (w/v) skim milk (Sigma)/PBS at RT for 2 hours. Mouse serum samples diluted 1:10 in 2% (w/v) skim milk were added into the wells, 50µl/well and incubated for one hour at RT. Plates were washed once with PBST 0.05% and two times with PBS. Goat anti-human IgG Fc cross-adsorbed (minimal cross-reactive with mouse serum proteins) secondary antibody, HRP-conjugate (Pierce, diluted 5000-fold in 2% (w/v) skim milk was added and incubated for 1 hour. Plates were washed as described above and TMB substrate (Pierce) was added. The reaction was stopped with 0.1 M sulfuric acid. Absorbance was read at 450nm by spectrophotometer.

3.8.7.2 <u>Sandwich ELISA for the detection of mouse antibodies against DENV1</u>

To measure the levels of mouse anti-DENV1 antibodies in mouse serum, 96-well plates (Nunc MaxiSorp® flat-bottom) were coated with 14C10 at 5µg/ml overnight, at 50 µl per well. Plates were washed once with PBST 0.05% and two times with PBS and blocked with 4% (w/v) skim milk (Sigma)/PBS at RT for 2 hours. The strain and batch of the DENV, which was used for the mice inoculation, was added at 10^6 PFU/ml, 50µl per well. Plates were

washed once with PBST 0.05% and two times with PBS. Serum samples diluted 1:10 in 2% (w/v) skim milk were added into the wells at 50µl/well and incubated for one hour at RT. Plates were washed three times with PBST 0.05%. Goat anti-mouse IgG (H+L) cross-adsorbed (minimal cross-reactive with human serum proteins) secondary antibody, HRP-conjugate (Pierce, 1:5000 in 2% (w/v) skim milk) was added and incubated for 1 hour. Plates were washed as described above and TMB substrate (Pierce) was added and the reaction was stopped with 0.1 M sulfuric acid. Absorbance was read at 450nm by spectrophotometer.

3.8.7.3 Sandwich ELISA for the detection of mouse antibodies against PR8

To measure the levels of mouse anti-PR8 antibodies in mouse serum, 96-well plates (Nunc MaxiSorp® flat-bottom) were coated with of human HA4 IgG1 antibody at 5µg/ml (50 µl per well) at 4°C overnight. Plates were washed once with PBST 0.05% and two times with PBS and blocked with 4% (w/v) skim milk (Sigma)/PBS at RT for 2 hours. The strain and batch of the PR8, which was used for the mice inoculation, was added at 10^6 PFU/ml, 50µl per well. Plates were washed once with PBST 0.05% and two times with PBS. Serum samples diluted 1:10 in 2% (w/v) skim milk were added into the wells at 50µl/well and incubated for one hour at RT. Murine HA4 IgG1 antibody in PBS starting with 40ug/ml with 2-fold dilution (50 µl per well) was a positive control. Plates were washed three times with PBST 0.05%. Goat anti-mouse IgG (H+L) cross-adsorbed (minimal cross-reactive with human serum proteins) secondary antibody, HRP-conjugate (Pierce, 1:5000 in 2% (w/v) skim milk) was added and incubated for 1 hour. Plates were washed as described above and TMB

substrate (Pierce) was added and the reaction was stopped with 2M sulfuric acid. Absorbance was read at 450nm by spectrophotometer.

3.9 Assessment of the total DENV1 in mouse serum

Serum samples were tested for the presence of infective viral particles with plaque assay. To compare neutralization versus clearance we needed to utilize an assay, which has the capacity to measure the total viral component of the serum including non-infective opsonized or fragmented viral particles. Thus, we carried out the real-time reverse transcriptase Polymerase Chain Reaction or quantitative PCR (real time RT-PCR or qPCR). The assay protocol was based on the previously published research papers (Lai, Chung et al. 2007, Gurukumar, Priyadarshini et al. 2009, Leparc-Goffart, Baragatti et al. 2009), which described the development of a quantitative real-time PCR assay for the detection of various Dengue serotypes in the serum from patients in the early stage of the disease.

3.9.1 DENV1 RNA extraction from mouse serum

Total nucleic acid extraction from the viral standard was performed using the DNA and Viral NA Small Volume kit (sample volume 200µl, elution volume 100µl) and the Viral NA Universal SV protocol on the MagNA Pure 96 Instrument (Roche Diagnostics). The plasmid standard constructed according to Lai *et al.* (2007) was a kind gift from Dr Eng Eong Ooi. For extraction of the plasmid standards (10^6 to 10^0 copies/µl) 100µl of the standard was added to 100µl PBS before extraction. For extraction of viral standards (10^5 to 10^1 PFU/ml)

standards were made in naïve mouse serum at 50µl then brought to 200µl with PBS before extraction.

3.9.2 Quantitative real-time PCR assay for DENV1

The PCR assay described in Lai et al. (2007) utilized a set of FRET probes (Den1-FL and Den1-LC) and two primers (Pan-dengue forward and Pan-dengue reverse) for the detection and serotyping of DENV1. However, the probe chemistry and PCR platform used in the study was not compatible with the existing PCR platform. Therefore, DENV1-FL fluorescent probe was modified into a dual-labeled probe called pan Den-Den1-P(5'-FAM-CAGGATACAGCTTCCCCTGGTGGTG-BHQ1-3') to be used with the same two primers for 5' nuclease PCR assay. This was followed by sensitivity testing to ensure that the modified PCR assay was suitable for subsequent use. The PCR set up was performed using

SuperScript III$^{®}$ One-Step RT-PCR System with Platinum$^{®}$ Taq DNA, which consisted of 1x Reaction Mix, 0.4µM of primers, 0.3µM of probe and 5µl of template in a total reaction volume of 20µl. Reverse transcription was carried out at 60°C for 15 min followed by Taq polymerase activation at 95°C for 2 min. A 45-cycle PCR amplification was performed with denaturation at 95°C for 5s, annealing and extension combined at 60°C for 45sec. The fluorescence emitted from the assay was captured at the end of extension phase of each cycle and the results analysed with the ABI 7500 Fast PCR System software version 2.0.5.

4 Chimerization and rational mutation of a human monoclonal antibody with potent Dengue 1 neutralizing activity

4.1 Background of the study

Whilst the Fab portion of an antibody determines its specificity, affinity and neutralizing potential, the constant region plays an important role in the outcome of an interaction between an antibody and an antigen (DiLillo, Tan et al. 2014, Mak, Hanson et al. 2014).

Based on elegant studies published in Nature and Science there was an assumption that the mutations of 14C10 would have an impact on the activity in vivo. In these studies of influenza infection, neutralizing antibodies required FcgR interactions for protection against influenza virus in vivo. Mice were treated with fully functional and mutated versions of anti-influenza antibody FI6 at 10 mg/kg or 3mg/kg. LALA mutation compromised the neutralization of an antibody suggesting that Fc mediated functions are very important to clear the virus from the system (Corti, Voss et al. 2011).

In another publication by DiLillo *et al.* mouse IgG variants and mutants were used to examine the role of FcγRs in the antibody-mediated neutralization of influenza virus. Only IgG2a antibody protected mice from weight loss and death. IgG1 antibody (which binds pref to inhibitory FcgR), D265A antibody and PBS treated animals were not protected. Hence, binding of activating FcγRs was required for antibody-mediated protection (DiLillo, Tan et al. 2014). Here, we designed constructs of 14C10, a fully human antibody that potently neutralizes DENV1 (Teoh et al. 2012), to study the effect of these mutations on the DENV1 protective efficacy.

4.2 Engineering and expression of 14C10 with altered Fc-region functionality

4.2.1 Human 14C10 WT antibody and its LALA mutant

The 14C10 WT antibody is a fully human monoclonal antibody with potent neutralizing activity against DENV serotype 1 (Teoh et al 2012). It does not trigger heterotypic antibody dependent enhancement (ADE) *in vitro*, but can mediate homotypic ADE at sub-neutralizing concentrations (Teoh, Kukkaro et al. 2012). ADE is hypothesized to happen when antibodies at low (sub-neutralizing) concentrations create complexes with the virus that lead to increased cell entry via FcγRs (Hu, Thoens et al. 2013). To determine the interactions between the Fc domain of the 14C10 and the FcγRs, we designed a human mutant antibody 14C10hG1LALA, which does not bind to human FcγRs. In the 14C10hG1LALA mutant, the leucine L234 and L235 in the CH2 domain are substituted with two asparagines (Figure 16). These substitutions abolish binding to the human FcγRs, which could reduce or eliminate the potential homotypic ADE effect (Balsitis, Williams et al. 2010, Williams, Sukupolvi-Petty et al. 2013, Arduin, Arora et al. 2015).

4.2.2 Mouse-human chimeric G1 construct and its D265A mutant (mG1D265A)

The mouse IgG1 antibody preferentially binds to low affinity FcγRs. In the mouse-human chimeric 14C10mG1 antibody (14C10-hCH1mG1HCH2-3) the hinge, CH2 and CH3 regions are substituted with the mouse equivalent IgG1 antibody as projected in Figure 17A. The full light chain and the variable region of the heavy chain remain unchanged as compared to the 14C10 WT (Figure 15). The D265A mutant of the mouse-chimeric IgG1 antibody (hCH1mG1HCH2-3-D265A) is a human-mouse chimeric equivalent of the 14C10-hIgG1LALA that is null for FcγR binding. The hCH1mG1HCH2-3-D265A construct was originally created for a study which used alanine scanning across the entire mIgG1 constant region to identify a mutant with defective FcγR binding (Clynes, Towers et al. 2000). The mutation is located within CH2 and abrogates FcγR binding in mIgG2a and mIgG2b (Baudino, Shinohara et al. 2008). The hCH1mG1HCH2-3-D265A point mutation substitutes aspartic acid with alanine (Figure 17B). This results in a complete loss of interaction with FcγRs (Nimmerjahn, Bruhns et al. 2005, Tan, Leon et al. 2016).

4.2.3 Mouse-human chimeric G2a and G2aEXT (mG2a and mG2aEXT)

To evaluate the effect of antibody subclasses on virus neutralization and the ability to trigger homotypic antibody dependent enhancement *in vivo*, an IgG2a mouse variant of the 14C10 antibody was produced (Figure 20C). IgG2a mouse antibodies bind all FcγRs (Nimmerjahn and Ravetch 2008). The mouse-human 14C10 hCH1mG2a antibody consists of human CH1, followed by mouse hinge and mouse CH2 and CH3 (hCH1mG2aHCH2-3). The purification process showed that the chimeric IgG2a construct was not folding properly (Figure 20C). Therefore, we exchanged the mouse hinge with a human hinge. Eventually the mouse-human chimeric G2aEXT antibody with the human hinge (hCH1mG2aHCH2-3EXT-14C10) was qualified as a suitable construct for further testing. It consists of human CH1, followed by human hinge, mouse CH2 and CH3 and it fully bound to FcγRs (Figure 20D).

4.3 Sequence analysis of 14C10 WT antibody and its human and chimeric constructs

The sequences of 14C10 WT and its constructs made in this project are shown in Figure 15 and Figure 18.

4.3.1 Sequence of fully human 14C10 WT antibody
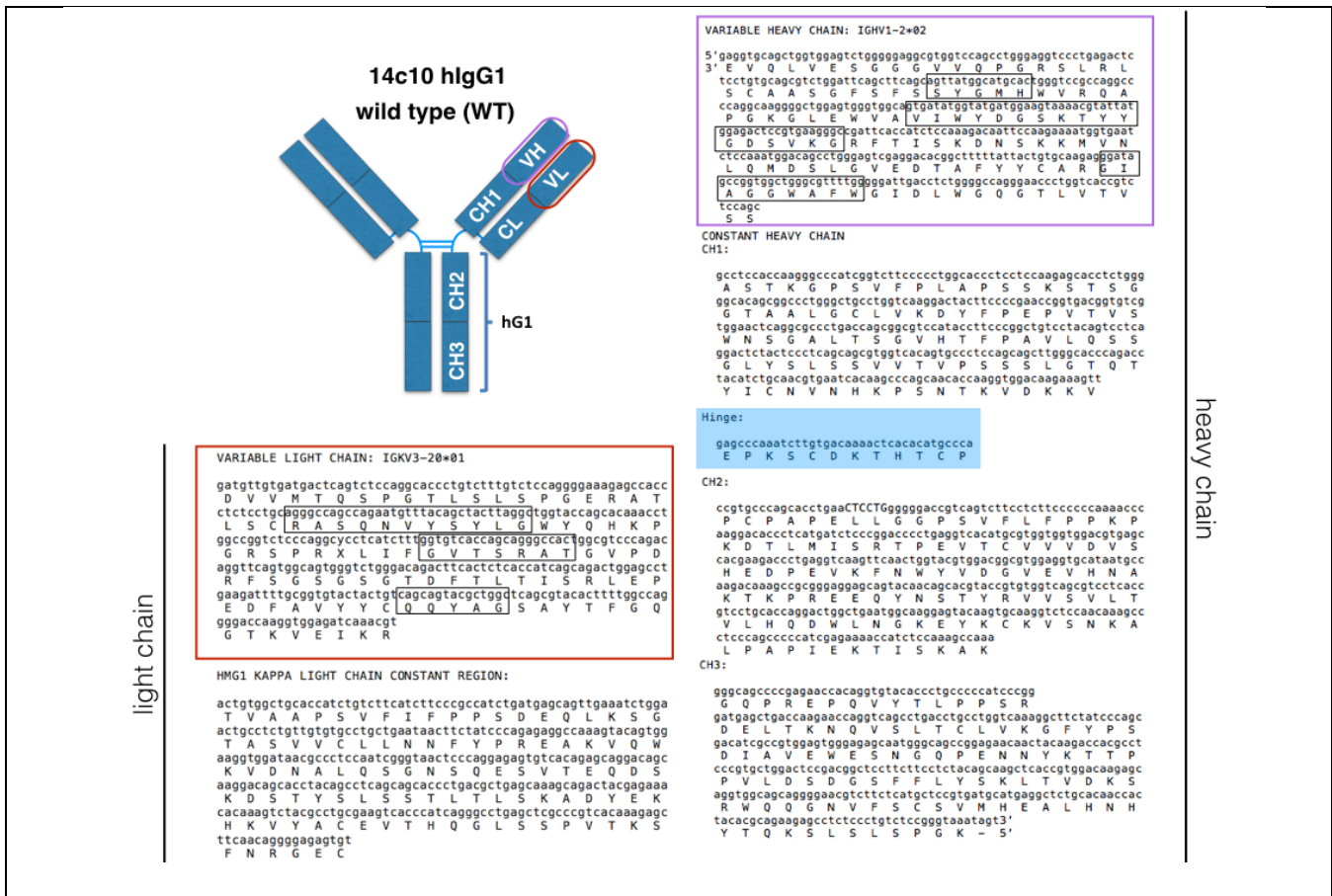
Figure 15. Sequence of 14C10 hG1 wild type (WT) antibody.

The light chain of 14C10 hIgG1 consists of the variable light chain (VL) – highlighted by a red box and the standard constant kappa light chain (CL). The heavy chain of 14C10 hG1 consists of the variable heavy chain (VH) – highlighted by a purple box and the constant region of the standard heavy chain of the human IgG1 antibody including hinge – highlighted in blue. The complimentary determining regions (CDRs) VL and VH, namely CDR1, CDR2 and CDR3 which bind them to DENV1 are highlighted in black boxes. The nucleotide sequence is accompanied with the complimentary amino acid sequence underneath.

4.3.2 Sequence of the LALA mutant of the 14C10 antibody

The 14C10-hG1LALA antibody is a fully human antibody with an altered CH2 domain of the heavy chain. The LALA mutation substitutes two leucines with two alanines in the CH2 domain (Figure 16). The other regions of the heavy chain and the light chain remain unchanged as compared to 14C10 WT antibody (Figure 15).

Figure 16. Sequence of 14C10 hG1LALA construct.

Schematic of 14C10 hG1LALA presented with the sequence of mutated CH2 domain of the constant heavy chain (highlighted in red). The other parts of the antibody remain unchanged as compared to 14C10 WT (Figure 15).

**In the CH2 region of the heavy chain of 14C10 hG1LALA two leucines are substituted with two alanines. The LALA mutation abrogates binding to the FcγRs (Arduin, Arora et al. 2015).

4.3.3 Schematic and sequence of hCH1mG1 and hCH1mG1D265A mouse-human chimeric constructs of 14C10 antibody.

The hCH1mG1 (hCH1mG1HCH2-3) construct is one of the three chimeric (mouse-human) constructs of the 14C10 antibody used in this study. In the hCH1mG1 construct, the hinge, CH2 and CH3 regions are substituted with the mouse equivalents from mouse IgG1 (Figure 17A). The CH1 region and the variable region remain unchanged. The light chain is unchanged as compared to 14C10 WT antibody (Figure 15).

In the hCH1mG1D265A (hCH1mG1HCH2-3D265A) construct the light chain remains unchanged as compare to the 14C10 WT antibody (Figure 15). The hinge, CH2 and CH3 regions are substituted with the mouse equivalents from the mouse G1 and in the CH2 there is a point mutation at D265A (Figure 17B). In the heavy chain, the variable region and the CH1 region of the constant chain remain unchanged. In summary, this construct is the same as hCH1mG1 except the point mutation at D265A in the CH2 domain of the mouse IgG1.
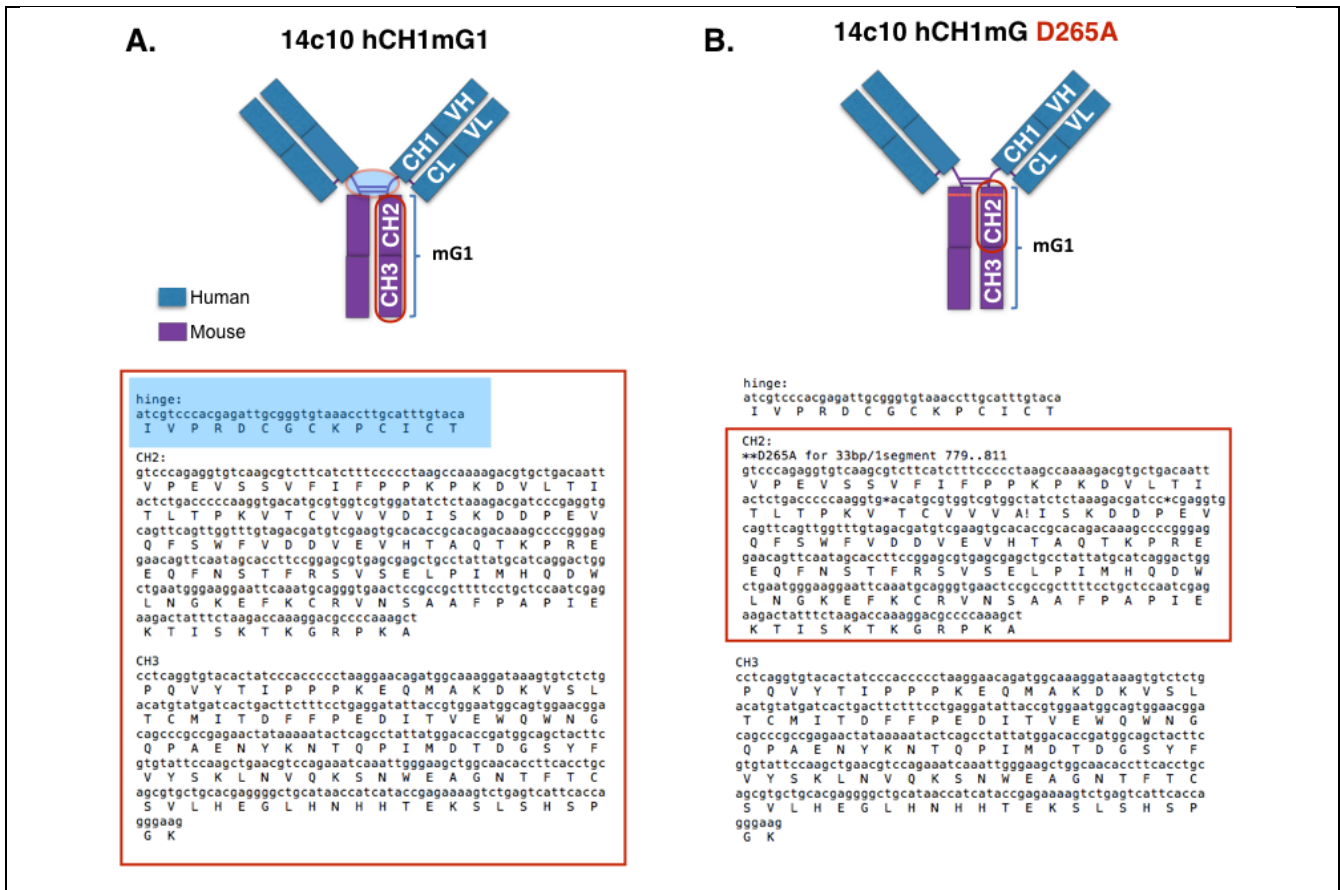
Figure 17. Sequences of 14C10 hCH1mG1 and 14C10 hCH1mG1D265A constructs.

A. The nucleotide sequence and the amino acid sequence of the G1 mouse-human chimeric variant of the 14C10 antibody (hCH1mG1HCH2-3). Red box: hinge, CH2 and CH3 regions are substituted with mouse equivalents. Mouse G1 hinge is highlighted in blue.

B. The sequence of the mouse IgG1-D265A mutant of the mouse IgG1 chimeric construct of the human 14C10 antibody (hCH1mG1HCH2-3-D265A). Red box: CH2 region with point mutation at the point mutation at D265A**.

4.3.4 Sequence of the mouse-human chimeric hCH1mG2a constructs of 14C10

In the hCH1mG2a (hCH1mG2aHCH2-3) antibody, the hinge and the CH2 and CH3 regions of 14C10 WT are substituted with mouse regions of the mouse IgG2a, the variable region, as well as the CH1 region of the heavy chain remain human. The light chain remains unchanged in full as compared to the 14C10 WT antibody (Figure 15). In summary hCH1mG2aHCH2-3 has the human CH1, followed by mouse G1 hinge, mouse CH2 and mouse CH3 regions (Figure 18).

In the hCH1mG2a_Ext (CH1mG2aHCH2-3_Ext), only CH2 and CH3 regions are substituted with the mouse IgG2a equivalents. Other parts of heavy change and the light chain remain unchanged as compared to 14C10 WT (Figure 15). In summary, the heavy chain consists of human CH1, followed by the human G1 hinge, then the mouse CH2 and CH3 (Figure 18).
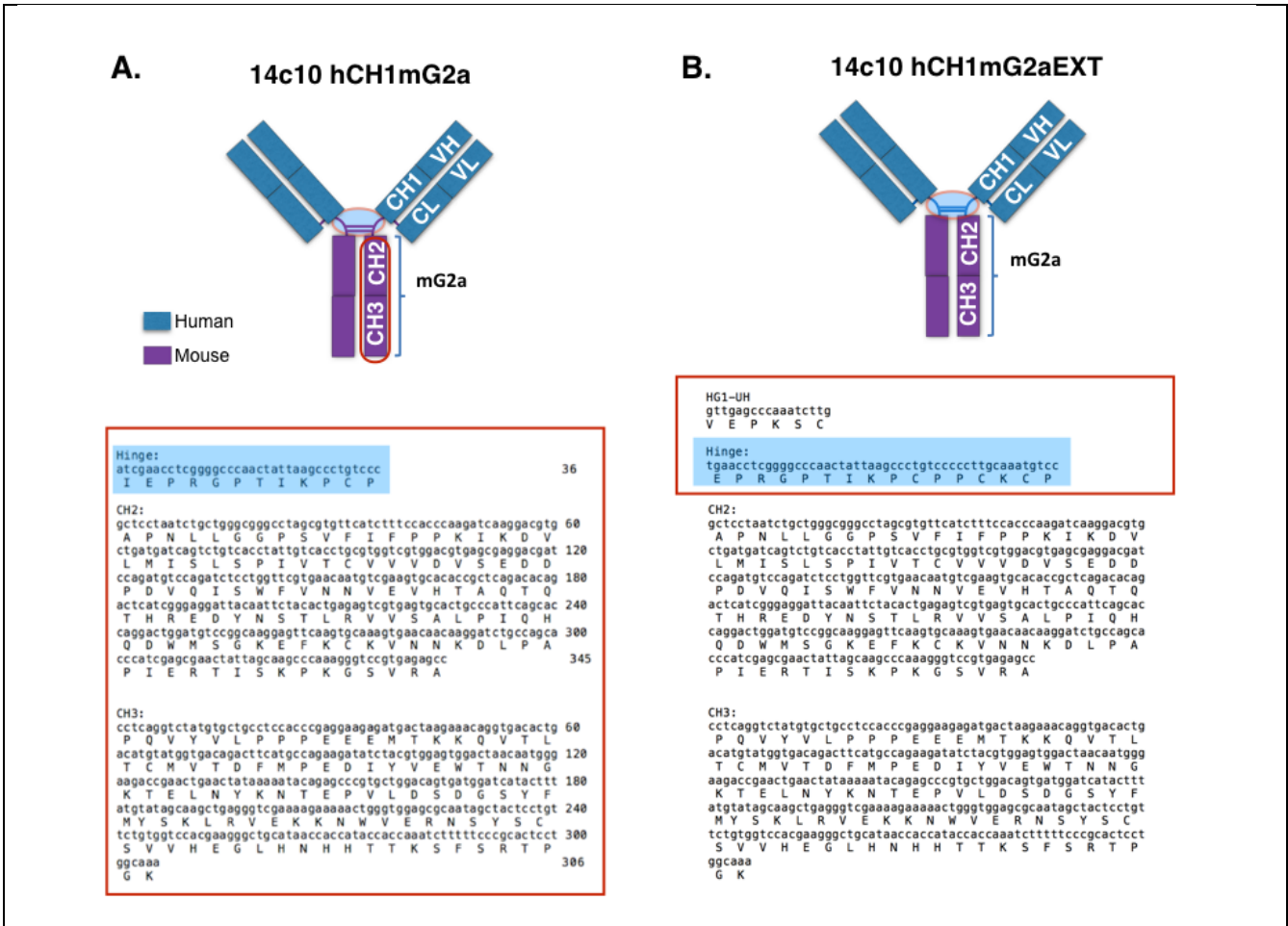
Figure 18. Sequences of 14C10 hCH1mG2a and 14C10 hCH1mG2a_Ext constructs.

The nucleotide sequence and amino acid sequence of CH2 and CH3 regions of mouse-human chimeric construct hCH1mG2a (hCH1mG2aHCH2-3). In hCH1mG2a, the CH2 and CH3 regions of the constant chain were substituted with mouse IgG2a equivalents (A. red box), and the hinge was substituted with a mouse G2a hinge (A. highlighted in blue). In hCH1mG2a_Ext, the human G1 hinge was left unchanged as compared to 14C10 WT (**B.** highlighted in blue).

4.4 Purity of the expressed constructs

The constructs were electrophoresed by 10% NuPAGE gel in order to check their purity (

Figure 19

Figure 19, Figure 20).

The hCH1mG2aHCH2-3 construct, which has human CH1, followed by the mouse G1 hinge, mouse CH2 and CH3 was found not to be folding properly as shown in Figure 20C. That is why its modification, hCH1mG2aHCH2-3_Ext, which has the human CH1, followed by the human hinge, then the mouse CH2 and CH3, was designed and produced (Figure 20D).

Eventually, four constructs qualified for further studies: 14C10-hG1LALA, hCH1mG1HCH2-3, hCH1mG1HCH2-3-D265A and hCH1mG2aHCH2-3_Ext.
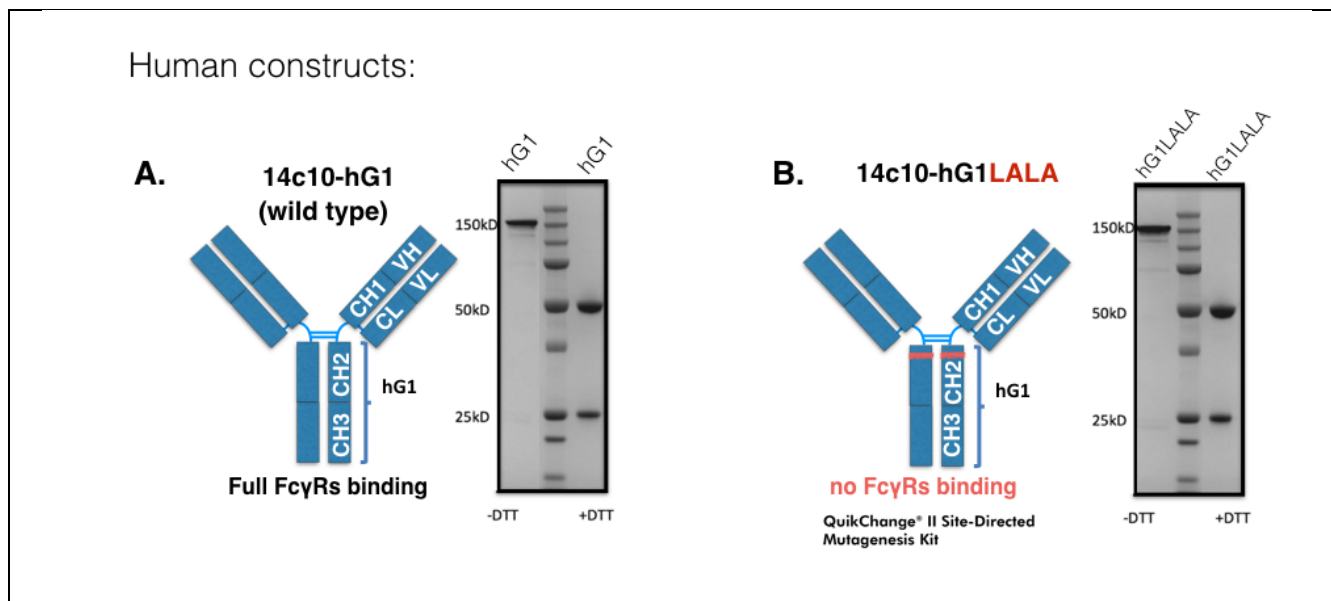
Figure 19. Schematics of human constructs (A. 14C10hG1 and B. 14C10hG1LALA).

Presence of the heavy and light chain bands at 150 kD (non-reducing conditions) or 50 kD and 25 kD (reducing conditions) when compared with the marker confirms proper folding of the respective parts of the antibodies.
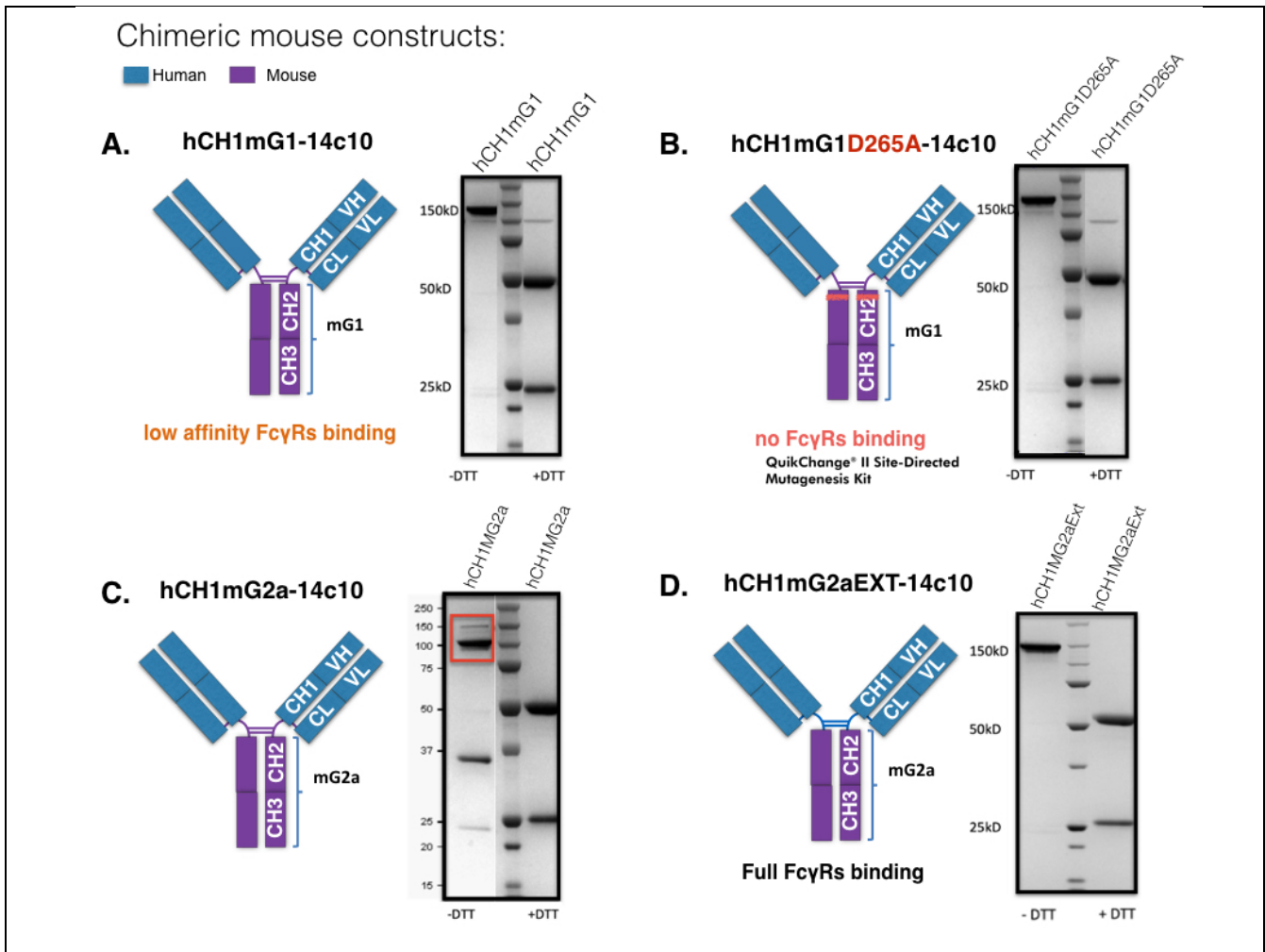
Figure 20. Schematics of mouse-human chimeric constructs A. hCH1mG1HCH2-3, B. hCH1mG1HCH2-3-D265A, C. hCH1mG2aHCH2-3, D. hCH1mG2aHCH2-3_Ext.

Standard gel electrophoresis of purified antibodies showed that construct hCH1mG2a was not folding properly as shown in the figure above (**C.** red box). The presence of the heavy and the light chain bands at 150 kD (non-reducing conditions) or 50 kD and 25 kD (reducing conditions) when compared with the marker for the antibody **A. B.** and **D.** confirm the proper folding.

4.5 Functional tests of the 14C10 antibody and its human and chimeric constructs

To confirm that all the changes that were made during the antibody production did not affect the binding-neutralization activity of the constructs I carried out multiple tests described below.

4.5.1 Binding specificity and affinity of the 14C10 antibody constructs

An ELISA assay was carried out to confirm DENV1-specific binding and to characterize binding affinities of the produced constructs. Binding affinities were compared to that of 4G2, a murine monoclonal IgG1 antibody specific for a Flavivirus group antigen or 3H5, a recombinant chimeric clone of the murine monoclonal IgG1 antibody, specific for DENV2. 14C10hG1LALA and the 14C10 WT antibody had similar binding affinities to DENV1. All mouse constructs had a higher binding affinity to DENV1 than the human 14C10 WT proving the retention of binding affinities in all the constructs generated. The DENV1 binding affinities of the constructs can be ranked as follows: hCH1mG1D265A > hCH1mG1 > hCH1mG2aEXT > 4G2 > hG1. None of the constructs bound to DENV2 (Figure 21).
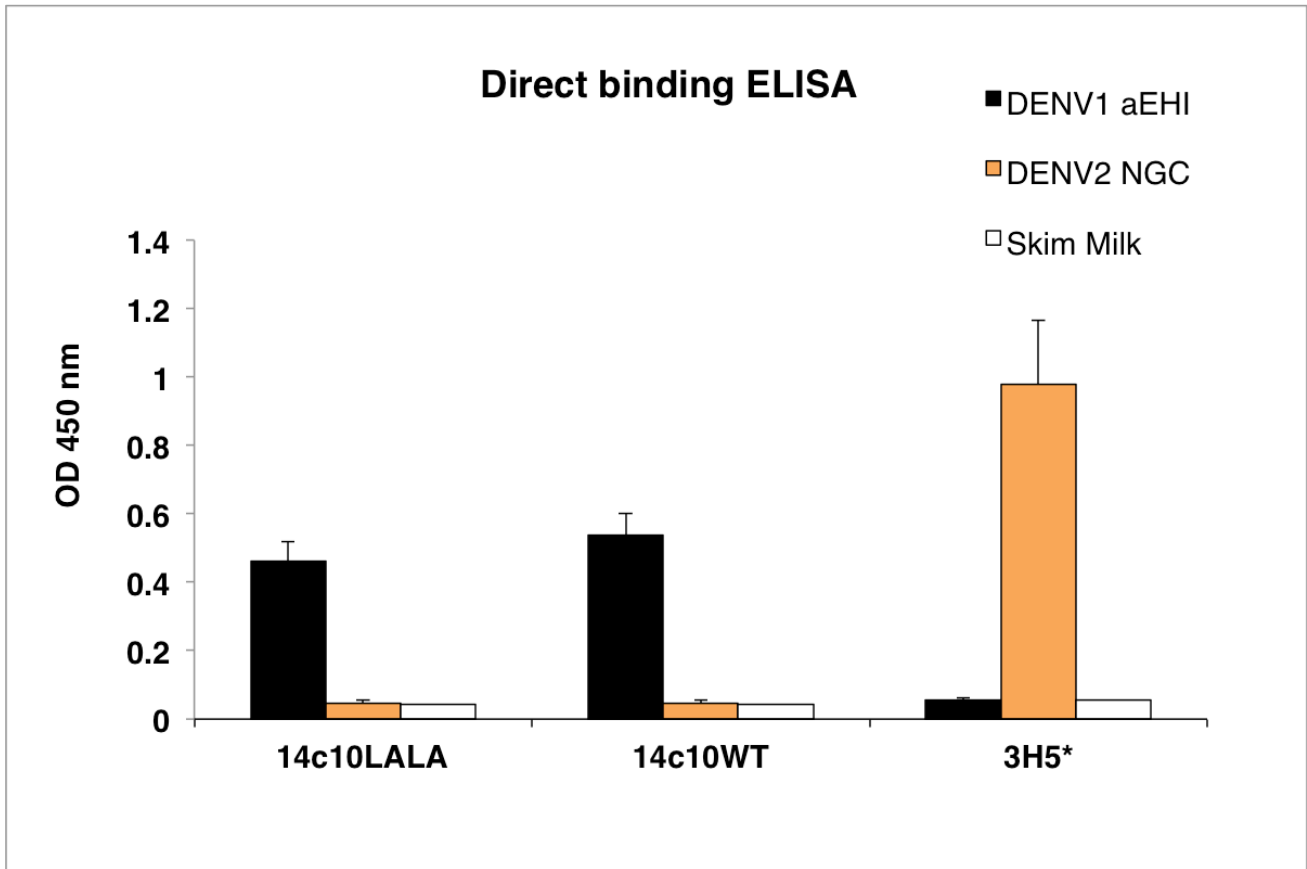
Figure 21. Binding specificity of the 14C10 hG1LALA construct versus 14C10 WT.

Binding specificity of 14C10 hG1LALA and 14C10WT were compared by direct ELISA assay. Purified DENV1 EHI or DENV2 NGC (specificity control) was coated on Nunc MaxiSorp® plates and 4% (w/v) skim milk (Sigma)/PBS was used as a negative control. After blocking with 4% (w/v) skim milk, samples of purified antibodies (14C10 WT/14C10hG1LALA/3H5) were applied onto the wells and incubated for 1 hour. 3H5* is an antibody specific for DENV2. Goat anti-mouse IgG (H+L) cross-adsorbed, HRP-conjugate (Pierce, 1:5000 in 2% (w/v) skim milk) was used as a secondary antibody. After final wash TMB substrate (Pierce) was added and the reaction was stopped with 0.1 M sulfuric acid. The reading of absorbance was taken at 450nm by spectrophotometer. Results are averaged from two independent experiments and error bars represent SD between experiments.
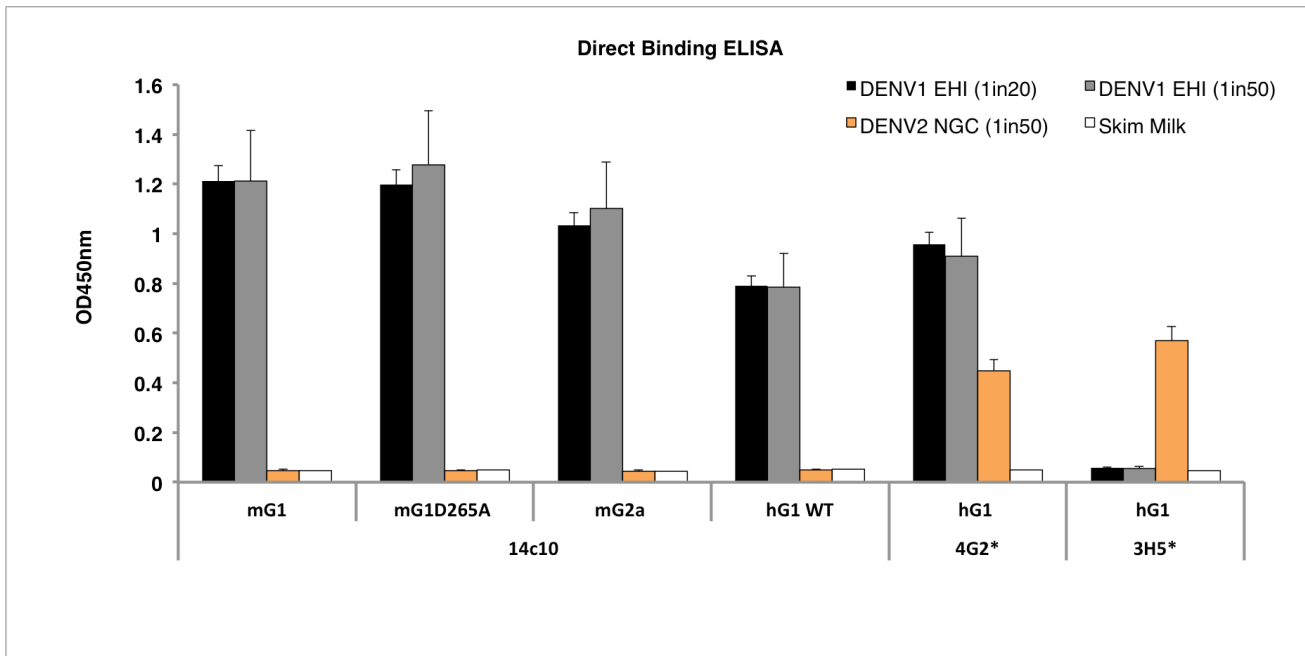
Figure 22. Binding specificity of the mouse-human chimeric constructs versus 14C10 WT.

Binding specificity of mG1, mG1D265A, mG2a mouse constructs of 14C10 antibody and the wild type hG1 14C10 were compared by direct ELISA assay. First, purified DENV1 EHI (dilutions 1 in 20 or 1 in 50) or DENV2 NGC (diluted 1 in 50) was coated onto Nunc MaxiSorp® 96-well plates at 50µl/well and incubated overnight at 4°C. The virus was washed off and the wells were blocked with 4% (w/v) skim milk at RT for 2 hours. Samples of purified antibodies were probed onto washed wells and incubated for 1 hour at RT. Then plates were washed again, and the anti-mouse HRP antibody or anti-human HRP antibody (1:5000) was added and incubated for 1 hour at RT. Subsequently, TMB substrate (Pierce) was added and the reaction was stopped with 0.1 M sulfuric acid. The reading of absorbance was taken at 450nm by spectrophotometer. 4G2* is a murine monoclonal IgG1 specific for Flavivirus group antigen; 3H5* is a recombinant chimeric clone of the murine monoclonal IgG1 specific for DENV2. Results are averaged from two independent experiments and error bars represent SD between experiments.
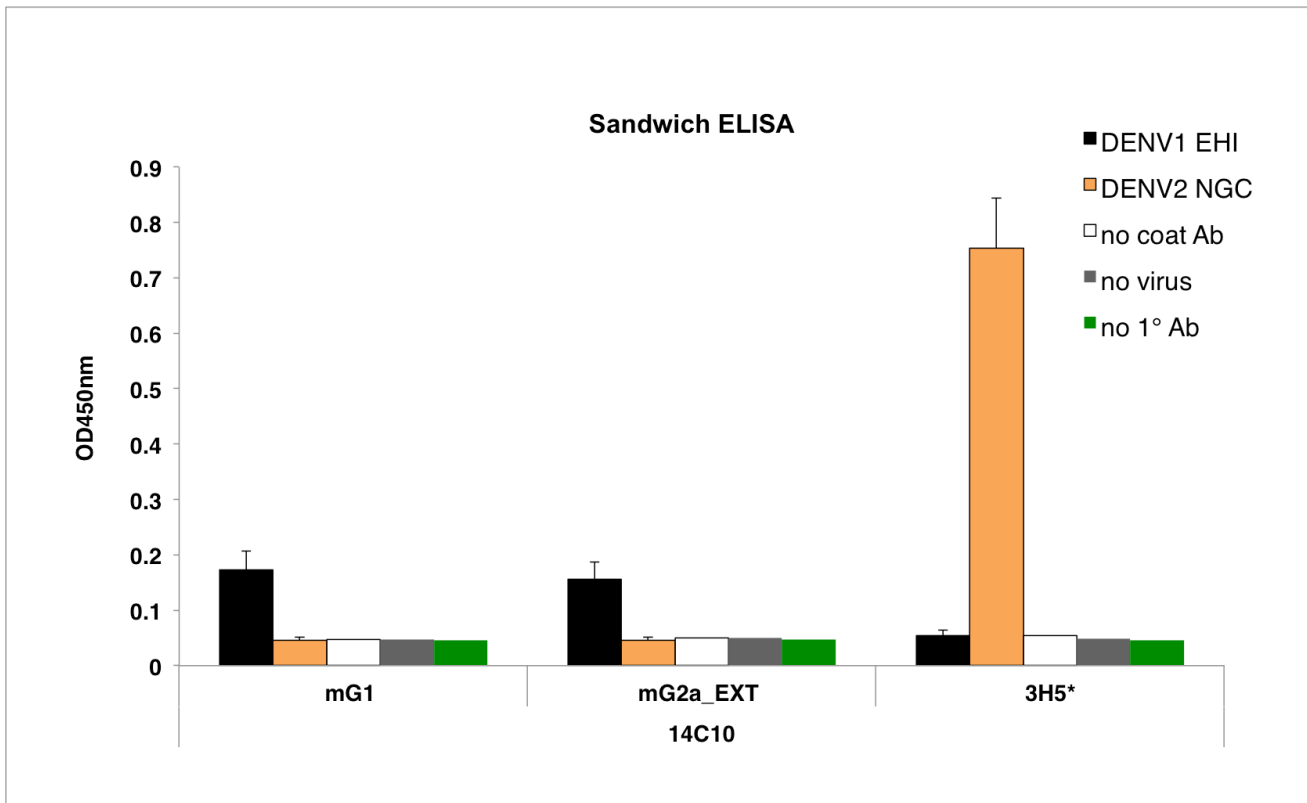
Figure 23. Binding specificity of mG2a_Ext and mG1 versus 14C10 WT.

Sandwich ELISA was carried out to check the binding characteristics of mIgG1 and mIgG2_aEXT. The primary antibody (human 4G2 IgG1) was coated at 5µg/ml, 50µl/well overnight at 4^oC onto Nunc MaxiSorp® flat-bottom 96-well plates. The wells were then washed once with PBST 0.05%, twice with PBS and blocked with 4% (w/v) skim milk (Sigma)/PBS at RT for 2 hours. Then non-purified DENV1 EHI or DENV2 NGC was added at 10^6 PFU/ml in 4% skimmed milk, 50µl/well. After an incubation of 1 hour at RT, the wells were washed and probed with the samples of purified antibodies. The 3H5* antibody used as control, is a murine monoclonal recombinant chimeric clone of the murine monoclonal IgG1 specific for DENV2. Goat anti-mouse IgG (H+L) cross-adsorbed (minimal cross-reactive with human serum proteins) secondary antibody, HRP-conjugate (Pierce, 1:5000 in 2% (w/v) skim milk) was added 50µl/well and incubated for 1 hour. After the final triple wash, TMB substrate (Pierce) was added at 50µl/well and the reaction was stopped with 0.1 M sulfuric acid 50µl/well. Absorbance was read at 450nm by spectrophotometer. Results are averaged from two independent experiments and error bars represent SD between experiments.

4.5.2 Analyzing the neutralization activity of the engineered 14C10 constructs

Neutralizing activities of the engineered constructs of 14C10 were tested against DENV1 EHI (Genotype 1) and DENV1 WP74 (Genotype 4) as shown in Figure 24 to 27. First, to determine an influence of the LALA mutation on the neutralization based on the Fab–virus binding, PRNT was carried out in BHK cells using 4-fold serial dilution of the antibodies with 30 µg/ml as the highest concentration. The results expressed as the percentage neutralization showed no significant differences between the neutralization activity of the LALA mutant versus WT antibody. We next tested the neutralization potency of the mouse-human chimeric IgG1 and its D265A mutant as well as the mouse-human chimeric IgG2a. All tested constructs of 14C10 exhibited neutralization activity for EHI and WP74 genotypes of DENV1.

antibody	EHI - $PRNT_{50}$ (µg/ml)	WP74- $PRNT_{50}$ (µg/ml)
14C10WT	1.97	0.35
14C10hG1LALA	1.72	0.43
14C10mG1	0.78	0.30
14C10mG1D265A	1.11	0.40
14C10mG2a	1.14	0.28

Table 1. The concentration of an antibody needed to reduce the number of plaques by 50% is termed the $PRNT_{50}$ value. The $PRNT_{50}$ values (µg/ml) presented in the table were determined with Prism by non-linear regression. Values represent an average of triplicates results.

There was no significant difference between neutralization activities of all the mutants and variants of 14C10 versus WT antibody. The results demonstrate that *in vitro* neutralization based on the Fab–virus binding is maintained in all constructs.
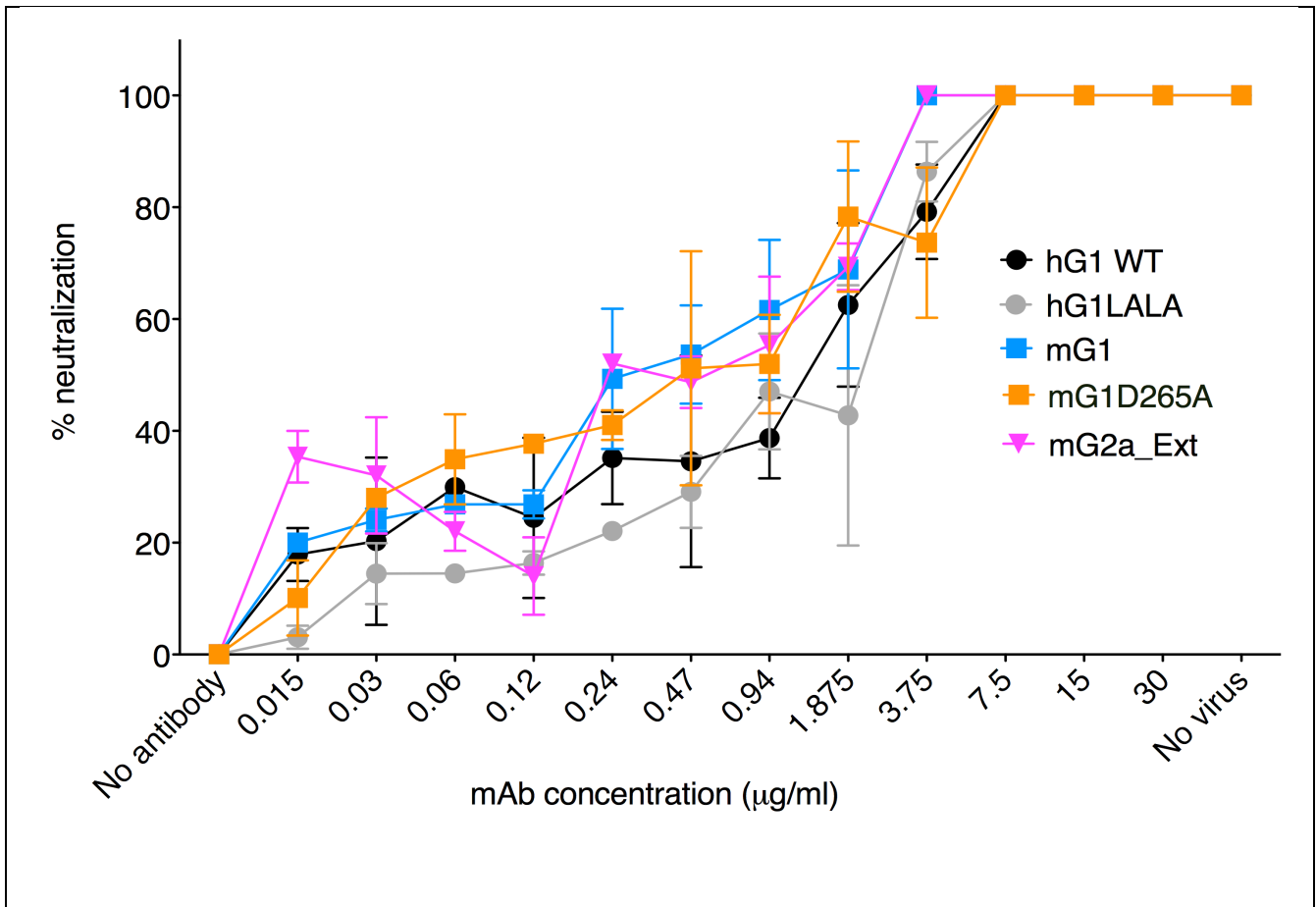
Figure 24. Neutralizing activities of 14C10 constructs for DENV 1 isolate <u>EHI</u> genotype 1.

A standard PRNT assay was carried out. Serially diluted 14C10 antibody was incubated with DENV1 EHI for 1 hour at RT and applied onto BHK cells for an additional hour at 37°C. The cells were covered with carboxymethyl cellulose for a 5-days incubation at 37°C. Afterwards, cells were fixed and stained with PFA and crystal violet. The plaques were counted. The $PRNT_{50}$ values were calculated using nonlinear regression on GraphPad Prism. Data points represent the average of three independent experiments performed in triplicates. Error bars represent SDs.
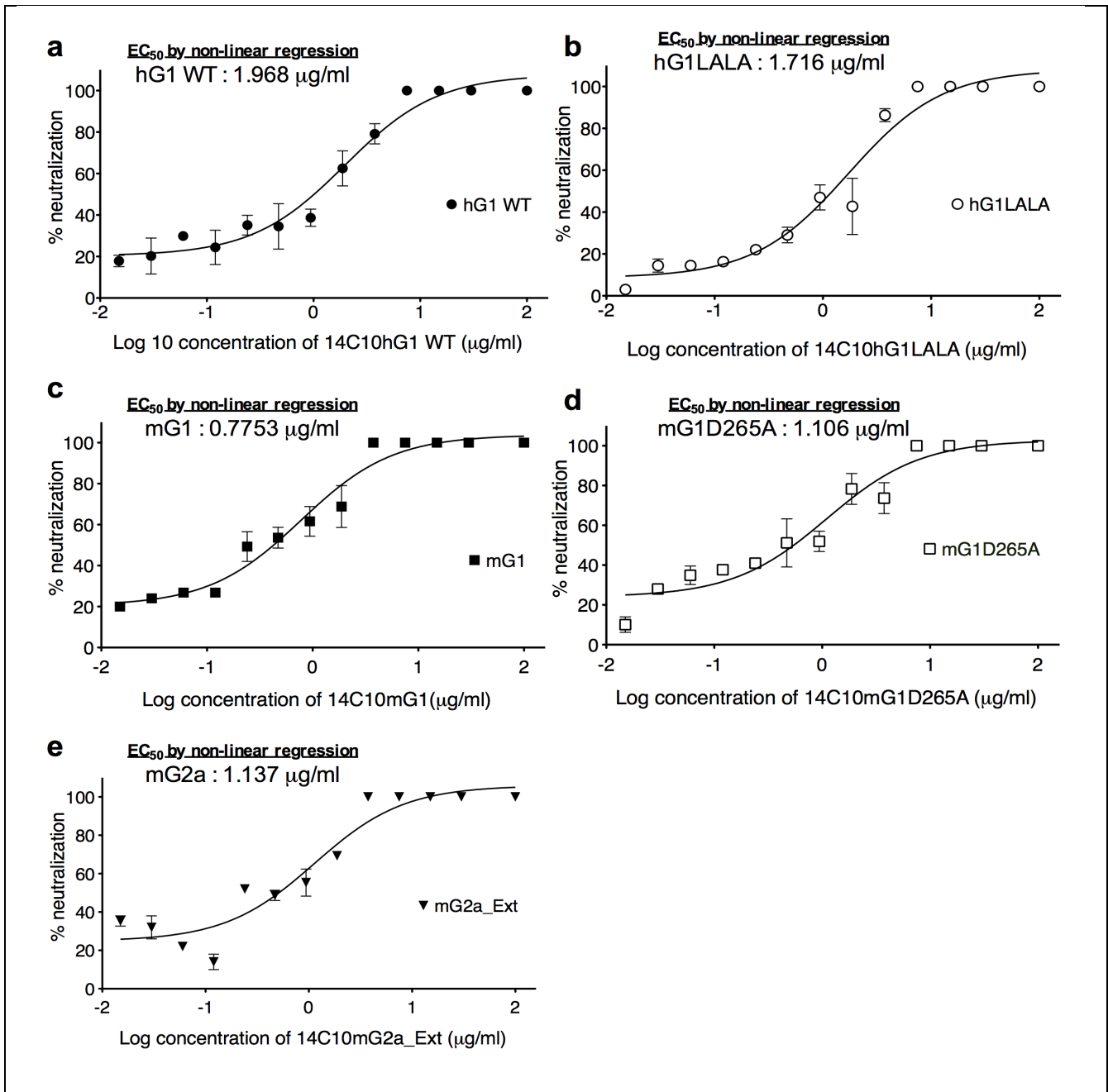
Figure 25. Comparison of neutralization activities of 14C10 constructs for DENV1 EHI *in vitro.*

(**a**) 14C10 WT (**b**) 14C10hG1LALA, (**c**) 14C10mG1, (**d**) 14C10mG1D265A, (**e**) 14C10mG2aEXT. Data points represent the average of at least 2 independent experiments performed in triplicates. Error bars represent SDs.
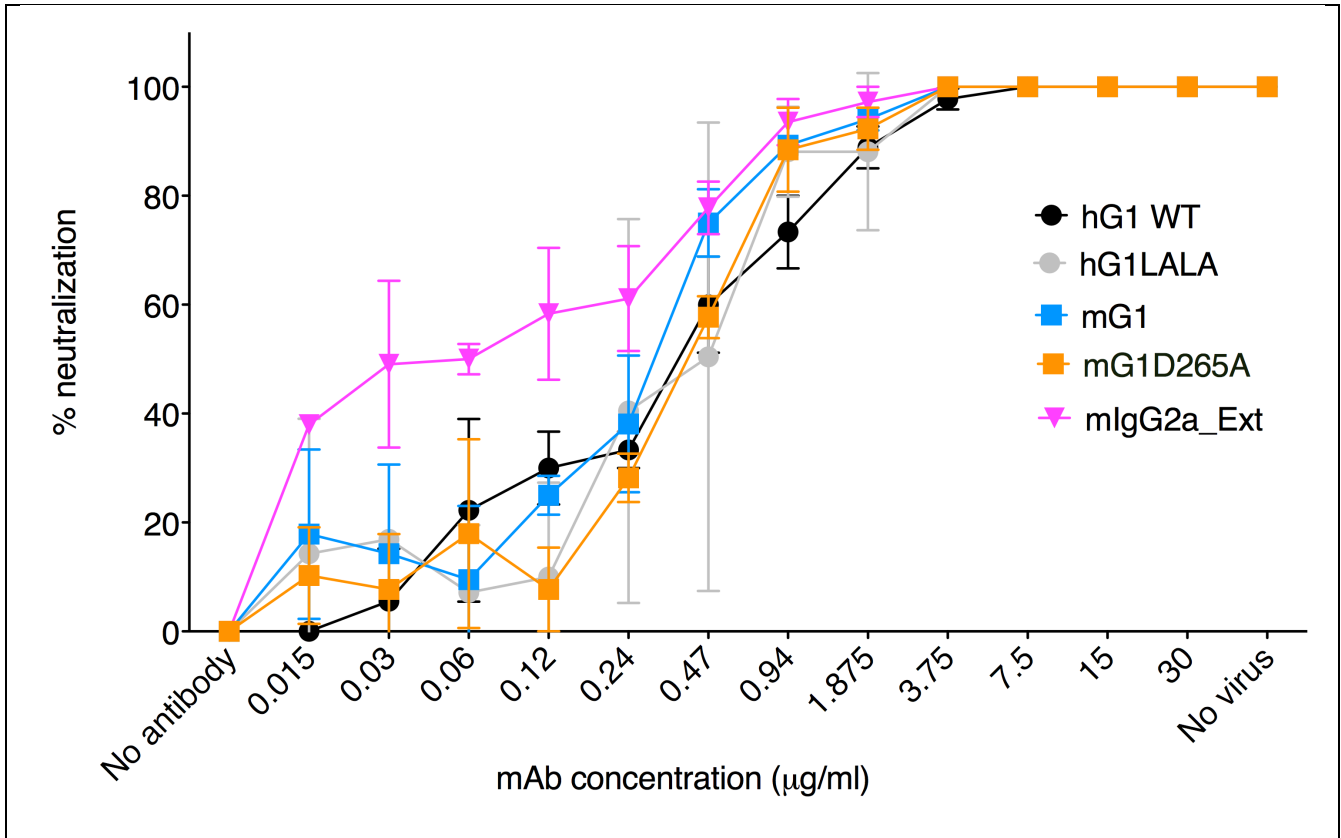
Figure 26. Neutralization profile of 14C10 constructs for DENV1 isolate <u>WP74</u> genotype 4.

The results show small but not significant differences in neutralization activities of five 14C10 constructs. All the constructs neutralized DENV1 WP74 to varying degrees. This shows that *in vitro* neutralization based on the Fab – virus binding is maintained regardless of 14C10 construct tested. The results are representative of three independent experiments. Error bars represent SDs of triplicate samples
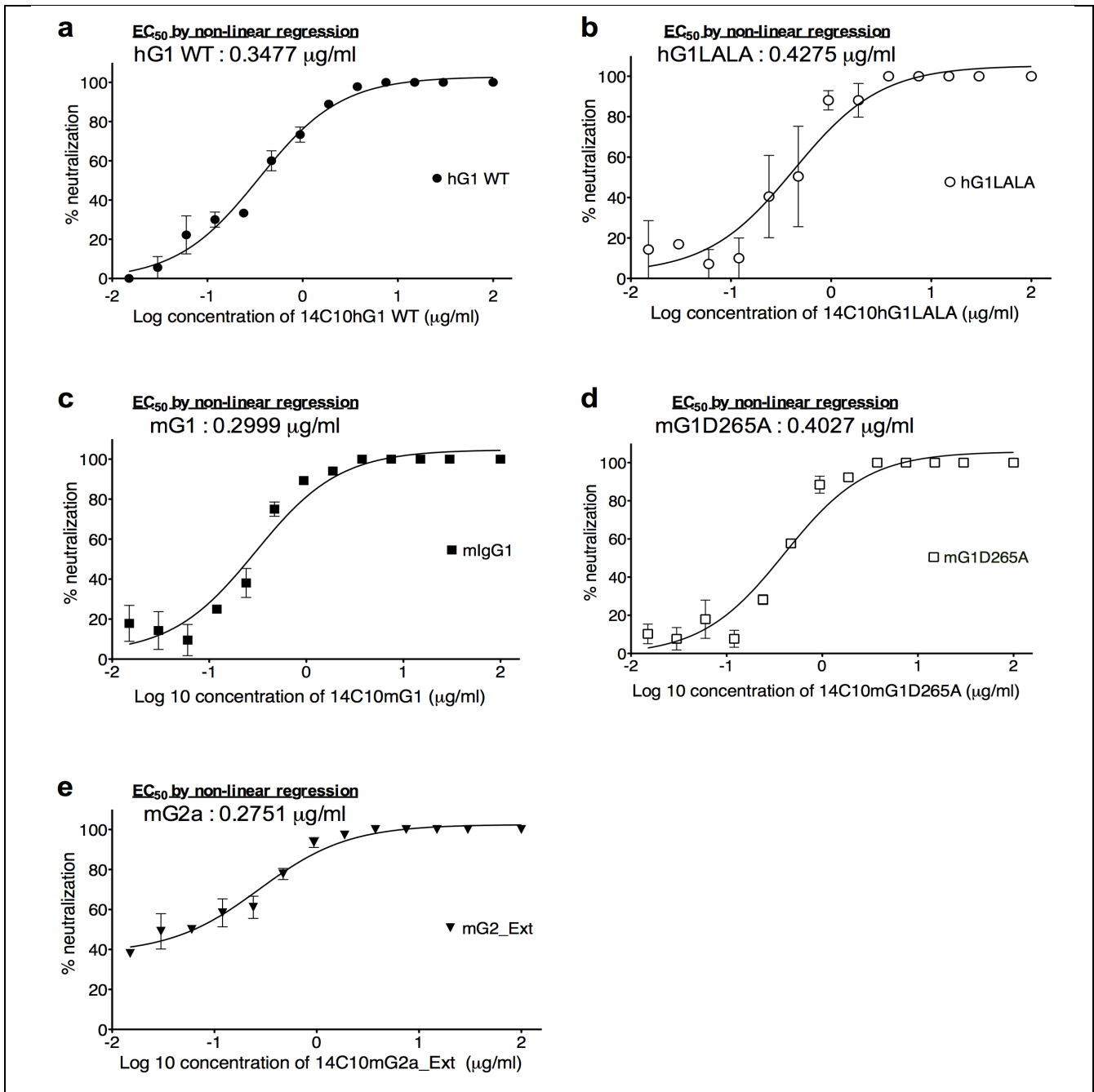
Figure 27. Comparison of neutralization activities of 14C10 constructs for DENV1 WP74 *in vitro.*

All constructs of 14C10 neutralize DENV1 WP74 in BHK cells as shown in the figure, (**a**) 14C10 WT (**b**) 14C10hG1LALA, (**c**) 14C10mG1, (**d**) 14C10mG1D265A, (**e**) 14C10mIgG2aEXT. The results represent the average of at least 2 independent experiments performed in triplicates. Error bars represent SDs.

4.5.3 Antibody dependent enhancement (ADE) *in vitro* mediated by the 14C10 constructs

To assess the levels of *in vitro* ADE and determine the infection-enhancing effects of each of the constructs at sub neutralizing concentrations, the gold standard assay for ADE *in vitro* in K562 cells was employed. The results showed a clear ADE effect mediated by 14C10 WT, hCH1mG1 and hCH1mG2aEXT. The ADE effect was absent in LALA and D265A mutations (Figure 28).
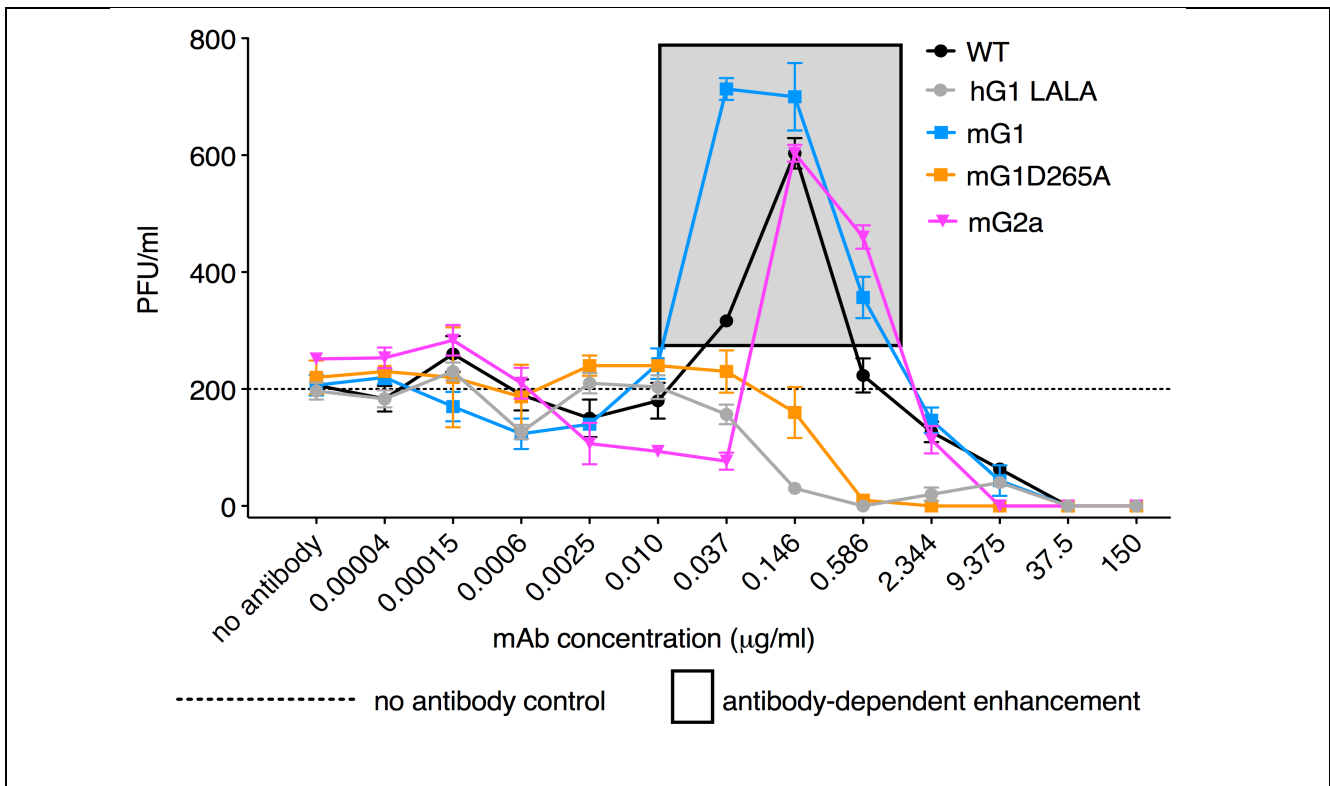
Figure 28. LALA and D265A mutations eliminate the ADE effect *in vitro.*

DENV1 EHI isolate was used to compare the constructs of 14C10 in the context of an antibody dependent enhancement (ADE) *in vitro*. The ADE effect was eliminated in the presence of LALA and mG1D265A constructs of 14C10, while WT, mG1 and mG2aEXT constructs of 14C10 triggered ADE and caused higher viral load in K562 cells (highlighted in a box). The results are representative of three independent experiments. Results represent the average of 3 independent experiments. Error bars represent SDs.

5 Defining the role of the Fc-region of Immunoglobulin in Dengue virus neutralization and clearance

5.1 Background of the study

It was suggested in previous published studies that the neutralizing mechanisms of anti-influenza antibodies are mediated entirely through their variable regions (Nelson, Palermo et al. 2007). However, DiLillo et al. examined the *in vivo* roles of the interaction between Fc portion of anti-influenza antibodies and their cognate Fcγ receptors and found that only five out of eight neutralizing antibodies required the above-mentioned interaction to confer protection from lethal H1N1 challenge (DiLillo, Tan et al. 2014). The five antibodies that required the Fc-FcγR interaction for protection from viral infection *in vivo* were broadly neutralizing monoclonal antibodies (bNAbs). Introduction of a D265A mutation, which abolishes Fc binding, significantly reduced their *in vivo* potency comparing to wild type antibodies (Figure 29). Three remaining antibodies were evenly protective regardless of the presence of the Fc-FcγR interactions and all of them were strain specific monoclonal antibodies (mAbs) (Figure 30).

We therefore hypothesized that modification of the Fc portion of the DENV1-specific 14C10 monoclonal antibody would prevent any potential homotypic ADE effect without affecting its neutralizing activity. To further examine the role of Fc-FcγR interactions for 14C10 antibody neutralization of DENV1 we tested *in vivo* constructs described in chapter 3, namely 14C10 WT, 14C10hG1LALA, 14C10mG1, 14C10mG1D265A, 14C10mG2aEXT.
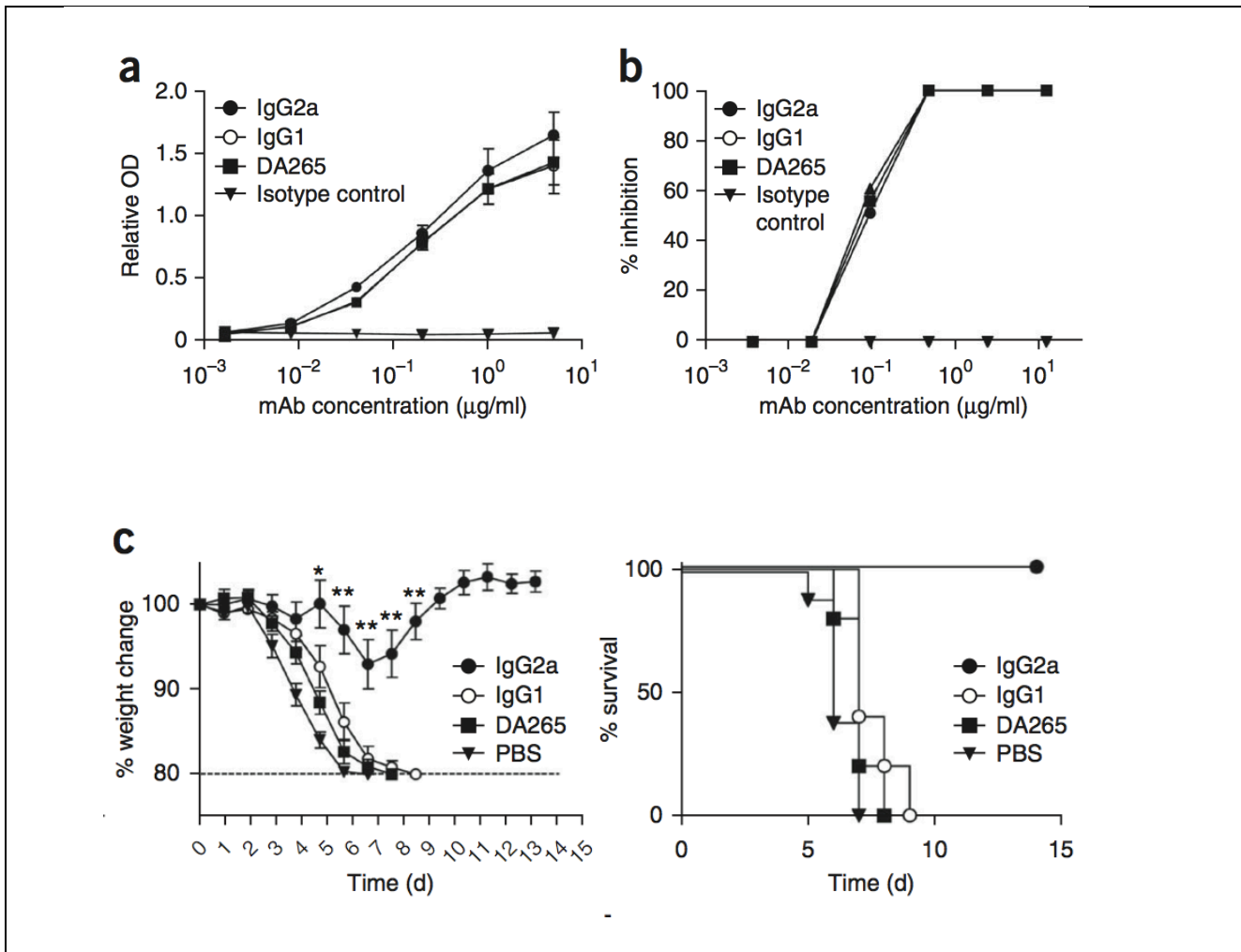
Figure 29. Fc-FcγR interactions are required for protection from influenza infection by bNAbs *in vivo*.

(**a**) Binding of 6F12 bNAb variants to PR8 HA. Mouse G2a, mouse G1 and DA265 mutant 6F12 bNAb and an IgG2a isotype control mAb diluted as indicated and tested for binding to PR8 HA by ELISA. Values represent mean ± s.e.m. relative optical density (OD) values from triplicate wells.

(**b**) *In vitro* plaque reduction neutralization by 6F12 bNAb variants. Values represent mean % inhibition, calculated by comparing plaque numbers in mAb-treated wells with wells receiving only PBS.

(**c**) Percentage weight change compared to day 0 (left) or percentage survival (right) in wild-type mice treated with mouse G2a 6F12 bNAb, mouse G1 6F12 bNAb, DA265 mutant 6F12 bNAb or PBS before infection with PR8 virus. Weight change values represent mean ± s.e.m. $n \geq 5$ mice per group. Reprinted by permission from Macmillan Publishers Ltd: Nature Publishing Group, advance online publication, (doi: 10.1038/sj.[NM], copyright (DiLillo, Tan et al. 2014)).
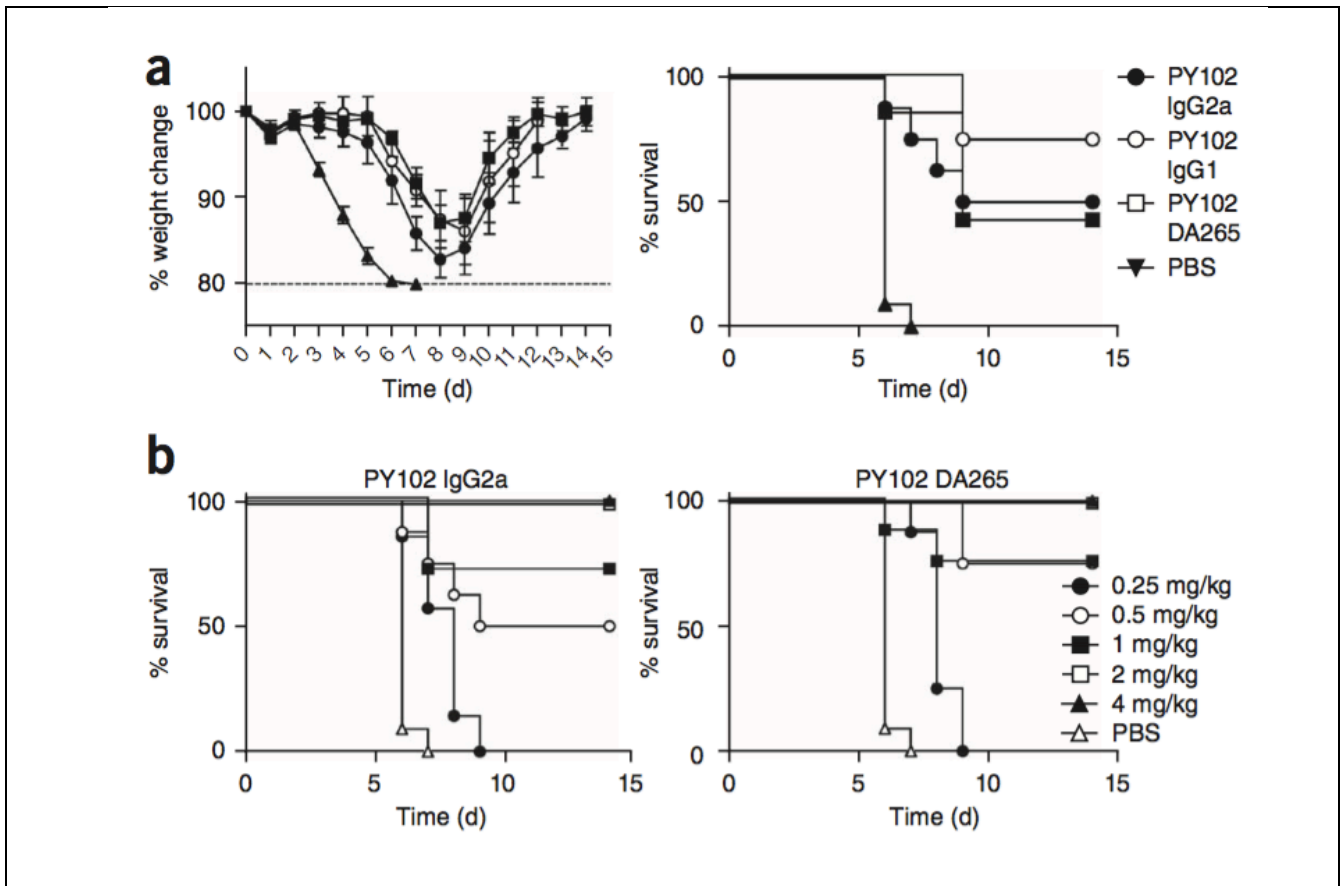
Figure 30. Strain-specific anti-influenza mAb does not require FcγR contributions during protection from viral infection *in vivo*.

(**a**) Percentage weight change compared to day 0 and survival over time in wild-type mice treated with mouse IgG2a, mouse IgG1 or DA265 mutant PY102 mAb or PBS before infection with PR8 virus. Weight change data in is expressed as mean ± s.e.m. *n* = 5–8 mice per group.

(**b**) Percentage survival over time in wild-type mice treated with the indicated doses of mouse IgG2a or DA265 mutant PY102 mAb, or PBS, before infection with PR8 virus.

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5.2 Setting up an *in vivo* model for DENV infection, neutralization and clearance

An AG129 mouse model was used to establish *in vivo* schemes for our studies as described in chapter 3.8. This is an asymptomatic model, and viremia levels are used as read-out of infection.

To assess the route of DENV1 infection, which results in the highest viremia, AG129 mice were inoculated with the virus intraperitoneally or subcutaneously and plasma viremia was compared by plaque assay. In mice infected with DENV1 EHI subcutaneously viremia levels were significantly higher (10^4 PFU/ml) than in mice infected via the intraperitoneal route (10^3 PFU/ml) as presented in Figure 31. Hence, the subcutaneous route of infection was chosen as a method of choice for virus inoculation for all *in vivo* experiments of this project. Time points for blood collection were standardized in the experiment presented in Figure 32. As a result days 3, 4, 5 and 9 were chosen for the future blood collection time points.

In the therapeutic scheme, animals were infected with DENV1 on day zero and treated with 14C10 antibody on day plus two post-infection. Viremia in mice infected with different genotypes of DENV1 was compared. In control mice infected with 200µl of 10^6 PFU of DENV1 EHI, viremia reached its peak between days three and four post-infection, with a serum titer of 10^4 PFU/ml. In mice treated with 30µg of 14C10 WT antibody, viremia decreased significantly to $10^{2.5}$ PFU/ml and was detected only on day three. The viral titer in the serum of control mice infected with 200µl of 10^7 PFU DENV WP74 reached its peak of $10^{3.5}$ PFU/ml on day four. In mice treated with 30µg of 14C10 WT antibody viremia was not

detectable at any time. The viremia levels in mice infected with DENV1 EHI (genotype 1) were higher, however the DENV1 WP74 (genotype 4) virus created clearer and easier to count plaques in BHK cells. Overall the results confirmed high neutralizing capacity of the wild type version of 14C10 antibody. Viremia in mice treated therapeutically with 14C10 was either not detectable at any time or decreased significantly. We concluded that both strains, namely EHI and WP74 representing most distinct genotypes of DENV1 should be used in the experiments of this project (Figure 32). In the prophylactic scheme, the 14C10 antibody was given one day before the subsequent inoculation of 200µl of DENV1 WP74 at 10^7 PFU/ml. By analogy to the therapeutic scheme, blood was taken on days 2, 3, 4, 5 and 9. In infected and non-treated control mice, viremia peaked at 10^3 PFU/ml on day four (Figure 34). In mice treated prophylactically with 10µg of 14C10 WT antibody, viremia was not detected at any time point, demonstrating that the prophylactic treatment is as efficient as the therapeutic treatment on day two. Based on these results, days 3, 4, 5 and 9 were chosen for the future blood collection time points.
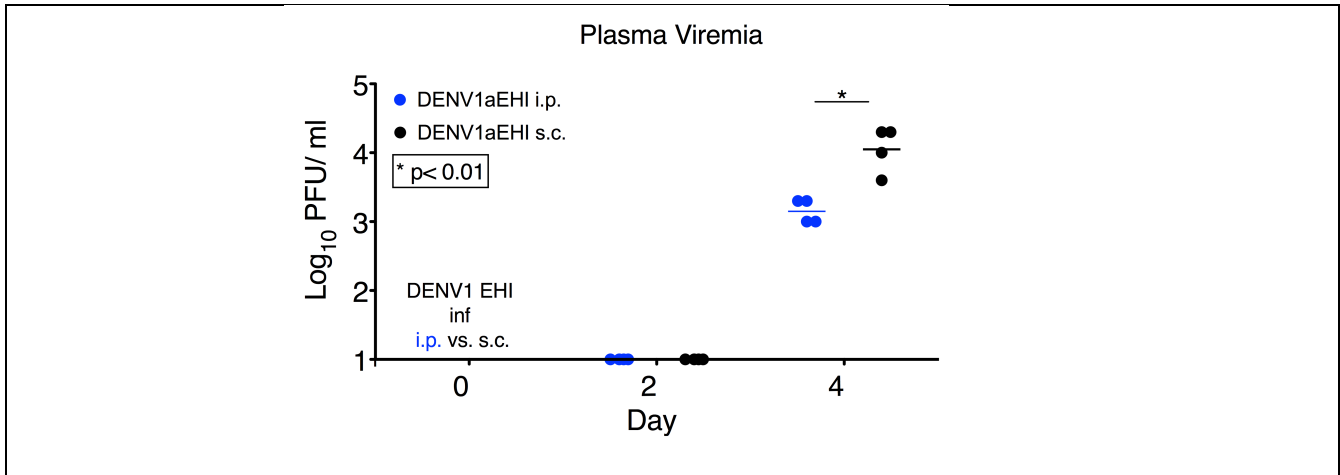
Figure 31. Mice infected with DENV1 EHI subcutaneously develop significantly higher viremia than mice infected via intraperitoneal route.

AG129 mice were infected with DENV1 EHI at 10^6 PFU in 200 µl/mouse via i.p. route (blue dots) or the s.c. (black dots) on day zero, (n=4). Blood samples were taken on days 2 and 4, and viremia levels were assessed by plaque assay and data points represent the averages of triplicate results. Lines represent mean of the plasma viremia in 4 animals, *p < 0.01.
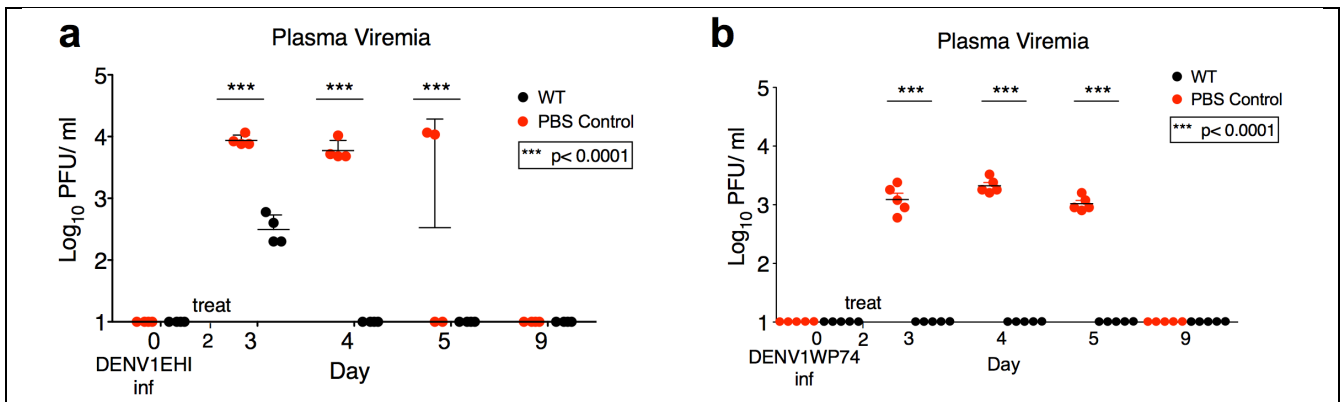
Figure 32. Plasma viremia in mice infected with different genotypes of DENV1

Mice were infected on day zero with 200µl of (**a**) DENV1 EHI at 10^6 PFU/ml, (n=4) or (**b**) DENV1 WP74 at 10^7 PFU/ml, (n=5). One group of mice (black dots) was treated with 30µg of 14C10 WT on day two (treat), second group of mice was given PBS instead of the antibody (red dots). Blood was collected on day 3, 4, 5 and 9 and viremia levels were assessed by plaque assay. Data points represent the averages of the triplicates of the plaque assay results from serum of each mouse. Lines represent the mean of viremia levels in all mice. ***p<0.0001

90
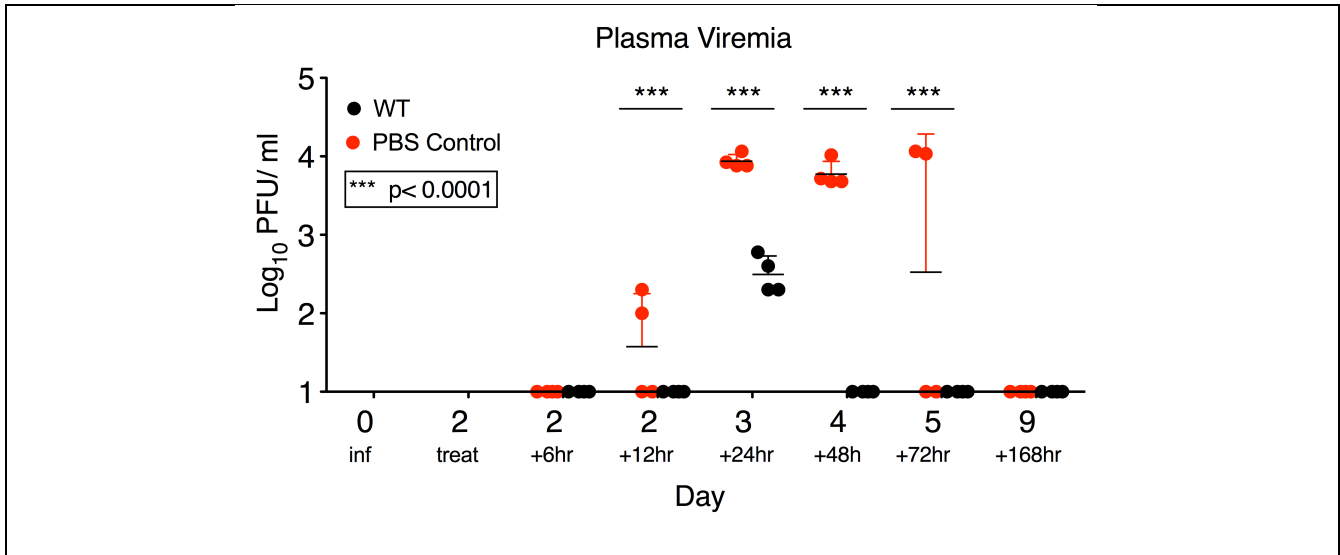
Figure 33. Establishing the 14C10 therapeutic scheme in the AG129 mouse model.

Mice (n=4) were infected with 200 µl of DENV1 EHI at $1x10^6$ PFU (inf) and treated (treat) with 30 µg of 14C10 antibody (black dots). Control mice were given PBS instead of the antibody (red dots). To examine the kinetics of viremia in serum, blood samples were collected at 6, 12, 24, 48, 72 and 168 hours post-treatment. Viremia levels (data points) were assessed with plaque assay. *** $p<0.0001$.
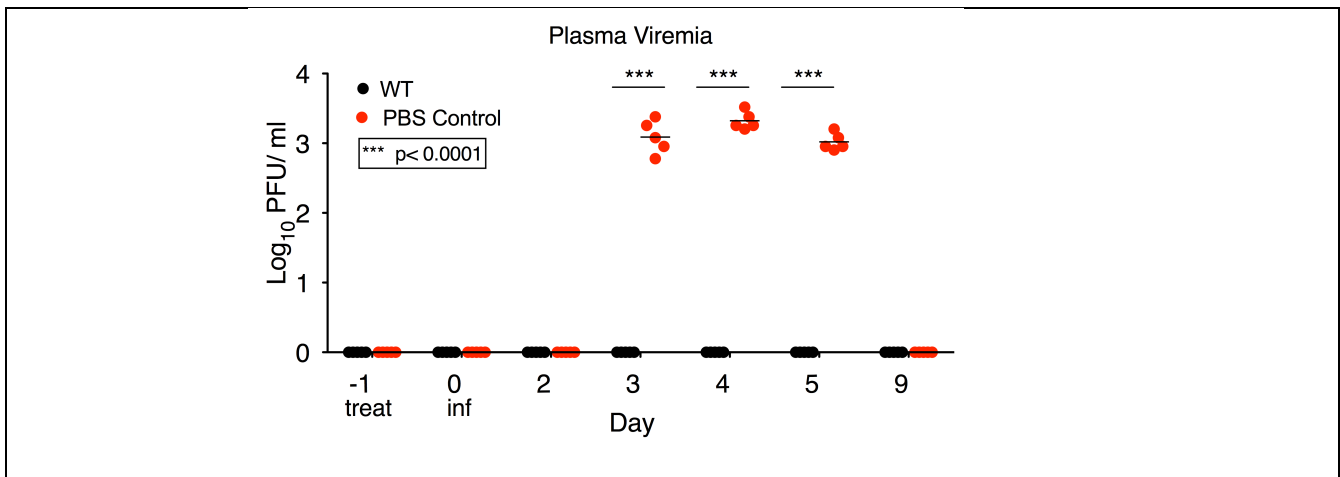
Figure 34. Establishing the 14C10 prophylaxis scheme in the AG129 mouse model.

On day minus one, mice were treated with 10µg of 14C10 antibody (treat), and on day zero DENV1 WP74 was injected at $1.3x10^7$ PFU at 200µl/mouse (inf). Blood was collected on days 2, 3, 4, 5 and 9. In mice given 14C10 antibody one day before the inoculation of DENV1, viremia assessed by plaque assay was not detectable at any time point (black dots). Control mice were given PBS instead of the antibody and they developed standard viremia for this experimental model (red dots). *** $p<0.0001$.

5.3 *In vivo* testing of 14C10 constructs

To assess *in vivo* efficacies of hG1LALA, mG1, mG1D265A, mG2aEXT and 14C10 WT, the therapeutic and prophylactic schemes described above were utilized in the AG129 mouse model.

5.3.1 Therapy of DENV1 infection by engineered derivatives of 14C10

To assess the efficacy of the antibody candidates as therapy for DENV1 infection, AG129 mice were inoculated subcutaneously with DENV1 on day zero and given one of the 14C10-derived antibodies, namely 14C10hG1LALA, mIgG1, mIgG1D265A or mG2aEXT on day two post-infection. Antibody dose 30 µg/mouse, was previously established neutralizing concentration for the 14C10 WT antibody (Teoh, Kukkaro et al. 2012). Since the results did not yield any significant difference between the *in vivo* efficacies of the 14C10-derived constructs used (data not shown), I employed lower concentrations of 10 and 6µg per mouse. In mice treated with 10 or 6 µg of the 14C10-derived constructs, virus was still not detectable at any time and there was no significant difference in the results regardless of the mutation or variation of the construct employed (Figure 35). In control group of non-treated mice, viremia lasted three days, and the peak was noted on day four. The lack of viremia in mice treated with one of five constructs suggested that the antibodies at the abovementioned concentrations saturated the system and thus, they were still too high to determine differences in the neutralization efficacy.
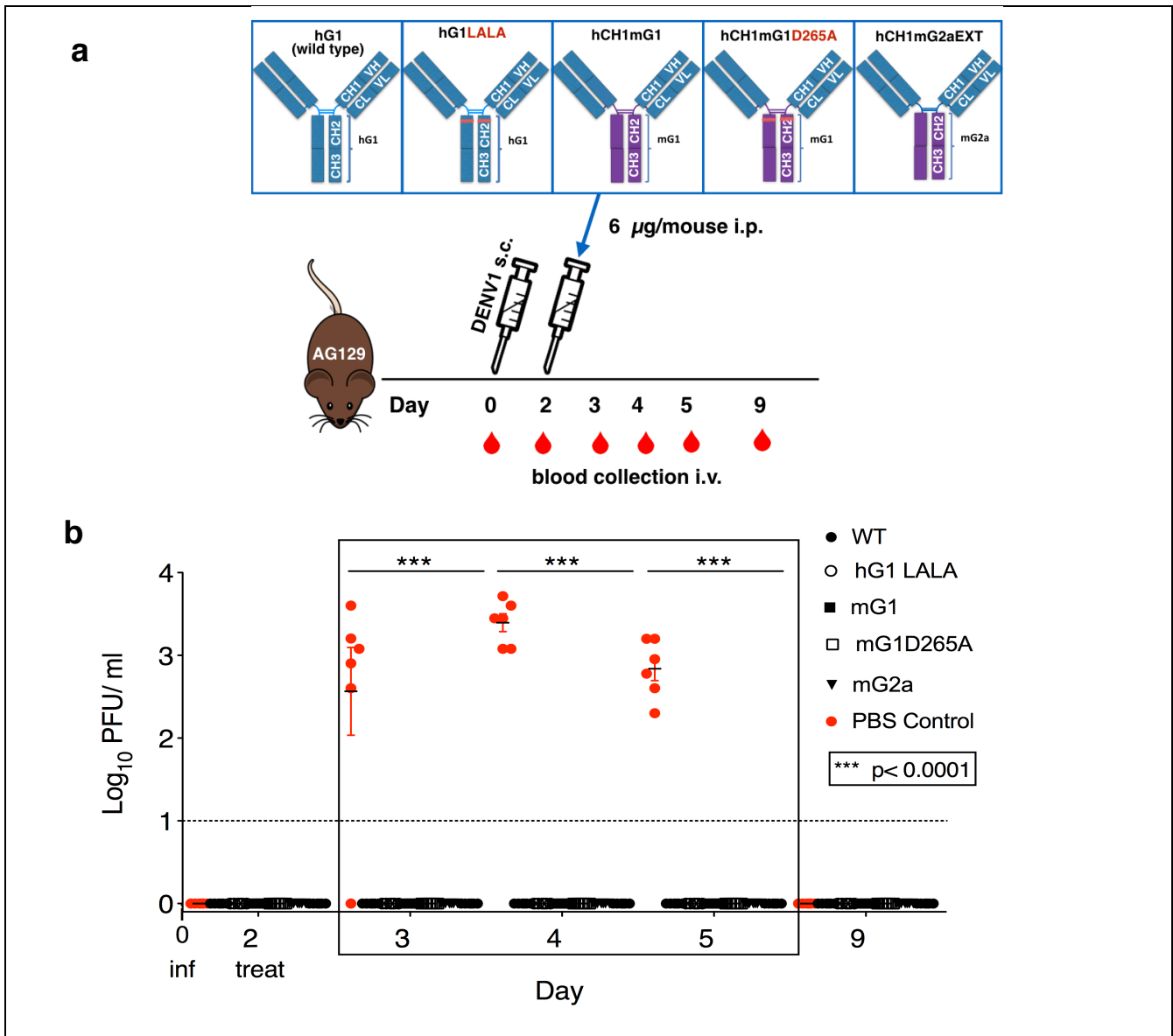
Figure 35. *In vivo* testing of the 14C10 constructs at 6 µg/mouse (therapy).

(a) Mice (n=6) were infected with DENV1 EHI $1x10^6$ PFU 200 µl/mouse on day zero (inf) and therapy with one of the 14C10 constructs was given on day plus two post-infection (treat). Blood was collected before an infection and treatment as well as on day 3, 4, 5 and 9.

(b) Standard plaque assay was utilized to measure the serum viremia. Serum samples were diluted 1:10 in RPMI and 100 µl of each sample was applied onto BHK cells in triplicates. After 1-hour incubation at 37^oC, the cells were covered with overlaid media and incubated for 7 days. Fixed and stained cells were used to count the plaques and calculate viremia levels. Each data point represents the average of the triplicate results, and the lines represent the mean of viremia in 6 animals.

5.3.1 Prophylaxis of DENV1 infection with engineered derivatives of 14C10

The experiments described above using a therapeutic scheme of infection and treatment showed a complete absence of plasma viremia in mice treated with any of the 14C10 antibody constructs. Concentrations as low as 30, 10 or 6 µg per mouse post infection resulted in complete virus neutralization (Figure 35). We next asked whether mice treated with 14C10 constructs prophylactically would still be protected against DENV1 infection. To test this, mice were treated with one of 14C10 antibody constructs one day pre-infection. Similarly all the mice treated with 14C10 WT or one of the constructs one day before DENV1 infection were protected and did not develop the viremia, unlike the control mice infected non-treated.
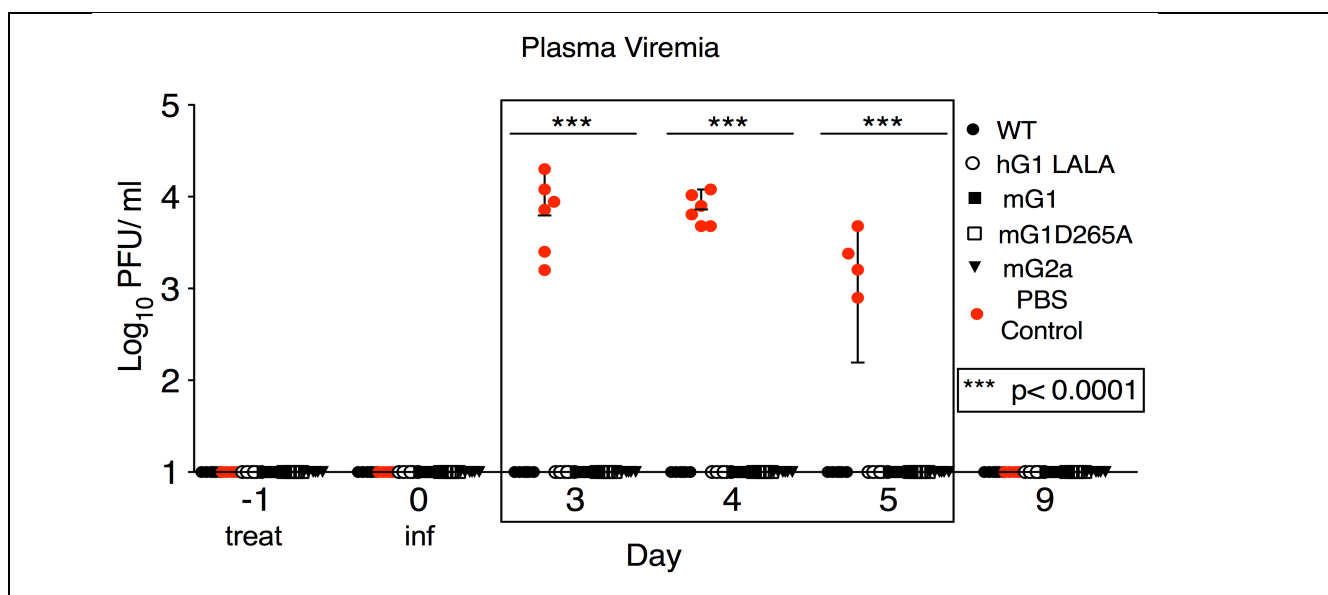
Figure 36. *In vivo* testing of the 14C10 constructs at 10 µg/mouse (prophylaxis).

Mice were treated with one of the 14C10 constructs on day minus one pre-infection (treat) and then infected (inf) with DENV1 EHI at 1×10^7 PFU, 200 µl/mouse on day zero, (n=6). Subsequently, the blood samples were taken on day 3, 4, 5 and 9. The serum was used to assess viremia levels by standard plaque assay. Each data point represents the average of the triplicates results, and the lines represent the mean of the viremia in 6 animals, ***p<0.0001.

5.4 Defining the role of the Fc-region of antibodies in Dengue virus neutralization and clearance

Although it was shown previously that the D265A mutation might significantly reduce *in vivo* potency of the mutant antibody comparing to the wild type antibody (DiLillo, Tan et al. 2014), (Corti, Voss et al. 2011), when the doses of 30, 10 or 6 µg of all five constructs of the 14C10 WT antibody were used *in vivo*, there was no significant difference in the viremia level (Figure 35). To further determine if changes made in the constructs interfered with neutralization *in vivo,* concentrations of 1 µg per mouse were administered to mice infected with DENV1. The serum samples were then tested by plaque assay for the presence of infective viral particles. Mice given the WT 14C10 displayed significantly lower viremia compared to control mice that were given PBS instead of 14C10 and it was decreasing on day three and four compare to day two. On day five, decreasing tendency changed and the viremia was higher compare to the previous days (Figure 37). This observed viral rebound could be explained as a result of sub-neutralizing antibody concentration, which first partially neutralises the virus, and later allows the virus to replicate. Another explanation could be an effect of the facilitation of the viral entry through Fc rec, which is unlikely since the result of LALA vs. WT 14c10 yield results, which are not significantly different (Figure 38 a). There were no significant differences in the neutralization potency between the four modified 14C10 constructs versus wild type 14C10 antibody (Figure 38).
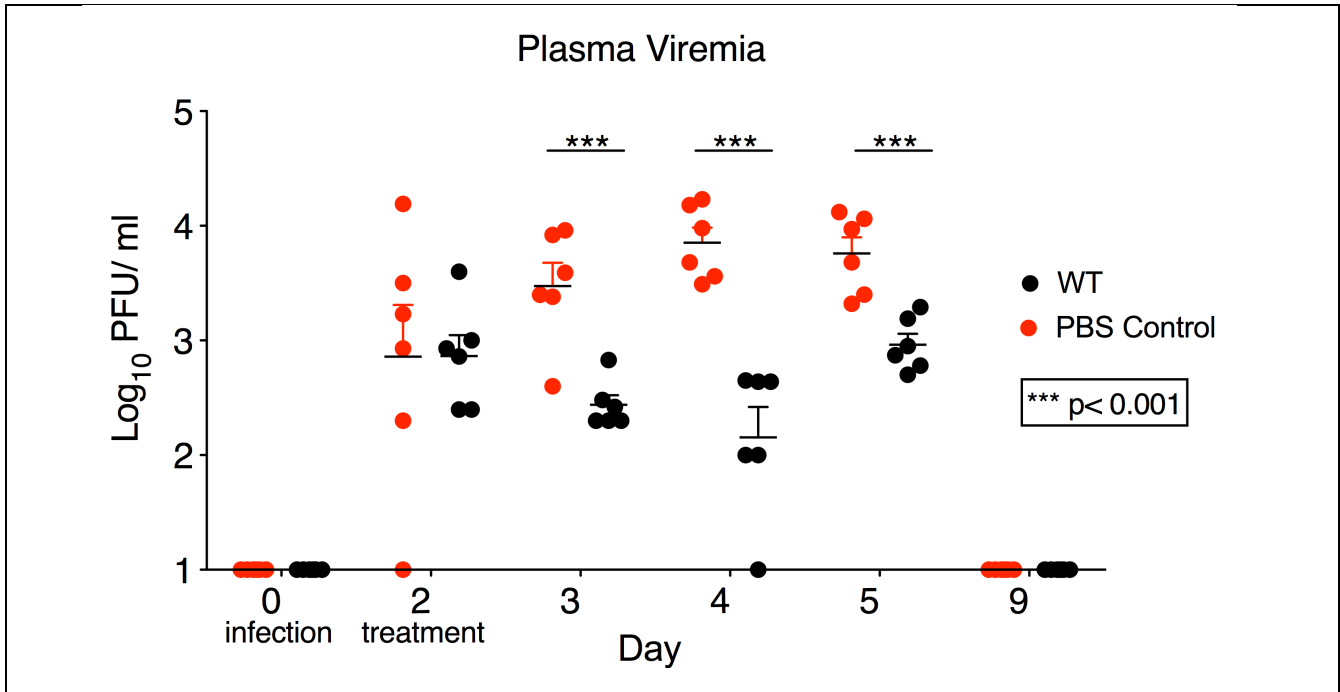
Figure 37. Viremia in mice treated with 14C10 WT at 1 µg/mouse.

Mice (n=6) were infected with 200 µl of DENV1 EHI at 1×10^{7} PFU/ml on the day zero and given 1 µg of 14C10 antibody on the day two post-infection (black dots). Blood was collected on days 3, 4, 5 and 9. Viremia levels in mouse sera were assessed by plaque assay. The results were compared with the control group, infected, PBS-treated (red dots). Viremia levels were tested by plaque assay. Serum samples were diluted 1:10 in RPMI and 100 µl of each sample was applied onto BHK cells in triplicates. After 1-hour incubation at 37°C, cells were covered with overlaid media and incubated for 7 days. Fixed and stained cells were used to count plaques and calculate viremia levels. Each data point represents the average of the triplicate results, and the lines represent the mean of viremia in 6 animals, ***p<0.0001.
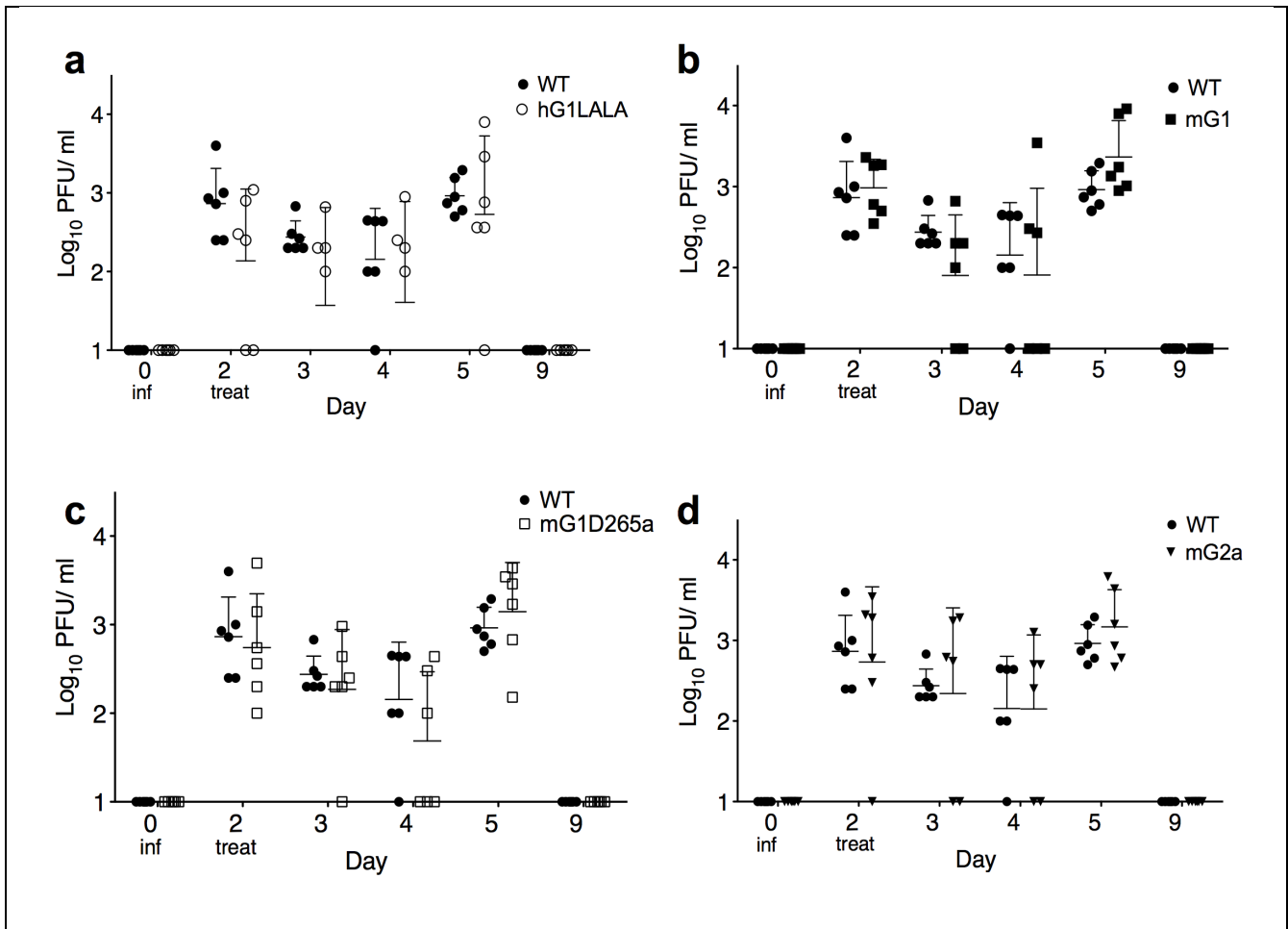
Figure 38. Plasma viremia in mice treated with 1 µg of 14C10hG1LALA, mIgG1, mG1D265A or mG2a assessed by plaque assay (Plaque Forming Units).

Mice (n=6) were infected with DENV1 EHI 1×10^{7} PFU/ml (200 µl/mouse) on day zero (inf) and then treated therapeutically with one of five 14C10 antibody constructs (1 µg/mouse) on day plus two post-infection (treat). All 14C10 constructs decreased the viremia like the wild type antibody. There was no significant difference between the *in vivo* efficacies of any of the 14C10 constructs versus 14C10 WT. Mouse sera were assessed for the presence of infective dengue particles by plaque assay. Data points represent the averages of triplicate results of each mouse and error bars represent SDs. The results of mice treated with 14C10 WT were compared to the results of mice treated with each construct, as follows (**a**) 14C10 WT vs. 14C10hG1LALA, (**b**) 14C10 WT vs. 14C10mG1, (**c**) 14C10 WT vs. 14C10mG1D265A and (**d**) 14C10 WT vs. 14C10mG2aEXT. No significant difference between four constructs and 14C10 WT antibody was detected.

5.4.1 Virus neutralization vs. clearance

Next, to address the question whether the virus neutralized with 14C10 WT antibody or the engineered constructs was cleared from the circulation, we employed the qPCR analysis of serum samples previously characterized by plaque assay and ELISA. While plaque assay detects infective viral particles, and the results can be used for the assessment of neutralization, qPCR assay detects both infective and non-infective viral particles, which translates into viral clearance.

The limit of detection for the quantitative real-time PCR assay was first determined using various dilutions of cultured DENV1 virus samples with known PFU titers, and a detection limit of 10^1 PFU/ml was determined (data not shown). Subsequent testing of the quantitative real-time PCR assay using quantitated plasmid controls showed that the limit of detection achieved was 100 copies/µl. A CT value of 43 or above was considered to be negative. The assessment of plasma viremia by plaque assay showed that all the constructs used, namely 14C10 WT, hG1LALA, mG1, mG1D265A and mG2aEXT neutralized DENV1. The outcome of the qPCR showed that not only was the virus neutralized but it was also cleared from the system by day plus nine post-infection (Figure 39, Figure 40). Thus, the comparison of the same samples with those two tests yielded the answer to the role of the Fc-region of tested antibodies in Dengue virus neutralization and clearance. As expected, the modifications of Fc portion of 14C10, which is a DENV1 specific antibody, did not affect its neutralizing capacity.
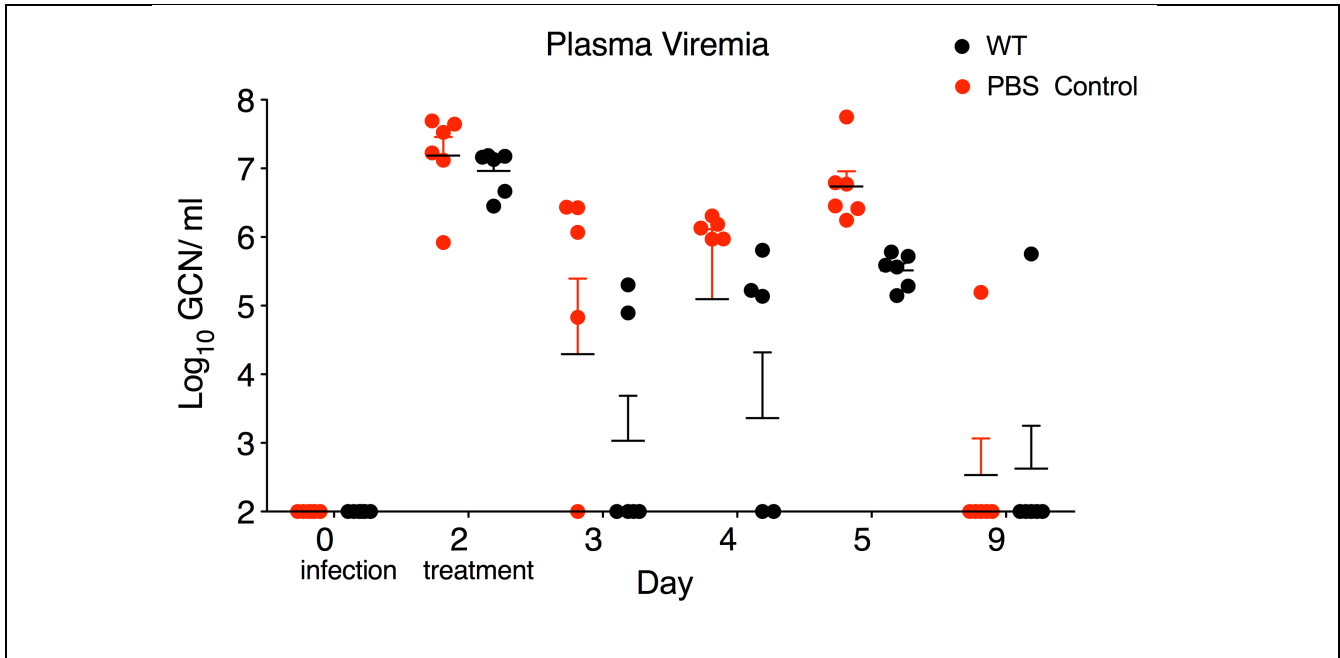
Figure 39. The level of total viral particles in mouse serum assessed by qPCR.

Mice (n=6) were infected on day zero with DENV1 EHI at 1 x10^7 PFU, 200 µl/mouse and treated with 14C10 WT antibody at 1 µg/mouse (black dots) on day two. Control mice were given 200 µl of PBS instead of 14C10 WT (red dots). Blood was collected on days 3, 4, 5 and 9. Mouse sera previously tested by plaque assay (Figure 37) here were tested with qPCR for total viral particles. Viral RNA was extracted and reverse transcription was carried out at 60°C for 15 min followed by Taq polymerase activation at 95°C for 2 min. A 45-cycle PCR amplification was performed with denaturation at 95°C for 5s, annealing and extension combined at 60°C for 45 sec. The fluorescence emitted from the assay was captured at the end of extension phase of each cycle and the results analysed with the ABI 7500 Fast PCR System software. Data points represent the avareage of the duplicate result and the error bars represent SDs.
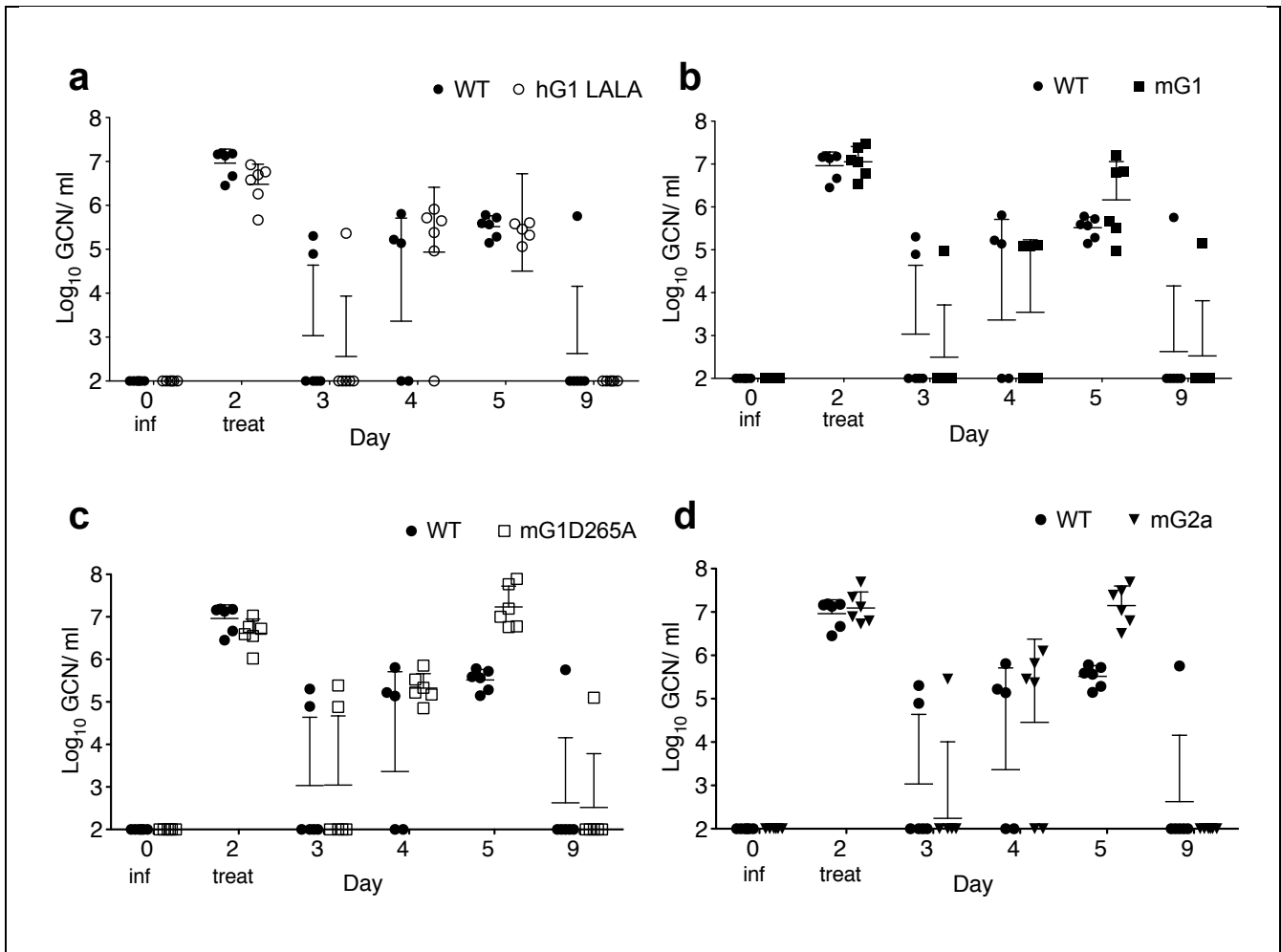
Figure 40. Total viral particles in mice treated with 1 µg of 14C10hG1LALA, mG1, mG1D265A or mG2a assessed with qPCR (Genome Copy Number).

Mice (n=6) were infected with DENV1 EHI at 1 x10^{7} PFU 200 µl/mouse on day zero (inf) and given sub-neutralizing concentrations of antibodies (1 µg/mouse) on day two (treat). Mouse sera were collected at indicated time points and were assessed for the presence of total (infective and non-infective) DENV1 particles by qPCR. The results for each of the 14c10 construct were statistically compared to 14C10 WT: (**a**) 14C10 WT vs. 14C10hG1LALA, (**b**) 14C10 WT vs. 14C10mG1, (**c**) 14C10 WT vs. 14C10mG1D265A and (**d**) 14C10 WT vs. 14C10mG2aEXT. No significant difference between the constructs and 14C10 WT antibody was detected. The difference for mG1D265A/mG2a compared to WT on day 5 is not significant (p>0.19).

6 The effect of antibody treatment on the induction of natural protective immunity

6.1 Antibodies as anti-infective treatment / prophylaxis modalities

There has been a significant revival in the employment of antibodies as anti-infectives in recent years, particularly for acute viral infections where alternative therapeutic modalities are unavailable. RNA viruses such as Zika, Ebola, SARS, Nipah, Influenza A and DENV are major sources of emerging, acute infectious diseases in human populations and thus represent optimal targets for this form of therapy (Morse and Schluederberg 1990, Casadevall, Dadachova et al. 2004, Caminade, Medlock et al. 2012, Casadevall and Pirofski 2015, Graham and Ambrosino 2015, Shriver, Trevejo et al. 2015, Malone, Homan et al. 2016). The mechanism of protection is proposed to be the neutralization and/or clearance of the viral pathogen from the circulation of the infected host with an associated reduction in the bioavailability of viral PAMPs and antigens. The impact that this form of intervention in the natural cycle of infection has on the development of a host long-term protective response remains unclear. We have addressed this question by analyzing passive antibody therapy for two genetically, morphologically and phenotypically distinct viral pathogens with fully human monoclonal antibodies derived from convalescent patients.

6.2 Establishment of a homologous re-challenge model for DENV

The evaluation of the *in vivo* efficacy of five different formulations of the 14C10 antibody revealed its kinetics and some of the effects on the immune system of the host. To examine whether 14C10 antibody treatment impairs the development of a natural immune response with associated long-term protection against the same serotype of DENV a novel re-challenge scheme was established. AG129 mouse model was employed for this study. Each group consisted of five to six AG129 mice at the age of eight-to-nine weeks. The mice were anesthetized and blood was taken from the jugular vein before all subsequent *in vivo* procedures. Viremia levels in mice were measured by plaque assay. Levels of 14C10 antibody in mouse sera and the endogenous protection response generated in mice were assessed by ELISA assay.

6.2.1 Therapeutic applications in DENV re-challenge model

In the dengue re-challenge therapy scheme, AG129 mice were inoculated subcutaneously on day zero and treated intraperitoneally with the therapeutic antibody on day plus two post-infection. Afterwards, the mice were re-infected subcutaneously on day 32 post-infection with the same strain of the virus as the primary infection. Blood samples were taken on days 3, 4, 5 and 9 post-infection to assess viremia, and on days 11, 14 and 29 to assess the levels of the delivered therapeutic antibody still in circulation, as well as the mouse anti-DENV1 antibody response. On days 35 and 37 re-challenged mice were bled to assess the potential secondary viremia and the levels of the given and endogenous antibodies. Control group (I) was injected with 200 µl of sterile PBS instead of therapeutic antibody treatment. Control group (II) was a group of naïve mice that were inoculated with the virus on day plus 32 post-infection. Mice infected with DENV1 and treated post-infection with the 14C10 antibody did not develop any plasma viremia, whereas in the control mice transient viremia at 10^3 -$10^{3.5}$ PFU/ml lasted from day three to five (Figure 42). Interestingly, the re-challenged mice remained protected after clearance of the administered antibody on day 32. As expected, control mice (II) infected on day 32 only developed transient viremia at 10^3 PFU/ml typical for this mouse model (day 35, 37).
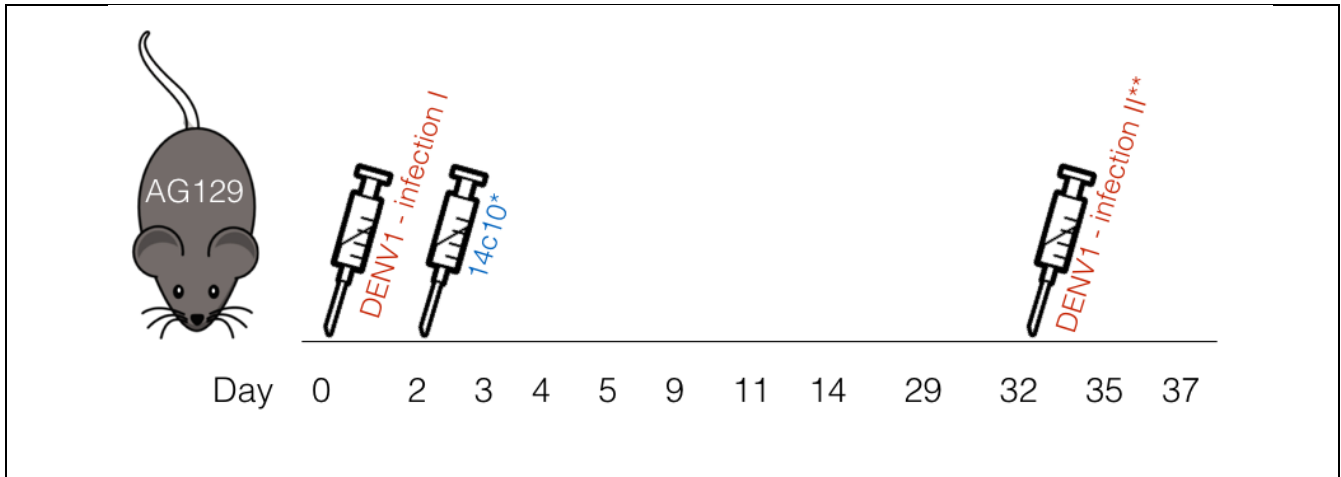
Figure 41. Therapy of dengue in the re-challenge scheme of infection.

Eight-to-nine weeks old AG129 mice (n=5) were infected with DENV1 at the established concentration on day zero. Thereafter they were treated with 14C10 WT antibody on day two. The blood samples taken on day 3, 4, 5, 9, 11, 14, and 29 were assessed for plasma viremia, levels of 14C10 still present in the mouse circulation and presence of endogenous mouse anti-DENV1 antibodies. Mice were then re-infected on day 32 with the same strain and quantity of DENV1 as the primary infection. Blood samples were taken on days 35 and 37 (day plus three and plus five after secondary re-challenge) and tested as previous samples. Control group (I)* was given PBS instead of antibody on day two. Control group (II)** was infected only once, on day 32.
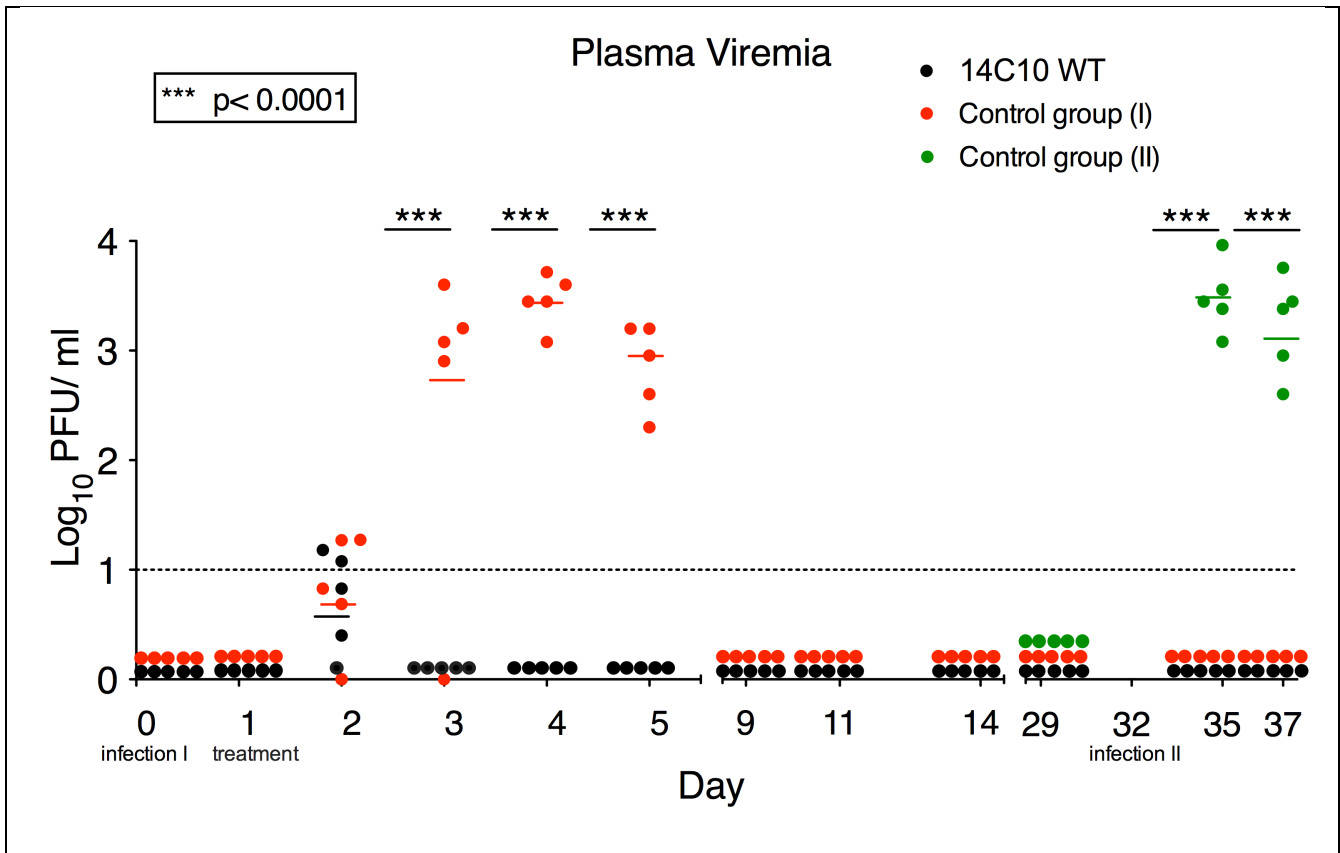
Figure 42. Plasma viremia in the re-challenge scheme of DENV1 infection and therapy with 14C10 WT antibody.

Therapeutic re-challenge scheme of infection (Figure 41) was used to measure viremia levels in mice infected and treated with 30 µg/mouse of 14C10 WT antibody (black circles). Viremia was assessed on days 2, 3, 4, 5, 9, 11, 14, 29, 35 and 37. The results were compared to the control group (I) – given PBS instead of antibody (red circles) and control group (II) – infected on day 32. The experiment was carried out twice with n=5 AG129 mice. Plaque assay was utilized to measure the blood viremia, presented as data points. The lines represent mean of the viremia in 5 mice, ***p<0.0001.

6.2.2 Prophylaxis of dengue in the re-challenge scheme of infection.

In the prophylaxis re-challenge scheme, the AG129 mouse model was treated on day minus one pre-infection with 14C10 given intraperitoneally (Figure 43). Control group (I) was injected with 200 µl of sterile PBS instead of the antibody. Next, on day zero the mice were inoculated subcutaneously with DENV1. A month after the initial infection, on day 32, mice were re-infected with the same strain of DENV1 as previously. Control group (II) were kept naïve until they were inoculated with DENV1 on day 32. Blood was taken from the jugular vein on days 3, 4, 5 and 9 to measure viremia, on days 11, 14 and 29 to measure the levels of 14C10 remaining in circulation, and on days 35 and 37 to assess the viremia and the levels of the antibodies. In mice given 14C10 antibody before the infection, viremia was not detectable at any time point of the experiment. Control group (I) developed viremia at 10^3 - $10^{3.5}$ PFU/ml which peaked on day four. After secondary infection, there was no detectable virus in the samples taken from mice treated and control group (I). On the contrary, control group (II) mice, infected only once on day 32, developed viremia (Figure 44).
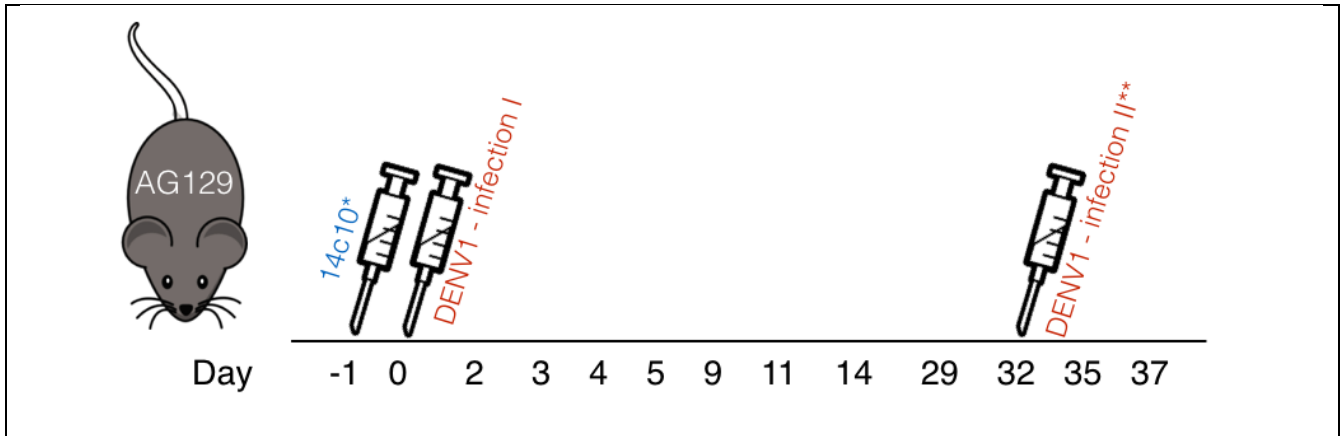
Figure 43. Prophylaxis of dengue in the re-challenge scheme of infection.

Mice were treated with the 14C10 WT antibody on day minus one pre-infection. Thereafter, on day zero they were infected with DENV1. On day 32, mice were re-infected with the same strain of DENV1 as in the primary infection. Blood samples were taken on days 3, 4, 5, 9, 11, 14, 29, 35 and 37 post-infection to assess the plasma viremia, levels of the given 14C10 antibody and presence of the endogenous mouse anti DENV1 antibodies.
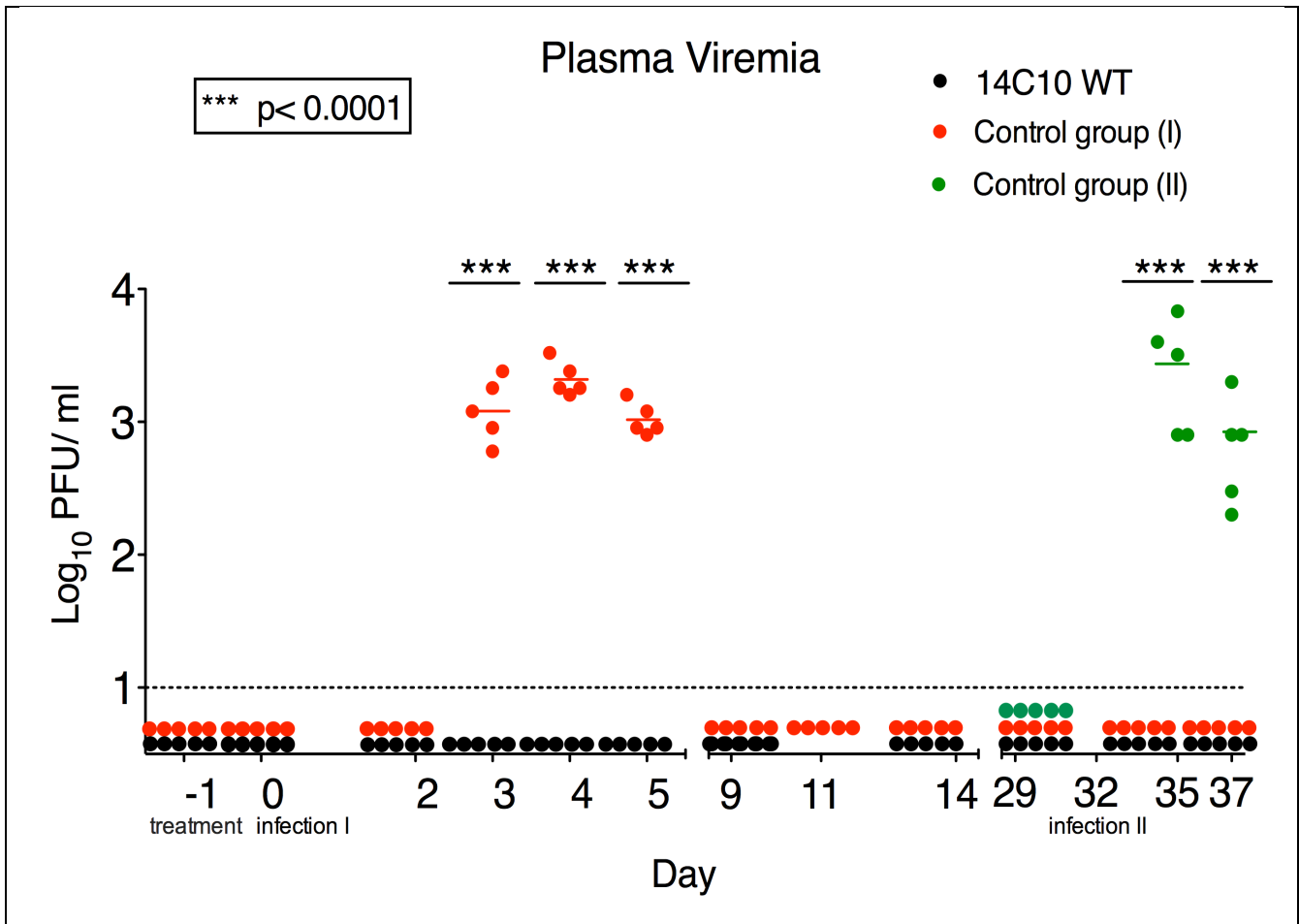
Figure 44. Plasma viremia in the re-challenge scheme of DENV1 infection and prophylaxis with 14C10 WT antibody.

Prophylactic re-challenge scheme of infection (Figure 43) was utilized to measure viremia levels in mice treated with 14C10 WT antibody at 30 µg on day minus one pre-infection. On day zero mice were infected with 200 µl DENV1 WP74 at 1.3×10^7 PFU/ml. Blood collected on days plus 2, 3, 4, 5, 9, 11, 14, 29, 35 and 37 was used to assess serum viremia by plaque assay presented as data points. Experiment was carried out twice with n=5 AG129 mice. Serum viremia in mice treated with 14C10 WT antibody (black dots) was compared to control group (I) - red dots, and control group (II) - green dots. Control group (I) was given PBS instead of antibody treatment on day minus one and challenged as described above for mice given 14C10. Control group (II) was kept naïve until infection on day 32. The lines represent mean of the viremia in 5 mice, ***p<0.0001.

6.3 Natural immune response in AG129 mice

Antibody therapy or prophylaxis may protect against development of viral infections, however it is not known how this potentially affects the ability of the host to protect themselves against subsequent re-exposure to the same pathogens after clearance of the administered antibody. To address this, we employed both pre- and post-infection models for delivering anti-DENV1 mAbs to AG129 mice then re-challenged the treated mice with the same viruses after the administered antibodies were rendered undetectable in their circulation.

6.3.1 Treatment with 14C10 does not inhibit the natural anti-DENV1 immunity

We found that mice treated with anti-DENV mAb after the primary infection, do not develop the viremia after secondary challenge (Figure 42). Thus, we hypothesized that the protection observed after the secondary challenge was a result of a murine immune response specific to the infecting agent, and that this occurs regardless of whether mice were given anti-viral antibodies. To exclude the possibility that the protection after subsequent infection observed in mice treated with anti-DENV mAb is not a result of the persistent presence of given antibody, we first measured the levels of administered anti-DENV mAb in mouse serum. In the mice treated with 14C10 WT, the antibody was detected only on day 3, 4, 5 (at 3.4 µg/ml) and on day 9 (at 1.1 µg/ml), and afterwards it was not detectable proving that it was cleared from the circulation. As expected, in the control

groups of mice (I) – PBS treated on day two and infected only on day 32, non-treated, 14C10 WT was not detected at any time point (Figure 45).

Levels of mouse endogenous anti-DENV1 antibodies were measured by ELISA. The results showed that mice infected and treated with anti-DENV1 mAb produced natural anti-DENV1 antibodies at the similar levels like mice infected and given PBS instead of mAb. The endogenous antibodies were present in the serum in both treated and untreated mice during secondary infection. In control group (II) –infected only on day 32, no endogenouse anti-DENV1 antibodies were detected (Figure 46).

Thus, our hypothesis was supported by undetectable levels of given mAb after day nine post-infection and the presence of mouse anti-DENV1 IgGs in the treated and control groups starting from day four and their presence during subsequent infection (Figure 46). Interestingly, the natural mouse IgG responses in mice given 14C10 WT post-infection were similar to control infected mice that were not given anti-DENV1 antibodies, suggesting that an introduction of 14C10 WT post-infection does not affect the induction of a natural anti-DENV1 antibody response.
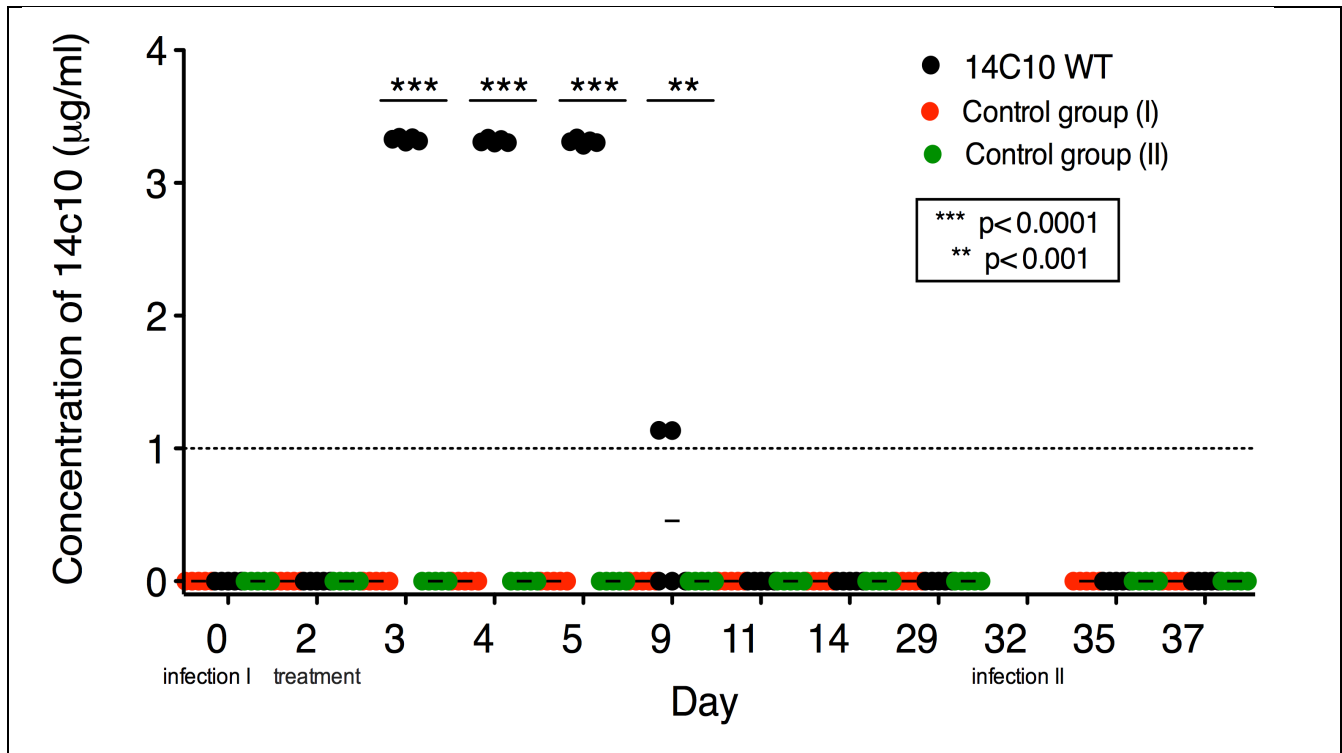
Figure 45. Concentration of 14C10 WT in mouse serum in dengue infected and mAb treated (therapy) mice.

Mice were infected with DENV1 WP74 at 1.3×10^7 PFU, 200 µl/mouse on day zero and 30 µg of 14C10 WT antibody per mouse was administered on day plus two post-infection (black dots). Mice were re-infected with the same strain of DENV1 as previously and the viremia was measured on day 35 and 37. Control group (I) – red dots, was given PBS instead of antibody treatment on day minus one and challenged as described above for mice given 14C10. Control group (II) – green dots, was kept naïve until infection on day 32. The experiment was carried out twice with n=5 AG129 mice. Blood samples were utilized to test levels of given antibody in mouse serum over the time of the experiment. Serum samples were tested by ELISA assay. Anti-idiotypic antibody E1 specific for 14C10 WT antibody was coated on ELISA plates, the serum samples (diluted 1:10) were probed onto blocked and washed plates and incubated for 1 hour at RT. After subsequent washing, anti-human HRP conjugate antibody was added at 5µg/ml, 50µl/well overnight. Plates were washed again and TMB substrate was added. The reaction was stopped with 0.1 M sulfuric acid. Absorbance was read at 450nm by spectrophotometer. Results are the averages of the duplicate reading, **$p<0.001$, ***$p<0.0001$.
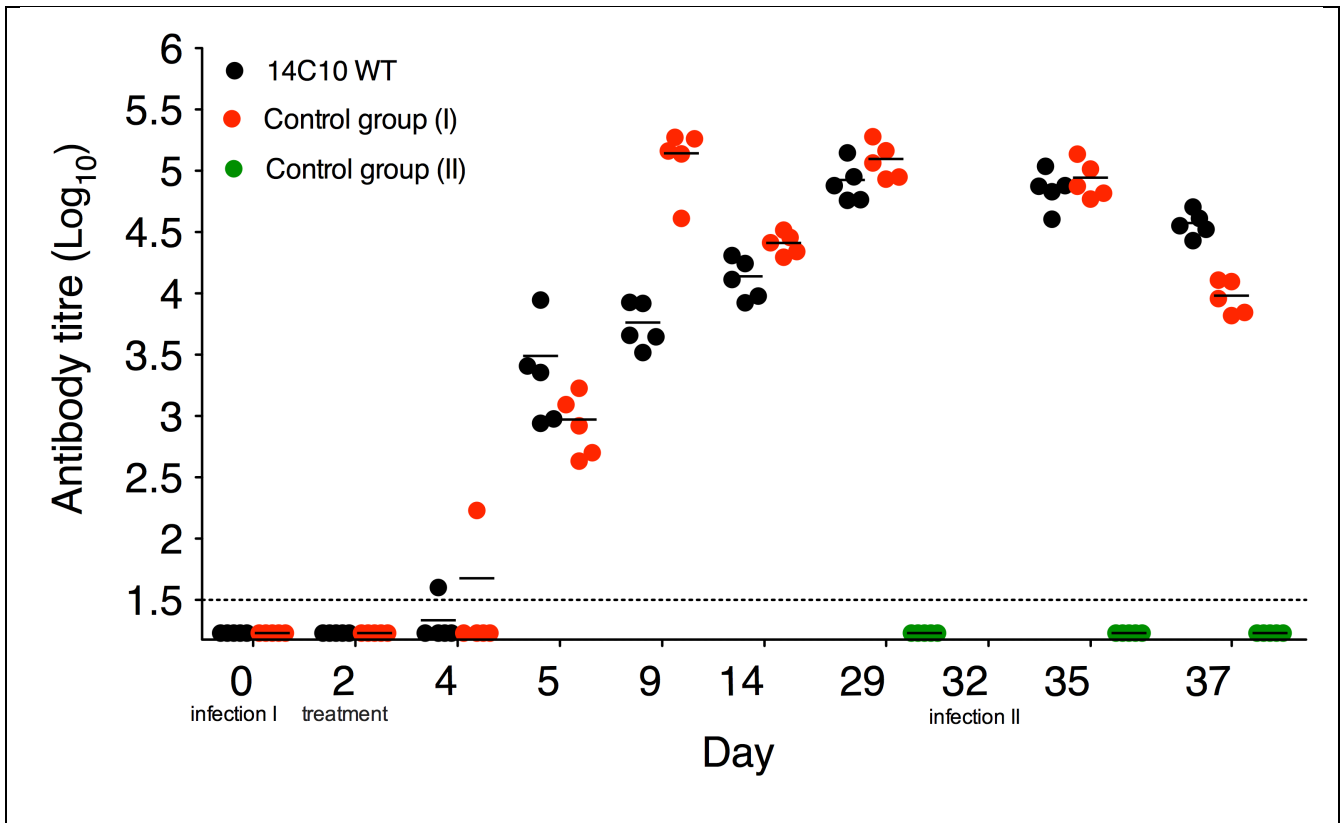
Figure 46. Concentration of mouse endogenous anti-DENV1 antibodies in dengue infected mice.

Blood samples collected in the previous experiment (Figure 45) were here utilized to measure levels of natural anti-DENV1 antibodies. ELISA plates were coated with 14C10 WT at 5µg/ml, 50µl/well overnight. Plates were washed and blocked with skim milk. The strain and batch of the DENV, which was used for the mice inoculation, was added at 10^6 PFU/ml, 50µl per well. Plates were washed and serum samples diluted 1:10 in skim milk were added onto the wells. After subsequent washes goat anti-mouse IgG cross-adsorbed secondary antibody, HRP-conjugate (1:5000) was added and incubated for 1 hour. Plates were washed and TMB substrate was added. The reaction was stopped with 0.1 M sulfuric acid. Absorbance was read at 450nm by spectrophotometer. Results are the average of the duplicate experiment.

6.3.2 Prophylactic administration of 14C10 antibody does not stop the immune system from producing anti-DENV1 antibodies

The experiment described above showed that therapeutic administration with the 14C10 anti-DENV1 antibody does not inhibit the induction of a natural protective anti-DENV1 immune response. The immune response in control mice (I) (DENV1 infected, non-treated), which recovered from primary infection, assured protection against DENV1 during secondary infection. Similarly, a DENV1-specific protective immune response was observed in mice treated therapeutically, which still produced antibodies against DENV1 regardless of the presence of 14C10. As a result, during secondary infection, when 14C10 WT antibody was no longer present in the treated mouse circulation the virus was still cleared from the system. This shows that 14C10 WT treatment does not inhibit the induction of a natural immune response against DENV1.

We next asked whether prophylactic treatment with 14C10 would affect the induction of a natural protective anti-DENV1 response. In this case the 14C10 WT antibody might bind to DENV1 immediately after administration, thus reducing the opportunity of the virus being 'visible' for immune cells. Surprisingly, the mice treated prophylactically did not develop the viremia after secondary re-challenge, at which time 14C10 is expected to be cleared from circulation (Figure 47). Hence, we hypothesized that mice treated with 14C10 WT pre-infection, were still able to mount their own anti-DENV1 antibodies. To exclude the possibility that clearance of the virus after secondary infection was not due to the presence of given antibody we confirmed that given 14C10 WT antibody was cleared from the mouse circulation before the secondary infection we carried out the Elisa. Administered 14C10 WT

antibody was present in the circulation up to day 14 after infection and it was not detectable on the day of secondary infection (day 32). As expected, in both the control groups 14C10 WT was not detectable at any time point. Control group (I) was given PBS instead of 14C10 WT. Control group (II) was infected on day 32 for the first time (Figure 47).

Mice treated with 14C10 antibody pre-infection, still produced an anti-DENV1 response at the comparable level to mice infected non-treated. In control mice (II) which were infected with DENV1 WP74 at 1.3 x 10^7 PFU 200 µl/mouse, three and five days after infection, no endogenous antibodies were detected (green circles). The result showed that presence of 14C10 WT antibody in mouse serum after prophylactic/pre-infection treatment lasted five days longer than after therapeutic/post-infection treatment. It is likely that lower serum levels of DENV1 occurred in the prophylactic model, where the virus replication was prevented was the reason that 14C10 WT persisted longer in the serum and was detectable till day 14. In the therapeutic model, however, the virus could replicate for two days before the post-infection treatment with 14C10 WT and all given 14C10 WT antibody was cleared from the system faster (by day 11 post-infection).
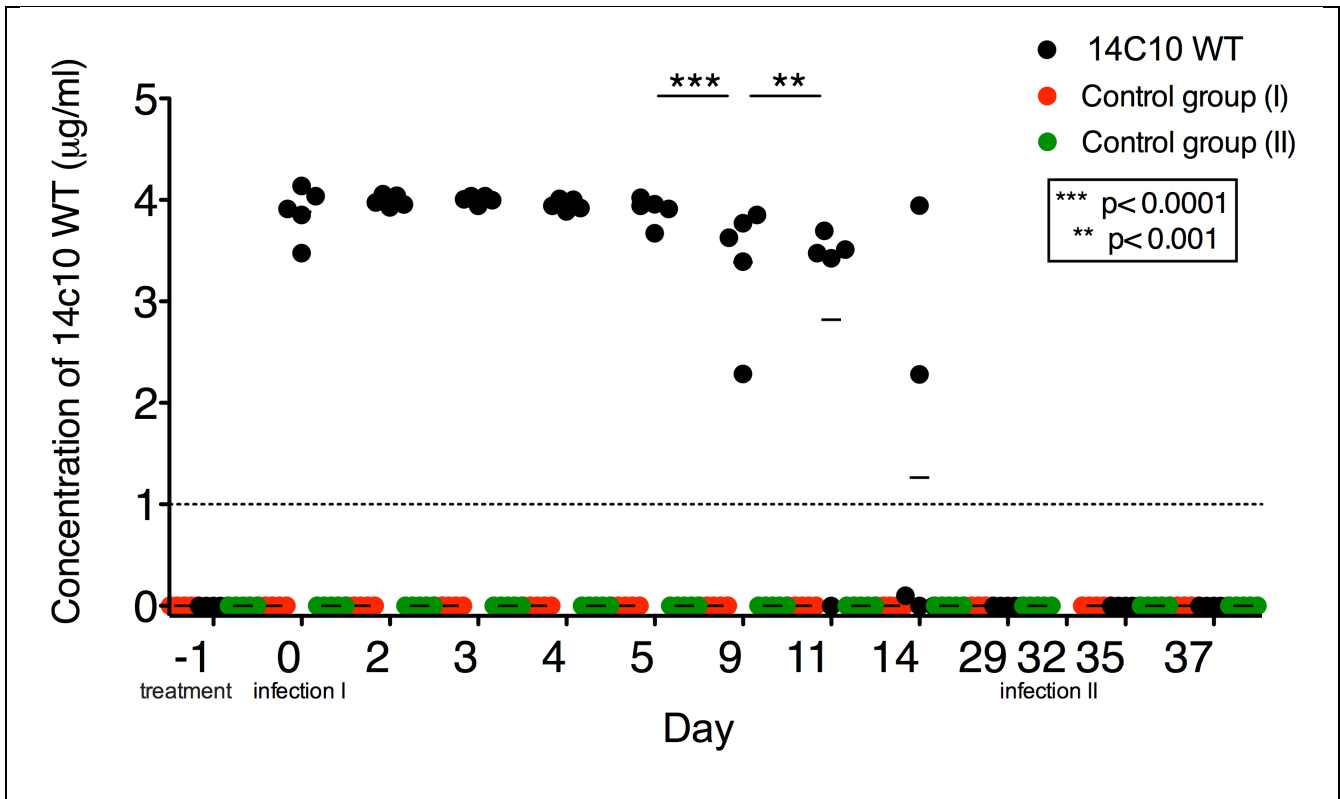
Figure 47. Concentration of 14C10 WT in mouse serum in dengue infected and mAb treated (prophylaxis) mice.

Mice were treated pre-infection with 30 µg of 14C10 WT antibody per mouse on day minus one (black dots). On day zero and on day 32 mice were infected with 200 µl of DENV1 WP74 at 1.3 x 10^7 PFU/ml. Control group (I) – red dots, was given PBS instead of antibody treatment on day minus one and challenged as described above for mice given 14C10. Control group (II) – green dots, was kept naïve until infection on day 32. The experiment was carried out twice with n=5 AG129 mice. Blood samples taken before all the procedures and on days 2, 3, 4, 5, 9, 11, 14, 29, 35 and 37 were used to measure levels of given 14C10 WT antibody. Serum samples were tested by ELISA assay with E1 antibody coated on ELISA plates. Serum samples were diluted 1:10, anti-human HRP conjugate antibody was used as a secondary antibody. Absorbance was read at 450nm by spectrophotometer. Results are the averages of the duplicate reading, **p<0.001, ***p<0.0001.
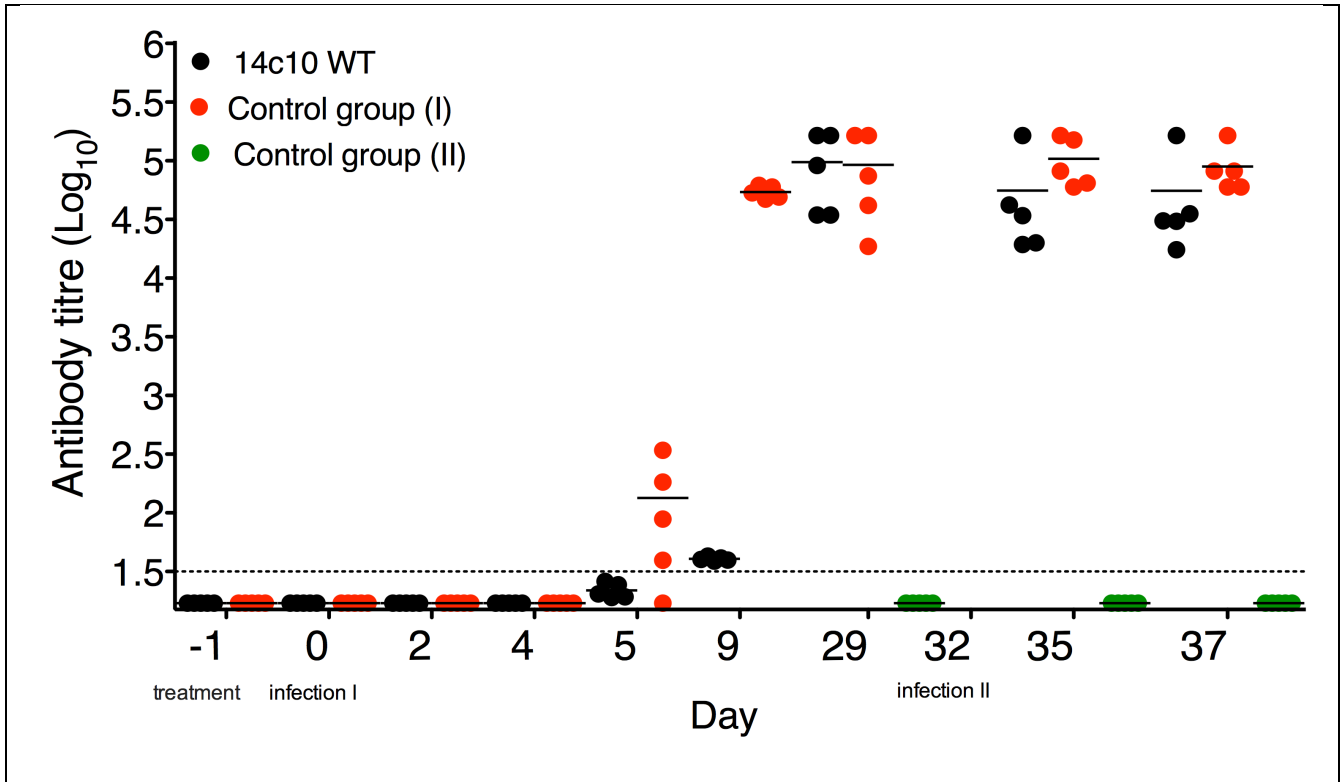
Figure 48. Concentration of mouse anti-DENV1 endogenous antibodies in dengue infected mice.

Blood samples collected in the previous experiment (Figure 47) were here utilized to measure levels of murine anti-DENV1 antibodies. The measurement was done by ELISA. Plates were coated with 14C10 WT overnight, then washed and blocked with skim milk. The strain and batch of the DENV, which was used for the mice inoculation, was added and incubated for 1 hour. Plates were washed and serum samples diluted 1:10 in skim milk were added onto the wells. After subsequent washes goat anti-mouse IgG cross-adsorbed secondary antibody, HRP-conjugate (1:5000) was added and incubated for 1 hour. Plates were washed and TMB substrate was added. The reaction was stopped with 0.1 M sulfuric acid. Absorbance was read at 450 nm by spectrophotometer. Results represent averages of levels of endogenous anti-DENV1 antibodies produced in duplicate experiment.

6.4 Analysis of the *in vivo* treatment with five engineered constructs of 14C10 antibody in the re-challenge experiments

The results obtained in the re-challenge experiments show that mice treated therapeutically or prophylactically with an anti-DENV1 antibody are still able to mount their own immune response against DENV1. In the experiment, mice treated therapeutically or prophylactically were re-challenged with the same strain of DENV1 after the 14C10 WT antibody was cleared from the murine system. The presence of mouse endogenous antibodies after the re-challenge strongly suggests that mice treated with 14C10 WT still produced an adaptive immune response equivalent to those recovered from dengue infection untreated challenged mice - control group (I). The next question that we wanted to address was the impact of the chimerisation and mutation carried out on 14C10 WT antibody on the endogenous immune response and long-time protection. Therefore, we measured and compared the mouse antibody responses in mice treated with five different constructs of 14C10 WT. Serum samples were tested for the presence of DENV1, persistence of administered antibody and natural anti-DENV1 mouse antibodies. Mice treated with one of the constructs LALA, mG1, mG1D265A or mG2a did not develop viremia, similarly to mice treated with 14C10 WT antibody Figure 49. Hence, we hypothesized that they produced endogenous anti-DENV1 antibodies regardless of which 14C10 construct was given. To confirm that the protection was not the result of the persistence of given 14C10 construct over the secondary infection, we measured the concentrations of given antibodies. The results confirmed that given antibodies were cleared from the mouse system by day of secondary infection (Figure 50 - day 32). Interestingly, the levels of endogenous murine

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anti-DENV1 antibodies were comparable in mice treated with any of 14C10 constructs and in non-treated infected mice, demonstrating that the presence of the 14C10 constructs does not inhibit the induction of a natural and protective anti-DENV1 response.
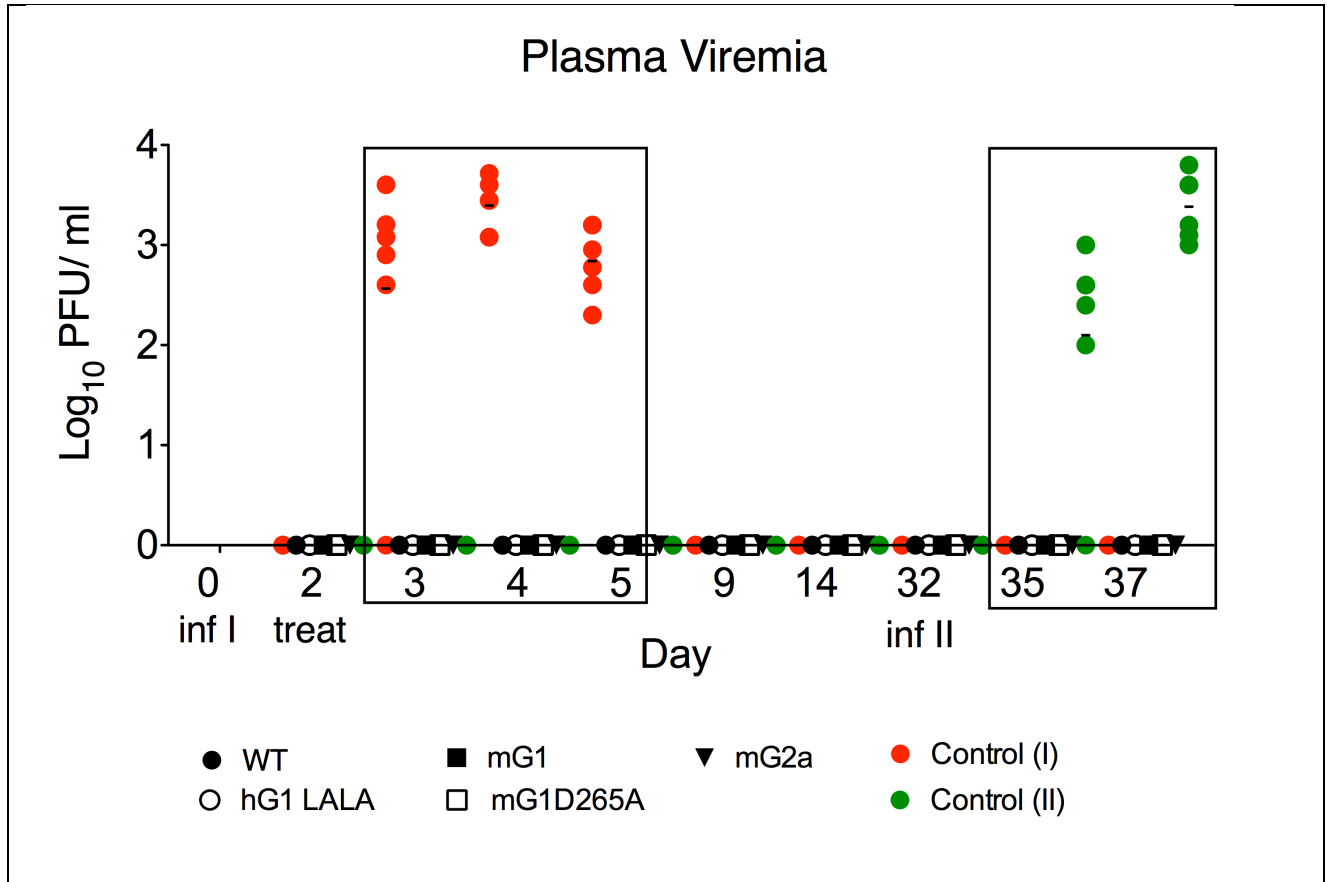
Figure 49. Plasma viremia in mice treated with constructs of 14C10 versus PBS control.

Mice were infected with DENV1 EHI at 1x 10^6 pfu 200 μl/mouse on day zero (inf I) and treated with respective antibody construct at 10 μg/mouse on day two (treat). Blood samples were taken on day 3, 4, 5, 9 and 14. Consequently mice were re-challenged with the same strain and quantity of DENV on day 32 (inf II). Control group (I) – was given PBS instead of an antibody (red circles) and control group (II) – was infected only once, on day 32 (green circles). As expected, plasma viremia was detected in both control groups of mice (I) and (II)– after primary infection in each of the groups, days three-five for control group (I) and days 35-37 for control group (II) – black boxes. Mice treated with one of the constructs (LALA, mG1, mG1D265A, mG2a) did not develop viremia just like mice treated with 14C10 WT antibody.
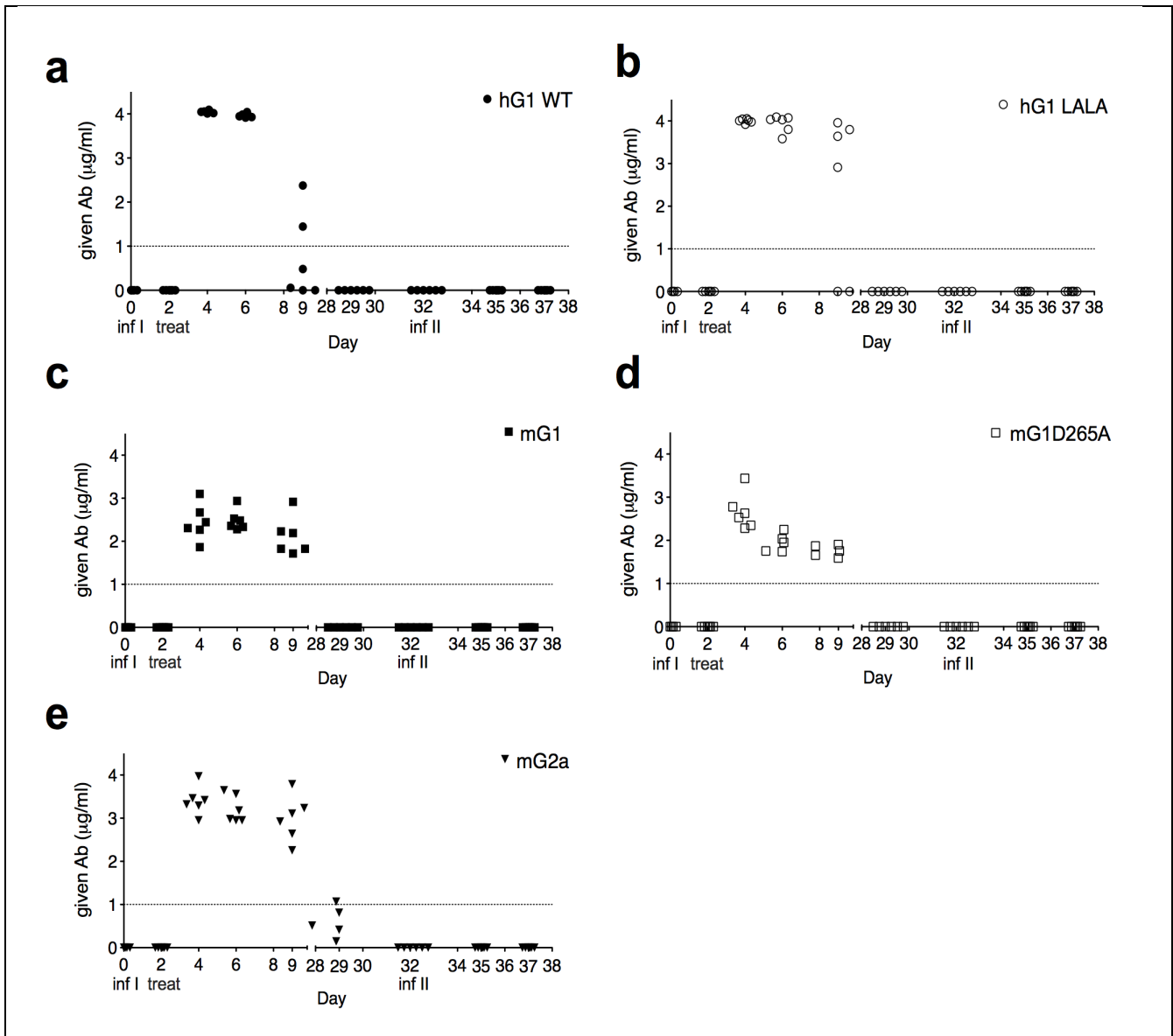
Figure 50. Concentrations of given antibodies in mouse serum over the course of primary and secondary infection.

Mice were infected with DENV1 EHI at 1x 10^6 pfu 200 µl/mouse on day zero (inf I) and treated with one of 14C10 antibody constructs (treat): (**a**) hG1 WT, (**b**) hG1LALA, (**c**) mG1, (**d**) mG1D265A, (**e**) mG2a, at 10 µg/mouse on day two. Blood samples were taken on day 4, 6, 9 and 29. Mice were re-challenged with the same strain and quantity of DENV1 on day 32 (inf II). Control group (I) – was given PBS instead of an antibody. In all groups given antibody was cleared before the secondary infection. That is why, we hypothesized that mice treated with one of the constructs and mice infected not treated did not develop viremia due to endogenous mouse immune response.
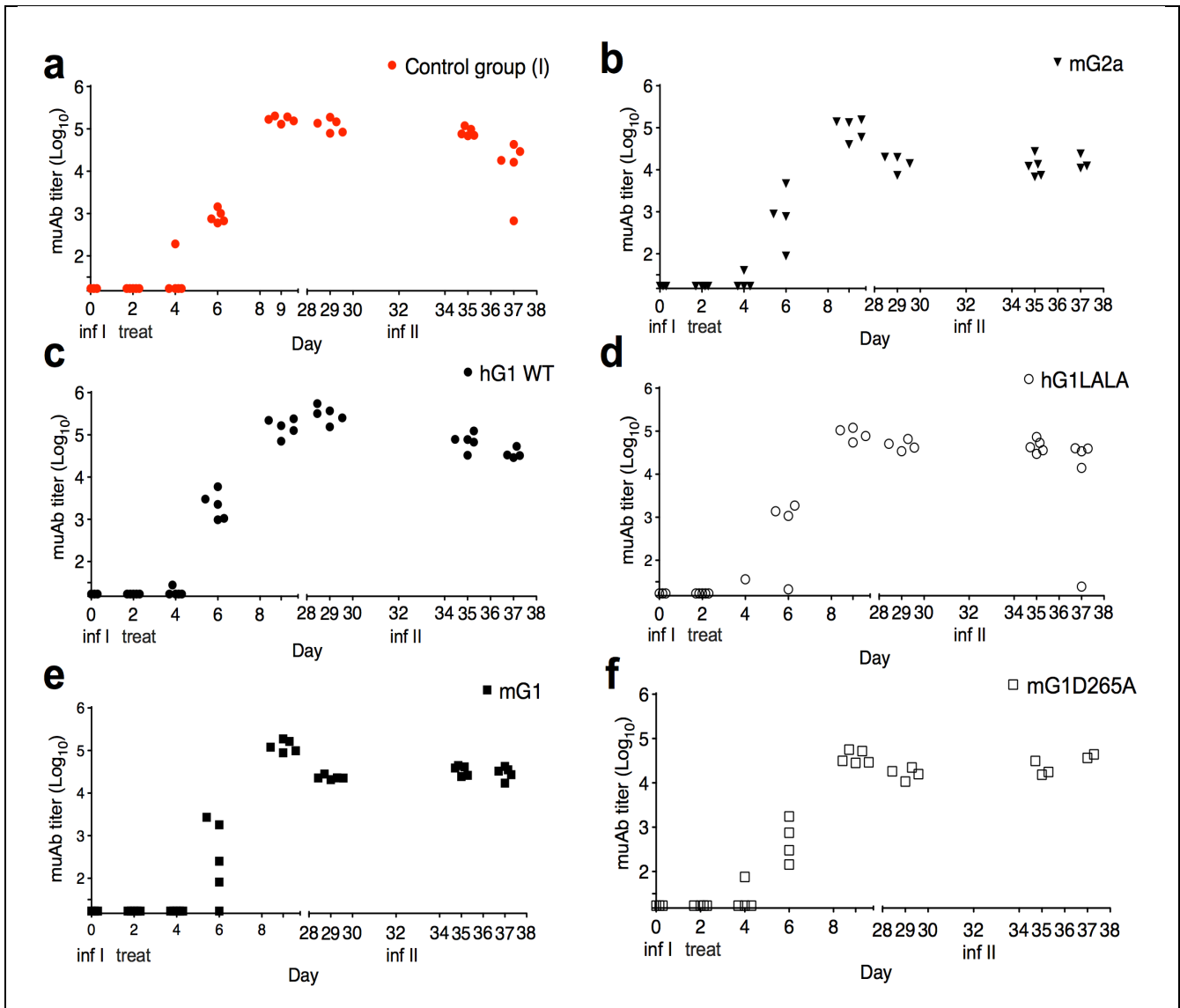
Figure 51. Murine anti-DENV1 endogenous IgG levels in the mouse serum over the course of primary and secondary infection.

Mice were infected with DENV1 EHI at 1x 10^6 pfu 200µl/mouse on day zero and re-challenged on day 32, antibody was given on day two post-infection with one of 14C10 antibody constructs at 10 µg: (**a**) Control group, treated with PBS, (**b**) mG2a, (**c**) 14C10 WT, (**d**) hG1LALA, (**e**) mG1 (**f**) mG1D265A. Murine anti-DENV1 antibodies in serum were assessed with ELISA assay and the averages of duplicate experiment are presented as data points.

6.5 Anti-human IgG response of the mouse immune system

Since the constructs of 14C10 antibody used in our studies were either chimeric human-mouse antibodies or fully human antibodies, we hypothesized that the immune system of the mouse would produce an anti-human IgG response. ELISA testing of the serum samples from the previous experiments confirmed that all mice infected with DENV1 and treated with one of 14C10 antibody constructs produced endogenous antibodies against the foreign antibody (Figure 52). It was not surprising that the endogenous anti-human response was stronger in mice treated with fully human antibodies (14C10 WT and hG1LALA) as compared to mice treated with human-mouse chimeric antibodies. There was no significant difference in the level of endogenous antibodies produced in mice treated with 14C10mG1 antibody and its D265A version. The lowest response was detected in mice treated with the mG2a subclass of 14C10 antibody.
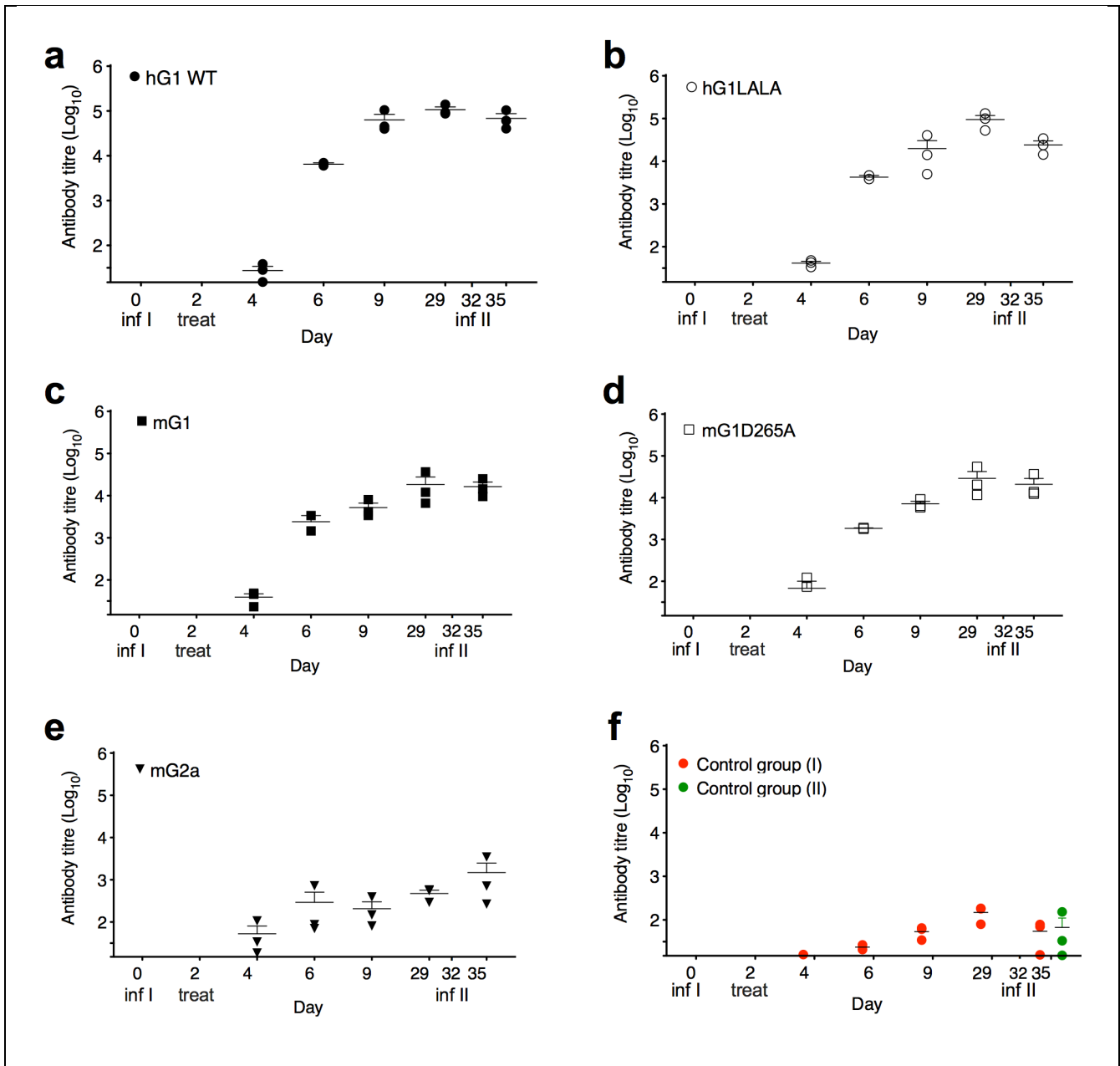
Figure 52. Anti human response of the mouse immune system.

To measure anti-human response in mice, which were infected with 200µl DENV1 EHI at 1x 10^6 PFU/ml on day zero (inf I) and day 32 (inf II) and then treated on day plus two with one of 14C10 antibody constructs at 10 µg (treat): (**a**) WT, (**b**) hG1LALA, (**c**) mG1, (**d**) mG1D265A, (**e**) mG2a serum samples were tested by ELISA. All mice treated with constructs (a-e) produced endogenous antibodies against the foreign antibody. The averages of duplicate ELISA experiments are presented as data points. Control group (I) – was given PBS instead of an antibody.

6.6 Effects of the 14C10 treatment vs. effects of the HA4 anti Influenza antibody treatment

Surprising outcome of the treatment with 14C10 antibody resulted in our general hypothesis that an administration of 14C10 antibody neutralizes the virus but does not stop the immune system of the host from making their own antibodies, which affirms the long-term protection. Thus, we approached our collaborators working on Influenza A virus and anti Influenza A antibodies to reproduce our experiments in the respective mouse model.

6.6.1 Influenza Virus

Influenza A is a prototypic, negative-sense RNA Orthomyxovirus that causes regular seasonal epidemics and periodic pandemics (Juergen and Mueller 2002, Palese 2004, Taubenberger* and Morens† 2006). Current anti-viral therapies have limited utility in outbreak situations (Lee, Yap et al. 2010) and the employment of M2 ion channel inhibitors and/or neuraminidase inhibitors usually results in the rapid development of resistance (Li, Guan et al. 2004, Tran Tinh Hien, Nguyen Thanh Liem et al. 2004, Hurt, Holien et al. 2009).

6.6.2 Vaccination and antibody therapy

The Influenza A vaccine requires yearly re-formulation for both Northern and Southern hemispheres, and this also greatly reduces its utility as a suitable response for new outbreaks (Li, Guan et al. 2004, Tran Tinh Hien, Nguyen Thanh Liem et al. 2004, Hurt, Holien et al. 2009). Passive antibody therapy is being actively explored as an alternative approach for DENV, Inf A and other emerging infections where current prophylaxes (vaccines) and specific therapies are limited (Casadevall, Dadachova et al. 2004, Hanson, Boon et al. 2006, Casadevall and Pirofski 2015, Shriver, Trevejo et al. 2015). This is based on the relative speed with which neutralizing antibodies can be isolated and/or cloned from infected / recovered patients (Pinna, Corti et al. 2009). The HA4 antibody is fully human antibody engineered by Brendon Hanson's lab, which was shown previously to protect mice infected with 5 x MLD50 dose of lethal strain of influenza A virus.

6.6.3 Influenza mouse model

Most influenza virus studies in mice engage the BALB/C or C57BL/6 mouse model and the lab adapted A/Puerto Rico/8/1934 (H1N1) [PR8] or A/WSN/1933 (H1N1) [WSN] influenza viruses (Bouvier and Lowen 2010). In our study, we employed the BALB/C strain in conjunction with A/Puerto Rico/8/1934 (H1N1) [PR8]. The model is highly susceptible to disease and death after intranasal inoculation of virus. Infected mice lose appetite and weight and require euthanasia by day 8 of infection. The read-out of the disease is weight loss and death/survival.

6.7 Reproduction of the re-challenge therapy and prophylaxis schemes in the Influenza model

In the DENV model, mice given 14C10 WT antibody one day before infection did not develop viremia. 14C10 WT was cleared by day 29, yet mice were protected against secondary infection with DENV1 on day 32. The measurement of murine anti-DENV1 response showed that treated mice produced their own IgG antibodies and were protected over subsequent infection. Here we carried out experiments utilizing another RNA virus in the analogous models (pre or post-infection schemes) to test whether the phenomenon found in dengue infection model can be translated into a more general rule that an administration of the anti-viral antibodies has no negative impact on the development of natural immunity.

6.7.1 Therapeutic influenza re-challenge scheme

By analogy to the previously described Dengue re-challenge model, an infection of BALB/C mice with influenza virus A/Puerto Rico/8/1934 [PR8] at 250 PFU per mouse on day zero was performed. Mice were infected intranasally on day zero and treated with an antibody via intraperitoneal route two days post-infection. It is a lethal model, and weight loss and death/survival rate are the read-outs of the disease.
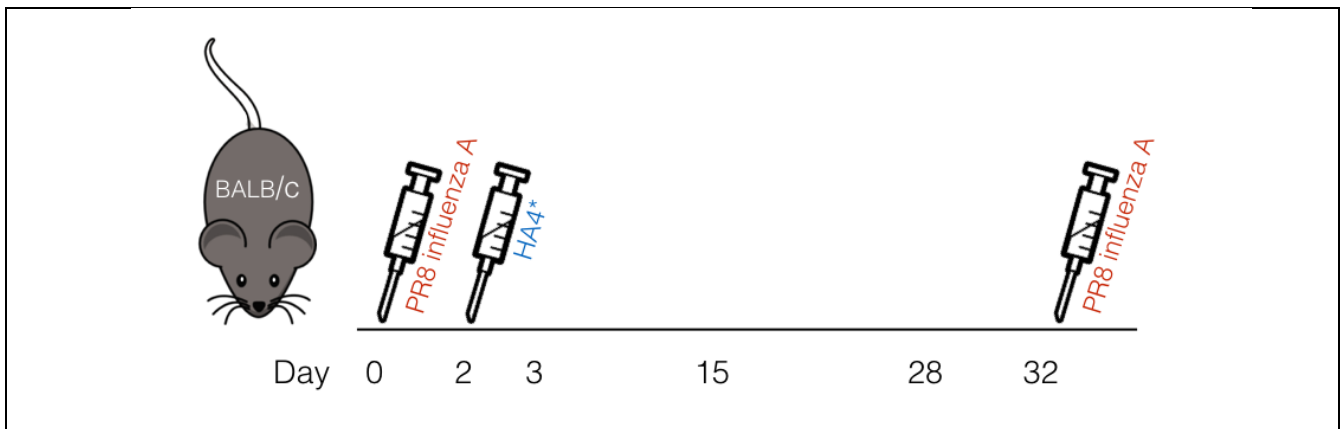
Figure 53. HA4 antibody therapy regime of mice infected with PR8 influenza A virus.

After intranasal infection with A/Puerto Rico/8/1934 virus [PR8] at 250 PFU on day zero, BALB/C mice were treated intraperitoneally with HA4 antibody at 450 µg per mouse on day two (n≥6). Control group (I) was given PBS instead of HA4 antibody. To measure the level of HA4 antibody and host's anti-influenza IgG level in the HA4 treated mice, blood samples were taken on day 3, 15 and 28. On day 32 mice were re-challenged with A/Puerto Rico/8/1934 virus [PR8]. Control group (II) was infected only once, on day 32.

HA4 treatment of mice infected with lethal strain of influenza virus [PR8] prevented weight loss (Figure 55) and resulted in 100% survival rate following primary infection. Most importantly, treated mice were protected from a re-challenge on day 32 (Figure 54). To confirm the hypothesis that the long-time protection was a result of the presence of natural anti-influenza IgG antibodies, we collected and analysed mouse serum for the presence of administered HA4 antibody and natural murine IgG antibodies. Administered antibody was undetectable in sera by day 15 (Figure 56a). At the same time, murine anti-influenza IgG antibody was detected on day 15. Thus, we concluded that treatment with PR8-specific antibody did not hamper the development of mouse anti-influenza IgG (Figure 56b). These results suggest that an administration of a therapeutic antibody does not impair the development of a natural immune response with associated long-term protection against the same strain of virus, suggesting that this phenomenon was not only DENV-specific.
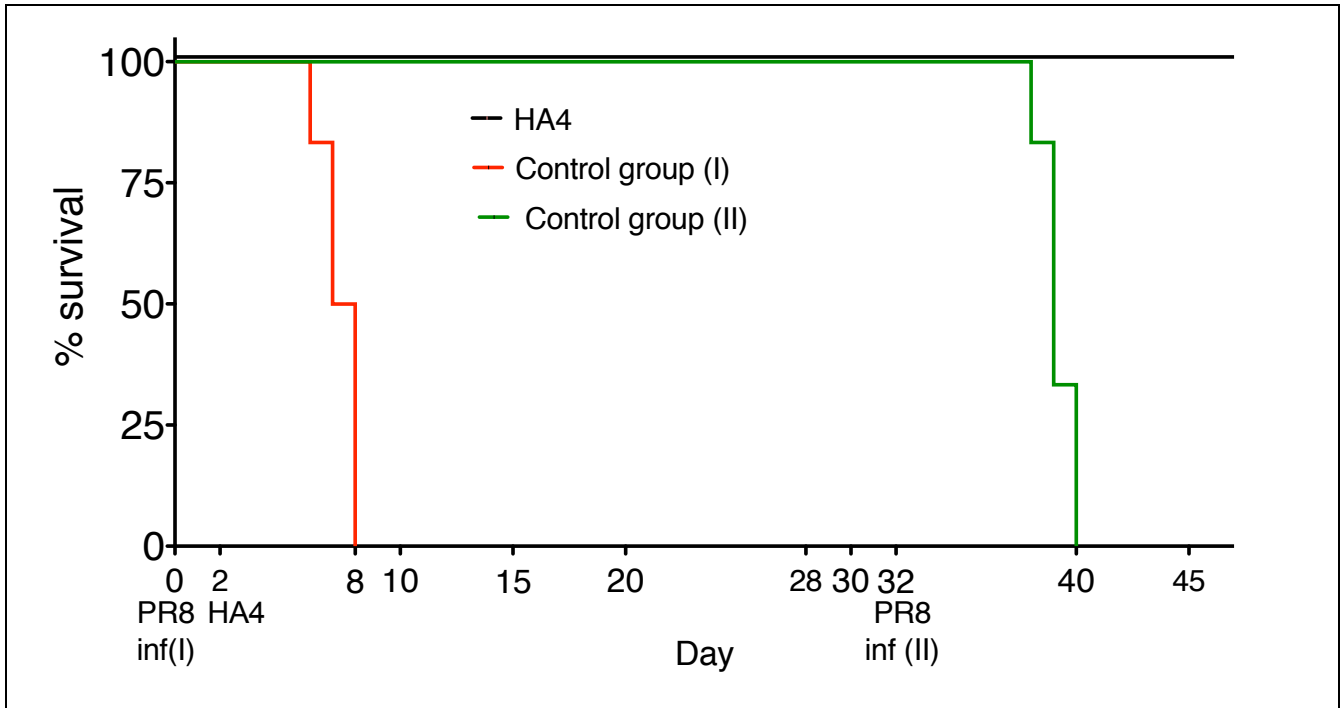
Figure 54. HA4 antibody thearapy protects PR8 infected mice from death.

Survival curve of mice infected with PR8 and treated therapeutically with HA4. Mice (n≥6) were infected with PR8 at 250 PFU on day zero [PR8 inf (I)] and treated with HA4 antibody at 450 µg on day two post-infection. All mice, which belonged to control group (I) were given PBS instead of HA4 antibody and all of them died by day 8. On the contrary, all HA4 treated mice survived the infection. On day 32 mice, which survived the primary infection were re-challenged with the same dose of PR8 [PR8 (II)]. All those mice survived the secondary infection. Control group (II) was infected only once, on day 32 and all mice from this group succumbed to infection within 8 days.

Figure 55. HA4 antibody therapy protects influenza infected mice from weight loss.

Percentage weight change in mice infected with PR8 virus and treated post-infection with HA4 antibody (in black) compared to mice infected, non-treated in red (**a**) and green (**b**). Weight change values represent mean ± s.e.m. n≥6 mice per group.

Figure 56. Anti-infectious antibody administered therapeutically does not stop hamper the development of mouse IgG.

Mice (n≥6) were infected with A/Puerto Rico/8/1934 virus [PR8] at 250 PFU on day zero and treated with HA4 antibody at 450 µg on day two post-infection. In vivo experiment showed that passive immunization with HA4 did not affect host's ability to mount its own immunity against influenza infection (shown in secondary challenge). Hence it was desired to analyse the amount of host's murine antibodies raised over the course of primary infection; when the mouse was treated with HA4 IgG1. To measure the levels of HA4 and murine natural IgG antibodies in the HA4 treated mice, blood samples were taken on day 3, 15 and 28. (**a**) HA4 antibody was detected on day 3 but not later on day 15 or 28. (**b**) On the contrary, murine anti-PR8 IgG was not detectable on day 3 but it was detected on day 15 and 28.

6.7.2 Prophylactic influenza re-challenge scheme

BALB/C mice were first treated with HA4 antibody intraperitoneally on day minus one. Subsequently, the animals were infected intranasally influenza virus A/Puerto Rico/8/1934 [PR8] at 250 PFU per mouse on day zero.
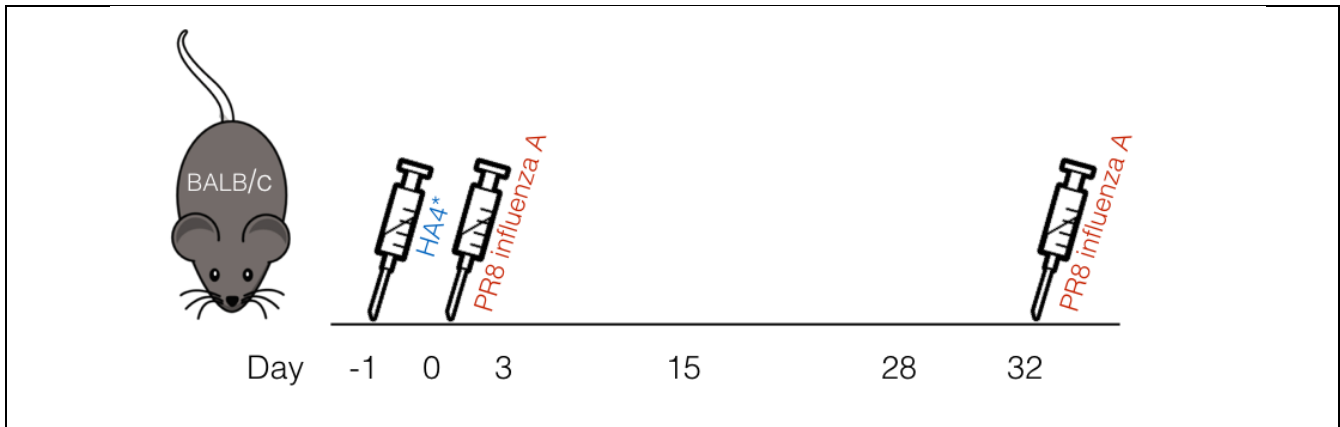
Figure 57. HA4 antibody prophylaxis regime of mice infected with PR8 influenza A virus.

BALB/C mice were first treated intraperitoneally with HA4 antibody at 450 µg per mouse on day minus one and then infected intranasally with A/Puerto Rico/8/1934 virus [PR8] at 250 PFU on day zero (n6). Control group (I) was given PBS instead of HA4 antibody. Blood samples were taken on day zero, 3, 15 and 28 to control the levels of HA4 antibody and host's anti-influenza IgG levels. On day 32 mice were re-challenged with A/Puerto Rico/8/1934 virus [PR8]. Control group (II) was infected only once, on day 32.

Mice administered with HA4 antibody one day before PR8 infection survived both primary and secondary infection (Figure 58) and maintained their body weight over the whole experiment (Figure 59). HA4 antibody was cleared in 8 out of 9 mice by day 28 (Figure 60a) and murine anti-PR8 antibodies were detected on days 15 and 28 (Figure 60b). These findings confirmed the previous finding that mice given anti-Inf A antibodies prophylactically still develop their own anti-influenza antibody response that protects them from subsequent infection.
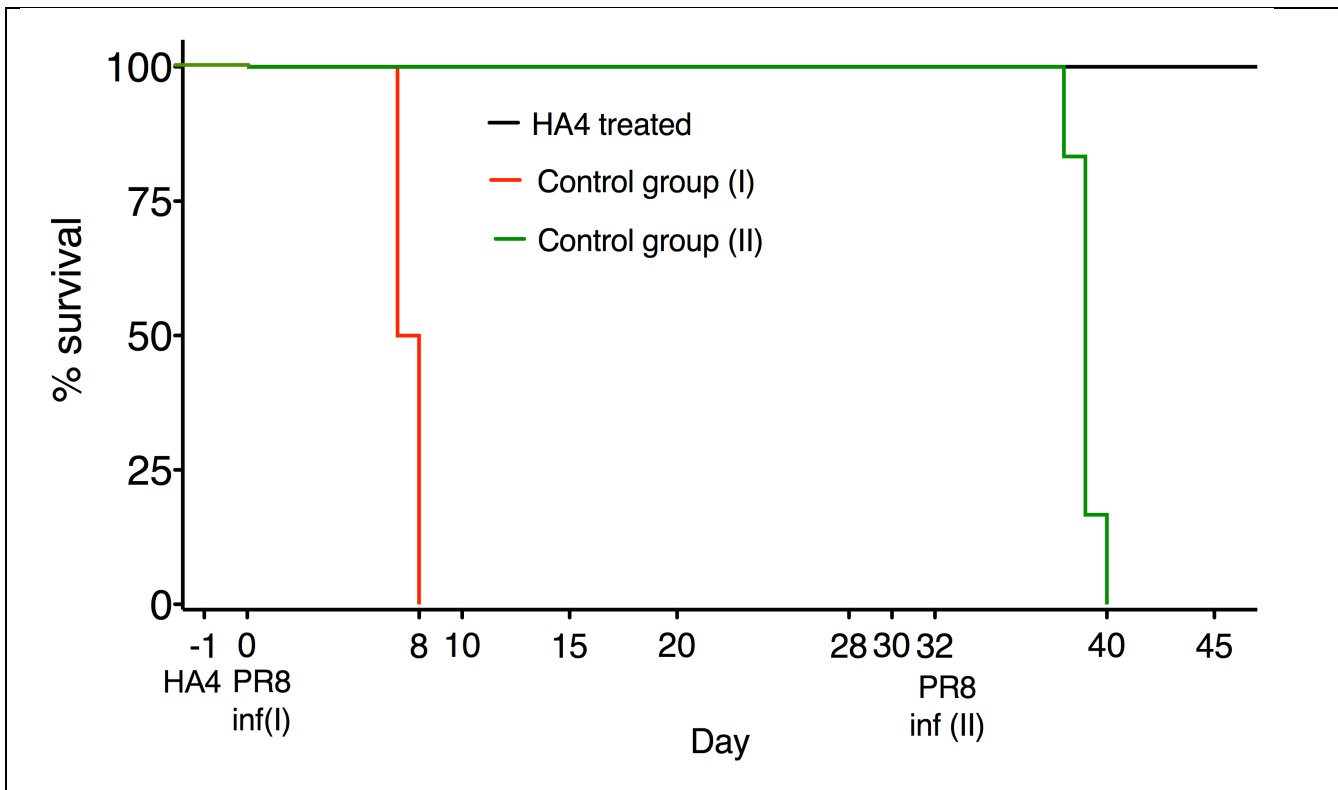
Figure 58. Survival curve of the mice infected with PR8 and treated prophylactically with HA4.

Mice (n≥6) were first treated with HA4 antibody at 450 µg per mouse on day minus one and then infected with PR8 at 250 PFU on day zero [PR8 inf(I)]. Control group (I) – red, was given PBS instead of HA4 antibody and all the mice died by day 8. On day 32 mice were re-challenged with PR8 [PR8 inf(I)]. Control group (II) –green, was infected only once, on day 32 and all the mice from this group died within 8 days (by day 40). All the HA4 treated mice survived the primary and secondary infection.
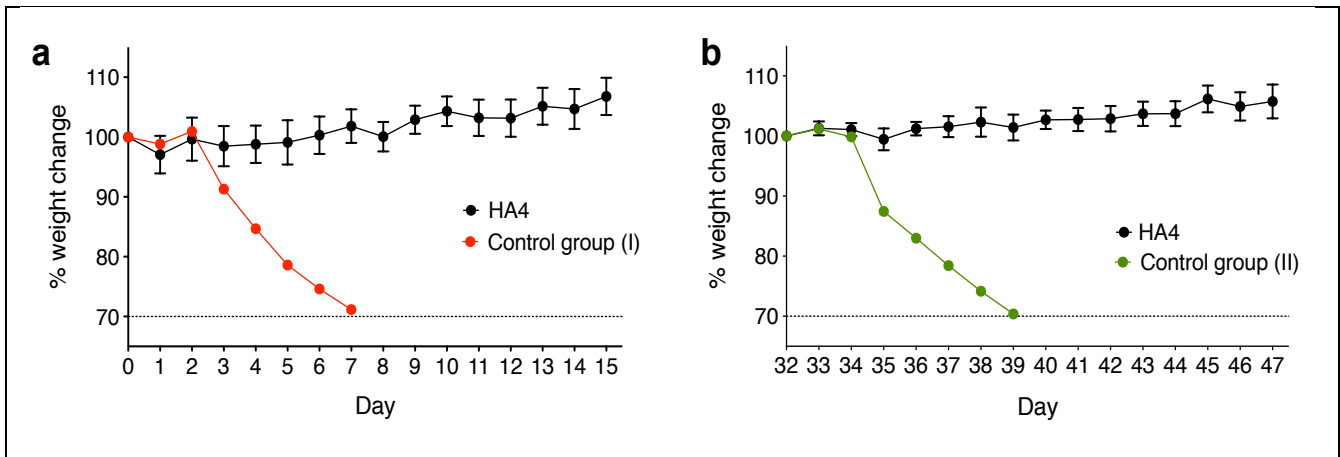
Figure 59. HA4 antibody prophylaxis protects influenza infected mice from weight loss.

Percentage weight change in mice infected with PR8 virus and treated with HA4 antibody (black dots) compared to mice infected, non-treated in red (**a**) and green (**b**). Weight change values represent mean ± s.e.m. n≥6 mice per group.
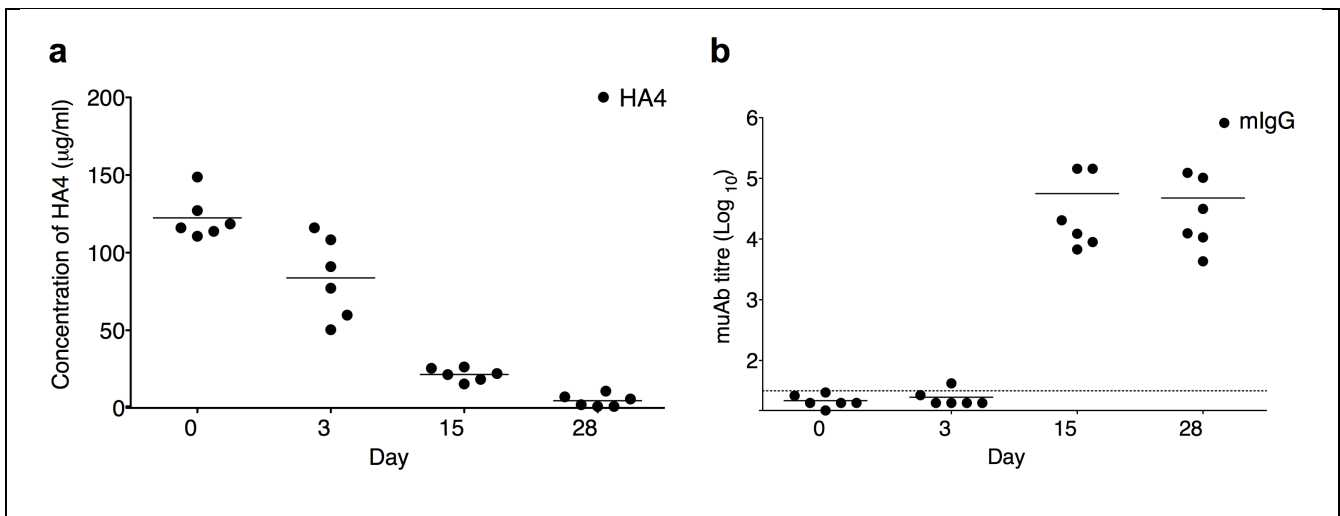
Figure 60. Anti-infectious antibody administered prophylactically does not stop hamper the development of mouse IgG.

Mice (n≥6) were treated with HA4 antibody at 450 µg per mouse on day minus one and then infected with A/Puerto Rico/8/1934 virus [PR8] at 250 PFU on day zero. Blood samples were taken on day 3, 15 and 28 to control the levels of HA4 administered antibody and host's anti-PR8 IgG levels. (**a**) HA4 antibody was detected on day zero and 3 but not on day 15 or 28. (**b**) The presence of HA4 antibody in the serum before introduction of the virus did not stop the immune system from producing natural murine anti-influenza IgG (detected on day 15 and 28).

7 Discussion

7.1 Complexity of the immune response against DENV and antibody therapy

The outcome of a DENV infection depends on a complex interplay of intrinsic (host) and extrinsic (virological) factors.

7.1.1 Correlation between viremia level and disease severity

Even though it is believed that a molecule which could be delivered into a patient and reduce viral load in the system might have an impact on the disease manifestation, one needs to be aware that the correlation between viral titer and clinical manifestation is still unproven. According to a study by Vaughn *et al.*, DENV quantity in blood correlates with dengue severity (Vaughn, Green *et al.* 2000). The team analysed four groups of around 50 patients at two hospitals in Thailand, each group infected with one of the DENV serotypes. The results showed that the severity of dengue disease depended on DENV level, virus serotype and antibody response pattern. Interestingly, the symptoms in patients infected with DENV2 were relatively more serious than in all the other groups. Moreover, in more than 80% of secondary dengue cases the symptoms were more severe when compared with primary dengue cases (Vaughn, Green *et al.* 2000). Another group utilized blood samples from pediatric patients of a hospital-based study in Thailand (Libraty, Endy et al. 2002). Fifty-four children with secondary DENV infections, all infected with DENV3, participated in the study. The analysis of viremia levels yielded a direct relation between higher mean viral load and severe outcome in the early stages of dengue disease including

plasma leakage and low platelet count. However, the high levels of cytokines such as IL-10, TNF related to thrombocytopenia and the elevated levels of ALT to plasma soluble IL-2 receptor levels independently of viral load (Libraty, Endy et al. 2002). Similarly, the results of a study conducted in Taiwan demonstrated clear correlation between viremia level and disease severity (Wang, Chao et al. 2003). The cohort consisted of 20 adults infected with DENV3, of which 9 were diagnosed with DF and 11 with DHF. During febrile illness, the blood of DHF patients showed higher viremia levels than the blood of DF patients. In addition, during defervescence, the viral RNA was undetectable in DF patients while in DHF patients it was still elevated (Wang, Chao et al. 2003). The same group carried out another study in Taiwan that included 103 DENV2-infected patients and reported consistent results that DHF patients had significantly higher DENV-containing immune complexes and lower clearance rate than DF patients (Wang, Chen et al. 2006).

In contrast, a more recent study showed lack of association between DENV viral load and dengue disease severity (Singla, Kar et al. 2016). This time the research was conducted in pediatric dengue patients experiencing primary or secondary DENV infection in New Delhi, India. Thirty cases were classified as non-severe dengue warning signs, 21 patients were diagnosed with DF, 46 children had severe dengue and 5 cases were fatal. High viremia was correlated with delayed recovery and low platelet count, but no relation with the disease severity was observed (Singla, Kar et al. 2016). Another study, which examined the role of interplay of intrinsic factors in the pathogenesis of severe dengue disease, was conducted in Vietnam in a cohort of 158 adults (Fox, Le et al. 2011). The authors did not observe a relation between viral load and disease severity and suggested

that it could be more prevalent during secondary infection while most of their cases were primary dengue disease (Fox, Le et al. 2011). However, the relation between primary or secondary DENV infection and disease severity also remains unclear.

Notwithstanding the described controversies, the current trend in dengue research is to pursue inhibitors against DENV and thus 14C10 antibody is a potential antiviral therapeutic for dengue disease caused by DENV1. The high viremia levels in patients with secondary infection and probable ADE effect might be correlated with severe clinical manifestation of the disease. Since the viremic peak of the virus in patients is relatively long (6 to 7 days), it gives a wide window for therapeutic intervention.

7.1.2 Antibody therapy

One of the key challenges in dengue therapy is treating the overwhelming inflammatory immune response (including a strong cytotoxic T cell response) that is engendered by acute DENV infection. This is why an ideal dengue therapy needs not only to focus on the viral load, but also should deal with the inflammatory response caused by the DENV. There are two potential ways of achieving this aim. One way would be an inhibition of the virus and subsequent control of inflammatory response, for instance by additional immunosuppression. The second, safer and more efficient way would be to block and clear the virus simultaneously, inducing decrease in bioavailability of viral molecular patterns driving the inflammatory response. Antibody 14C10 is a single molecule that could potentially accomplish both these goals. The main doubt related to 14C10 antibody treatment is the possible homotypic ADE effect. A mutation in the Fc portion might be a way

to overcome this concern. Nonetheless, this mutation might affect the number of processes, which are triggered via FcRs binding. My project aimed to determine the role of antibody FcR binding regions in DENV neutralization defined as the loss of infectivity through reaction of the virus with specific antibody and clearance of the virus from the system.

I described here engineering of five constructs of 14C10 antibody, namely 14C10 WT, 14C10hG1LALA, 14C10mG1D265A, 14C10mG1 and 14C10mG2aEXT (Chapter 4). 14C10hG1LALA and 14C10mG1D265A were utilized to study the role of FcγRs in the DENV neutralization and clearance. The study of the LALA and D265A versions of 14C10 antibody were based on the previous results of other groups (Balsitis, Williams et al. 2010, Corti, Voss et al. 2011, DiLillo, Tan et al. 2014). As an example, the group of Antonio Lanzavecchia studied the LALA modification of FI6 antibody. FI6 is a neutralizing anti influenza antibody, which binds to   Group 1 and Group 2 Influenza A Hemagglutinins and was shown to fully protect mice from lethal infection with A/Puerto Rico/8/34 (H1N1) virus after at 4mg/kg pre-infection and at 15mg/kg post-infection (Corti, Voss et al. 2011). The LALA modification of the Fc portion of the FI6 antibody did not hamper *in vitro* binding and neutralizing activity. The half-lives of the mutated and wild type versions were comparable since the mutation does not affect the FcRN binding. However, treatment of lethally infected mice with a 2.5 higher dose of 10mg/kg of FI6-LALA protected only 40% of the animals. Consequently, an administration of FI6-LALA at 3mg/kg protected only 20% of lethally infected mice. The results led to a conclusion that the lack of complement and FcR binding substantially decreases FI6-LALA functional activity *in vivo* (Corti, Voss et al. 2011). Fc-mediated functions seemed to be crucial for the clearance of the virus from the system.

Thus, no significant difference between 14C10 WT and 14C10hG1LALA *in vivo* efficacy was an unexpected result. Two other constructs, 14C10mG1 and 14C10mG2aEXT, representing mouse IgG1 and mouse IgG2 were used to determine the influence of the immunoglobulin subclass on neutralizing affinity. Similarly, the results showed no significant difference in the neutralizing affinity *in vivo*. It confirmed very high affinity of 14C10 antibody and suggested that 14C10hG1LALA might be an answer to the potential homotypic ADE.

Additionally, it seems probable that the mechanism of action of broadly neutralizing antibodies and strain specific antibodies are different. This could be the reason why the modification of the Fc portion of an antibody affects the *in vivo* efficacy of broadly neutralizing antibodies but not the specific ones. Our 14C10 WT antibody is a DENV1-specific antibody, and the modifications that we made did not change the neutralizing activities *in vivo.*

A phase I clinical trial for 14C10 is planned to start in 2017, and it will include testing the pharmacokinetics (PK) of the antibody. For the assessment of 14C10 WT concentration in serum an anti-idiotypic clone of anti-14C10 antibody (E1) was generated in our lab (Lim, Chan et al. 2015). E1 is a chimeric mouse-human antibody (mG2a/hG3) and was used to trace the 14C10 levels in the prophylactic and therapy models in the AG129 mice. The results showed that in mice infected and treated with 14C10 WT, the 14C10 WT antibody was cleared from the system between days 5 and 14, and no 14C10 was detected at the time of the subsequent infection. At the same time the endogenous protection response in mice was measured by sandwich ELISA. Regardless of the 14C10 antibody treatment post or pre-infection, mice generated their own antibodies against DENV1, and this immune

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response assured virus clearance during subsequent DENV1 infection. At the same time, the *in vivo* experiment complements the *in vitro* tests, thus E1 is a potential assay antibody, which can be used to determine the concentration of serum 14C10 antibody in clinical trials.

7.2 Molecular biology of DENV clearance, neutralization and post infective immunity

We looked into the potential mechanisms of neutralizing and clearing the virus as well as the development of post-infective immunity. We observed that the wild type antibody and its LALA or D265A versions, which do not involve the FcR binding in the neutralization or clearance process, still neutralize and trigger the clearance of the virus. The hypothetical cellular mechanism behind acquiring long-time protection against DENV1 regardless of the antibody treatment during the first infection are run by the complement components and low affinity antibodies like IgM, which are present in mouse circulation as fully functional antibodies. It is likely that the 14C10 antibodies bind and neutralize the virus through blocking the cells that DENV needs for a productive infection. Simultaneously, natural immune components attach to the virus and render it susceptible to uptake via complement receptors and low affinity Fc receptors leading to immune clearance. Thus, the immune complexes are processed regardless of the lack of the involvement of Fc binding of the administered antibodies.

7.3 Alternative vaccine strategy - Ring fence prophylaxis

Since our suggested alternative vaccine approach is dependent on individuals getting infected naturally, it would be useful to examine whether the same effect would be obtained if the antibody and virus, in an attenuated or inactivated form, were administered at the same time, so that a single injection would guarantee protection. Interestingly, in the prophylactic setup, human antibodies persisted for longer in the treated mice compared to the therapeutic setting. This may reflect the relative numbers of viral pathogens that require antibody/binding neutralization for the clearance effect. Under conditions of post-infection treatment, the virus is able to replicate and expand to greater numbers than is possible with prophylaxis. In the prophylactic model, the virus was neutralised almost immediately since 14C10 antibody was administered one day before an infection.

In the dengue model of infection, the levels of induced anti-DENV1 IgG antibodies in mice infected and non-treated were similar to those in mice treated with 14C10 WT pre-infection (Figure 48). Similarly, in the influenza model of infection, there was a significant increase in anti-PR8 antibody production by day 15 in mice treated with anti-Inf A antibody pre-infection (Figure 59). The induction of antibody responses in mice treated prophylactically with monoclonal antibodies demonstrate that passive immunization does not impair the induction of a host immune response against the specific virus.

These data suggest that passive immunization does not inhibit the induction of an antibody response in the host, even when given before infection, suggesting that antibodies can also be used in a prophylactic manner to prevent individuals from subsequent infection. For example, anti-DENV1 or anti-Inf A mAbs could potentially be administered to patients

diagnosed with DENV1 or with Influenza A (as post-infection therapies) as well as at-risk individuals in their proximity (ring-fence prophylaxis) to limit the spread of the viruses.

In our alternative vaccine strategy, people living in the proximity of the infected people would be protected with the administered antibody and in case of the natural exposure to virus they would develop protective immunity just like recovered (treated or non-treated) patients (Figure 61). The process of vaccine development is time-consuming. Our findings open the possibility on the formulation combining mAbs with an infective agent in a much quicker process. This necessitates further studies employing more viruses in the similar experimental models.
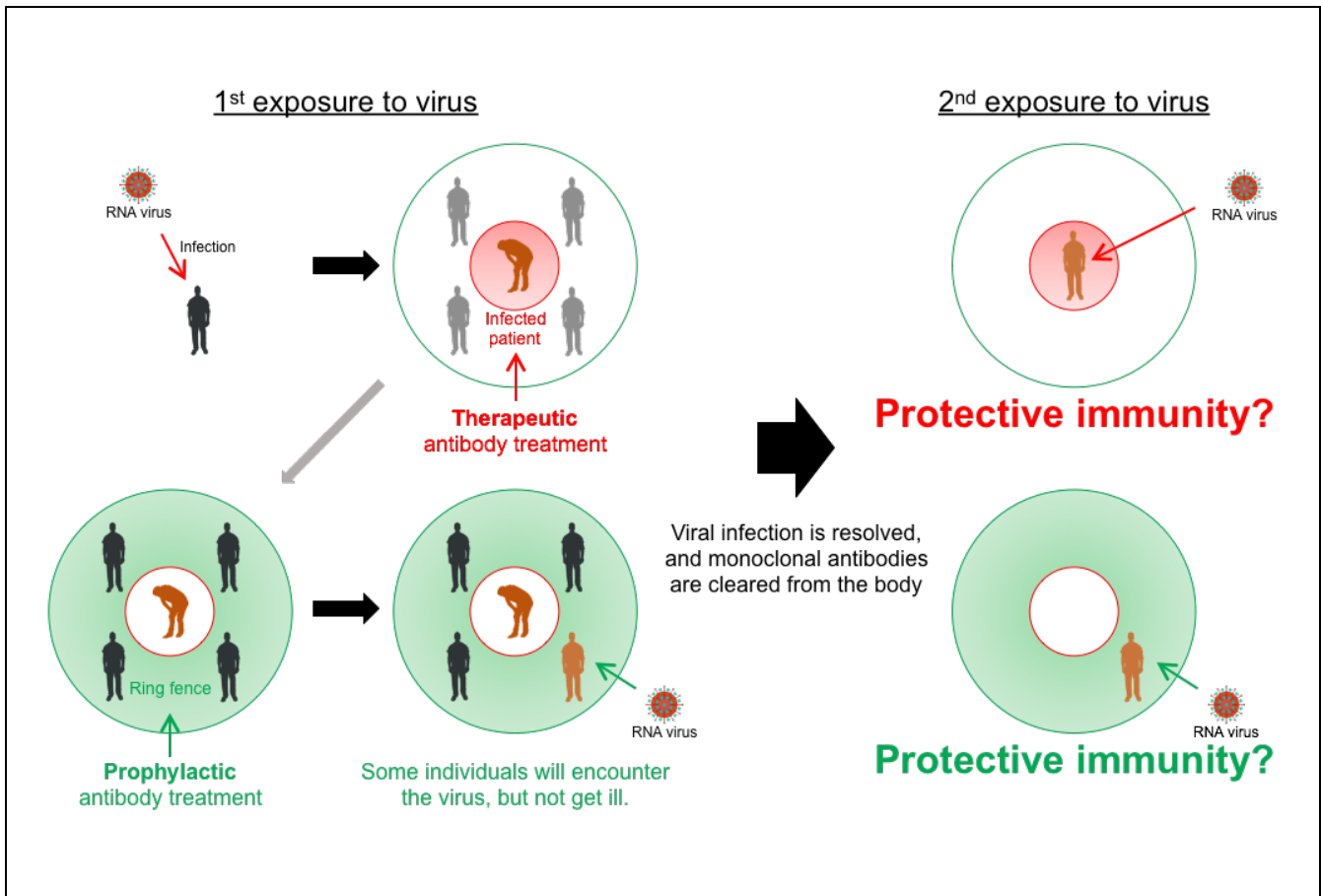
Figure 61. Ring Fence Prophylaxis with anti-viral antibodies.

RNA viral infections can be treated post-infection with neutralizing monoclonal antibodies and the treatment does not hamper the natural immune response and long-time protection over the subsequent contact with the same strain of virus. We hypothesize that prophylactic administration of monoclonal antibodies to people living in proximity of diagnosed individuals, would protect them from getting infected. More importantly, based on our findings we claim that in case of natural exposure to an infective agent, the immune system of a prophylactically treated person would still develop a protective immune response just like survivors of the infection. (Image contributed by Maria Lisa Knudsen).

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