筑　波　大　学

博 士 （ 医 学 ） 学 位 論 文

Analysis of genotype-specific and genotype cross-reactive neutralizing antibody response to dengue virus infection in a common marmoset *(Callithrix jacchus)* model

（デングウイルス感染における遺伝子型特異的及び遺伝子型交叉性抗体応答のマーモセットモデルを用いた解析）

２０１８

筑波大学大学院博士課程人間総合科学研究科

Nor Azila Muhammad Azami

THESIS ABSTRACT

Introduction

Neutralizing antibodies play an important role in protection against dengue virus (DENV) infection. Current tetravalent dengue vaccine against all four serotypes of DENV was developed using only single genotype from each serotype, with the assumption that infection with one genotype within the same serotype will induce protection against all genotypes within the same serotype. It is widely believed that despite antigenic differences, neutralizing antibody epitopes are conserved among the strains within the same serotypes and strain variation does not affect the ability of neutralizing antibodies to confer protection. Nonetheless, the efficacy of tetravalent vaccine is varied by infected virus, and the efficacy of DENV-2 vaccine is the lowest among four serotypes. Studies on the levels of genotype-specific and genotype cross-reactive neutralizing antibodies to genotypes of DENV are still limited. In addition, studies on correlation between antigenic differences in genotypes and levels of viremia and cell-mediated immunity during DENV infection are limited as well.

This present study aims to (i) determined the levels of genotype-specific and genotype cross-reactive neutralizing antibody in response to DENV-2 in primary, secondary, and tertiary DENV infections in common marmosets (*Callithrix jacchus)* model; (ii) determined levels of viremia and immune-related genes upon inoculation with different genotypes and serotypes of DENV in primary, secondary, and tertiary infections in common marmosets *(Callithrix jacchus)* model.

Materials and Methods

In study (i), a total of 59 plasma samples were obtained from 34 common marmosets that, were either inoculated with clinically-isolated virus strains (N=26), or inoculated with candidate vaccine strain (N=8). Plasma samples were obtained from marmosets after primary, secondary, or tertiary infection of DENV. There were 12 groups of marmosets that were inoculated with clinically-isolated virus strains and 5 groups of marmosets that were inoculated with candidate vaccine strains. The levels of neutralizing antibodies to DENV-2 Cosmopolitan, Asian I, and Asian/American genotypes and DENV-1 were determined using a conventional plaque reduction neutralization assay on day 0 (prior to virus inoculation), 4, 7, and 14 post-inoculation. Student's t-test was used to determine the differences in mean titers of neutralizing antibody between genotypes of each group.

In study (ii), a total of 19 plasma and peripheral blood mononuclear cells samples were collected on days 0 (prior to virus inoculation), 2, 4, 7, 10, and 14 post-inoculation from 7 groups of common marmosets. There were 2 groups of marmosets for primary infection (N=6), 3 groups of marmosets for secondary infection (N=7), and 2 groups of marmosets for tertiary infection (N=6). Viral RNA copy numbers and infectious virus titers were determined using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) and conventional plaque assay, respectively. Levels of immune-related genes including CD3ε, CD4, CD8α, IFNγ, IL-1β, IL-2, and IL-10 were determined using qRT-PCR. Expression levels of each gene were analyzed using comparative C_T method and normalized with levels of GAPDH as reference gene. Differences in levels of immune-related gene on days 2, 4, 7, 10, and 14 p.i were compared with those on day 0 as a reference using Student's t-test.

Results

Neutralizing antibodies induced during primary DENV-2 infection neutralized all three genotypes of DENV-2 (Cosmopolitan, Asian I, and Asian/American genotypes). In secondary infection with homologous genotype, levels of genotype-specific and genotype cross-reactive neutralizing antibodies to all three genotypes of DENV-2 did not differ from days 0-14 post-inoculation in marmosets that were inoculated with DENV-2 Cosmopolitan and Asian I genotypes but not in marmosets that were inoculated with DENV-2 Asian/American genotype. Higher levels of neutralizing antibodies to Asian/American genotype than those to Cosmopolitan and Asian I genotypes were observed in secondary infection with heterologous genotype and in tertiary infection. Single infection with DENV-1 did not induce serotype cross-reactive neutralizing antibodies to DENV-2, and following secondary DENV-1 infection, serotype cross-reactive neutralizing antibodies to DENV-2 were induced. Levels of neutralizing antibodies in marmosets that were inoculated with candidate vaccine strain were comparable as those marmosets that were inoculated with clinically isolated parent strain.

Levels of viremia in primary and tertiary DENV infection were different upon inoculation with different genotypes of DENV. In secondary infection, viremia was detected in marmosets that were inoculated with heterologous serotype but absent in those that were inoculated with homologous serotype. Levels of CD3ε, CD4, IFNγ, IL-2, and IL-10 in all groups of marmosets differed. Interestingly, levels of CD8α decreased in early phase of viremia and increased in late phase of viremia. Levels of IL-1β decreased in all marmosets that developed viremia following DENV infection.

Discussion

Neutralizing antibodies induced during primary infection were genotype cross-reactive but serotype-specific. Differences in levels of neutralizing antibodies during heterologous infection suggest heterogeneity in antigenic molecules among genotypes may lead to the induction of higher levels of neutralizing antibodies. Sequential infection induced broad cross-reactive neutralizing antibodies that neutralized multiple serotypes including non-infecting serotypes. Heterogeneity and homogeneity of infecting genotype influence the levels and cross-reactivity of neutralizing antibodies that were induced in following infections. Study on candidate vaccine strain suggested that while attenuation decreased the virus pathogenicity, immunogenicity patterns between genotypes retained. Differences in the levels of viremia may have been caused by antigenic differences. Nonetheless, the correlation between antigenic differences in genotypes of DENV and levels of immune-related genes remained inconclusive. Levels of CD8α inversely correlated with viremia levels, because CD8+ T cells may have been activated to control the viral replication. Decreased levels of IL-1β in marmosets maybe associated in the absence of vascular permeability symptoms.

Conclusion

Levels and patterns of genotype-specific and genotype cross–reactive neutralizing antibodies, and viremia levels were different in primary, secondary and tertiary DENV infection in common marmosets. Thus, identification of DENV strains that able to induce similar levels of neutralizing antibodies and offer protection against homologous or heterologous genotypes of DENV should be addressed in DENV vaccine development study.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ACKNOWLEDGEMENT

I would like to express my deepest gratitude to my supervisor and professor, Dr. Ichiro Kurane, for his excellent guidance, knowledges and advices throughout this research process.

I would also like to thank Dr. Tomohiko Takasaki from Kanagawa Prefectural Institute of Public Health and Dr. Meng Ling Moi from Institute of Tropical Medicine, Nagasaki University for their guidance and assistance in handling experiments.

I would like to express my appreciation to Dr. Masayuki Saijo, Dr. Chang-Kweng Lim, Dr. Shigeru Tajima, Dr. Satoshi Taniguchi, Dr. Fumihiro Kato, Mrs. Makiko Ikeda, Mr. Kenichi Shibasaki, and members of Department of Virology 1, National Institute of Infectious Diseases for their assistances, advices, motivations, and jokes during my time in NIID, Japan.

A big thank you to Dr. Yasushi Ami and Dr. Yuriko Suzaki from the Division of Experimental Animal Research, National Institute of Infectious Disease of Japan for their assistance in handling all the marmosets and their knowledge and expertise about marmosets.

I would like to thank Associate Professor Dr. Atsushi Kawaguchi as well for his administrative support to complete my doctoral study. Deepest appreciation to the thesis committee members, Professor Dr. Yukiko Wagatsuma, Professor Dr. Shigeyuki Kano, Associate Professor Dr. Kiong Ho, and Associate Professor Dr. Kaoru Takeuchi for the comments and suggestions. Not forgetting, Mrs. Flaminia Miyamisu for teaching me the correct usage of grammar during writing this thesis.

I would like to say thank you to my scholarship authority, Fellowship scheme of UKM Medical Molecular Biology Institute, Universiti Kebangsaan Malaysia. Not forgetting my friends both in Malaysia and Japan as well for their best wishes.

Finally, deepest love and highest appreciation to my parent, Mr. Muhammad Azami Mat Amin and Mrs. Zainab Abdullah Sani, my siblings, and my family, not forgetting my furkids (Minachi and Pinpin) for always supporting me.

All the praise and thanks to the most merciful Allah s.w.t.

ABBREVIATIONS

A1	Asian I genotype
AA	Asian/American genotype
ADE	Antibody-dependent enhancement
BHK	Baby-hamster kidney cell
cDNA	Complementary deoxyribonucleic acid
CD	Cluster of differentiation
CM	Cosmopolitan genotype
DENV	Dengue virus
DF	Dengue fever
DHF	Dengue hemorrhagic fever
DSS	Dengue shock syndrome
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
NH_4Cl	Ammonium chloride
NHP	Non-human primate
NS	Non-structural protein
p.i.	post-inoculation
PBMC	Peripheral blood mononuclear cell
PDK	Primary dog kidney cell
PRNT	Plaque reduction neutralization test
RNA	Ribonucleic acid
RT-PCR	Reverse-transcriptase polymerase chain reaction
qRT-PCR	Quantitative reverse-transcriptase polymerase chain reaction
SD	Standard deviation
vRNA	Viral ribonucleic acid

CHAPTER 1: LITERATURE REVIEW

1.1. Dengue virus

The word "Dengue" (DEN) is originated from the Swahili phrase "ki dinga pepo" which means a "kind of sudden cramp-like seizure from an evil spirit or plague"[1]. Dengue virus (DENV) belongs to genus *Flaviviridae* of the family Flavivirus. The genus *Flaviviridae* comprised of DENV, Japanese encephalitis virus, yellow fever virus and West Nile virus. This virus is usually transmitted to human by *Aedes aegypti* and *Aedes albopictus* mosquitoes[2,3].

DENV is a small, enveloped virus that contains a single-stranded, positive sense RNA genome[2,4–6]. The RNA genome of DENV (-11,000 nt) is made up of a single open reading frame that encodes for three structural proteins: capsid (C), pre-membrane (prM) and envelope (E), and seven non-structural protein: NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5[4,7,8]. The C and prM proteins mediate in the encapsidation of RNA genome, and the release of mature virion, respectively[9]. The E protein facilitates in virus binding, fusion of viral to host cellular membranes during viral entry and in the stimulation of host protective immunity[3,5,9]. Individual subunits of E protein composed of three beta-barrel domains: envelope domains I, II, and III (EDI, EDII, and EDIII, respectively)[10,11]. EDIII domain has been correlated with a strong neutralization activity[3]. The viral envelope contains two integral membrane proteins, E and prM protein, and these proteins are the main target of immune response during DENV infection[5,11]. The non-structural proteins were responsible in the replication of viral genome but not detectable in viral proteins.

1.2. Serotype of dengue virus

There are four serotypes of DENV; DENV1, DENV-2, DENV-3, and DENV-4. The definition of serotype is based on the observation that full protection was conferred against infection with homologous viruses but limited protection against heterologous viruses [9,12]. Differences in amino acid sequences in EDIII domain in E protein provide the basis for the antigenically distinct serotypes of DENV[10]. The homology among serotypes is less than 60% at the nucleotide levels and less than 80% at the amino acid levels[13,14]. All four DENV serotypes have similar clinical symptoms and co-circulate in endemic areas. Nonetheless, outbreaks of DENV-2 and DENV-3 were often associated with severe dengue and outbreaks of DENV-1 and DENV-4 were often associated with more mild diseases. DENV-2 is the most prevalent serotype in the current worldwide dengue epidemic[15].

1.3. Genotype of dengue virus

Phylogenetic analysis of the E protein revealed that each DENV serotype has 4-6 genotypes. Genotype is defined as clusters of DENV with sequence divergence not greater than 6% within the chosen genome region (E/NS1 junction). The homology among genotypes within the same serotype is about 94-97%[14]. Antigenic differences among genotypes have been speculated played an important role in the severity of the diseases, epidemic magnitude, viral fitness and evolution, and vaccine design[12,14].

Genotypes of DENV-2 differed in term of virulence, incidence, and vector competence[16–20]. DENV-2 is divided into six genotypes: sylvatic, Cosmopolitan, Asian I, Asian II, Asian/American, and American genotypes [15,16]. The Cosmopolitan genotype was associated with diverse range of geographical localities and severe dengue, and the

American genotype was associated with milder diseases [16,18]. The viral fitness in the Asian I and Asian/American genotypes differed. Infection with the Asian I genotype induced higher levels of viremia than infection with the Asian/American genotype[16,20]. The differences in viral fitness lead to displacement of the Asian/American genotype to the Asian I genotype in Vietnam, Thailand, and Cambodia[19]. In term of vector competence, the Asian/American genotype was efficiently infected the salivary gland of Aedes mosquitoes than the American genotype[17].

1.4. Epidemiology of dengue

DENV was first isolated in Nagasaki-Sasebo, Japan during the epidemic in 1942, by inoculating serum from acutely ill patients into suckling mice [21,22]. Dengue (DEN) has become major public health threat in tropical and subtropical regions including Southeast Asia, Africa, South Pacific, Central and South American and Eastern Mediterranean[23,24]. In recent years, dengue outbreaks were reported in temperate places such as France, Croatia, Japan and China[25–29].

DENV had infected up to 4 billion people worldwide and 390 million new DENV infection cases were reported annually[8,16,30,31]. The incidences of DENV infection had increased up to 30-folds for the past 60 years[14]. Population growth, global urbanization, ease of transportation, expansion of vector geographical habitats and inadequate mosquito controls were factors that facilitated the emergence of dengue incidence[28,32]. World Health Organization (WHO) aimed to reduce mortality and morbidity of DEN by at least 50% and 25%, respectively in 2020[33].

1.5. Clinical features of dengue

Infection with DENV can either be asymptomatic or symptomatic. The 2009 WHO DEN case classification categorized DEN cases based on disease severity levels[34]. Symptomatic cases of DENV infection ranged from mild febrile illness, dengue without warning signs, dengue with warning signs, to severe dengue and sometimes dengue-related death. There are three phases of DEN illness: febrile phase which last for 3-7 days, critical phase, and spontaneous recovery phase[14,35]. The symptoms usually start to manifest after the incubation period of 3-7 days[35]. While most patients recover after the febrile phase, a small proportion of the patients progress to severe dengue symptoms[27].

1.5.1. Dengue with or without warning signs

Dengue fever (DF) is characterized by high-grade fever, headache, arthralgia, myalgia, rash, gastrointestinal discomfort, and retro-orbital pain[27,36,37]. Minor hemorrhagic manifestation occurs in the form of petechiae, epistaxis, gingival bleeding[37]. The progressive decrease in total white cell count during full blood count is sign of DF. Patients who recover following defervescence are considered as dengue without warning signs. Patients that were diagnosed as dengue with warning signs showed symptoms including abdominal pain, persistent vomiting, fluid accumulation, mucosal bleeding, lethargy, liver enlargement, increasing hematocrit with decreasing platelets[34].

1.5.2. Severe Dengue

About 1-6% of the symptomatic cases of DENV infections progress to severe[38]. The mortality rate of severe dengue is 2.5%, particularly in infants and children[8,27]. The 1997 WHO DEN case classification categorized severe dengue into dengue hemorrhagic fever (Grades 1-2) and dengue shock syndrome (Grades 3-4). Dengue hemorrhagic fever (DHF) was diagnosed as DF with hemorrhagic manifestations including, plasma leakage resulting in shock, accumulation of serosal fluid leading respiratory distress, severe gastrointestinal involvement such as persistent vomiting, and severe organ impairment including acute liver failure and acute renal failure[14,35]. DEN patient is considered to have shock when the differences between the systolic and diastolic pressure is ≤20 mm Hg or has cold extremities, delayed capillary refill or rapid pulse rate. Platelets counts lower than 50,000 cells/mm^3 are considered sign of severe disease[27]. In 2009 WHO DEN case classification listed symptoms such as severe plasma leakage, severe hemorrhage, and severe organ impairment including myocarditis, hepatitis, and encephalitis as criteria for severe dengue[14,23,39].

1.6. Dengue diagnosis

DEN diagnostics detects either the viral components (genome or antigen) or the host response to the virus (serology testing) or both[14,35].

1.6.1. Detection of viral components

In early stage of infection, virus isolation, nucleic acid or antigen detection are used for DEN diagnosis. DENV can be detected in serum, plasma, circulating blood cells and tissues on days 4-5 after the onset of illness[27]. Reverse-transcriptase polymerase chain reaction (RT-PCR), quantitative RT-PCR (qRT-PCR) and isothermal

RNA amplification assay are used to detect the viral genome components in serum and plasma. In contrast, enzyme-linked immunosorbent assay (ELISA) are used to detect virus-expressed soluble non-structural protein-1 (NS1)[35]. Virus isolation is a cell culture technique that used mosquitoes cell line (C6/36) or mammalian cell lines (Vero, LLCMK2 and BHK21) as host cell to isolate DENV.

1.6.2. Detection of host response

Serology method is usually used to diagnosis DEN at the end of acute DEN phase[27]. ELISA assay is used to determine the presence of DENV-specific IgM or IgG antibody. In primary DEN infection, IgM antibody detected earlier than IgG antibody. In secondary DEN infection, IgG antibody detected earlier than IgM antibody. The IgM/IgG antibody ratio is used to distinguish between primary and secondary DENV infection.

1.6.3. Plaque reduction neutralization test

Plaque reduction neutralization test (PRNT) is a gold-standard technique to measure the titer of neutralizing antibodies in the serum. Neutralization assay detects and quantifies neutralizing antibodies in serum samples by calculating the percentage of reduction of virus activity, as the concentration of virus used is usually constant[40]. The PRNT and the micro-neutralization assay served as method to determine the protection levels by using levels of neutralizing antibodies as a proxy of protection[41]. These techniques are mainly used in vaccine development studies to determine the vaccine efficacy and safety.

1.7. Dengue pathogenesis

DEN is multifactorial disease that involved several factors including viral factors, cytokines storms/soluble factors, host genetic factors, transient autoimmunity, cross-reactive T- cell response, and antibody-dependent enhancement[5,11,37,42]. Because of the complexity of the DEN pathogenesis, one had to consider all the immunological, pathological, clinical and epidemiological features to understand DEN pathogenesis.

1.7.1. Viral factors

DENV are genetically diverse and classified into distinct genotypes with different geographical distribution and pathogenic potential[11,43]. Antigenic differences among genotypes and serotypes of DENV were associated with disease severity, epidemic cycling and viral evolution[20]. Certain strains and genotypes of DENV are able to induce high levels of viremia and associated with severe disease[16,18,37]. Primary DENV-1 infection is associated with overt cases while primary DENV-2 and DENV-3 infection are mostly asymptomatic[44]. Secondary DENV-2 infection is associated with severe disease with signs of plasma leakage[44–46]. Patient infected with DENV-4 usually has milder clinical profile[46].

1.7.2. Antibody-dependent enhancement

In most acute virus infection models, neutralizing and non-neutralizing antibodies correlates with control, elimination and protection of the host cells[37]. DENV infection with one serotype will induce long-lasting protection against homologous serotype. However, cross-reactive protection against other DENV serotypes wanes after few months[5]. However in DEN infection, DENV sub-neutralizing, infection-enhancing antibodies induced during primary infection was risk factor for disease severity in

secondary infection with heterologous serotype[47,48]. Antibody-dependent enhancement (ADE) theory is based on the observations that pre-flavivirus immune individual had higher risk to develop for severe disease during secondary infection because non-neutralizing cross-reacting antibody facilitated virus entry into the cells via Fc gamma (Fcγ) receptor, in turn, enhance viral infection, and lead to increase in viral load[7,49].

1.8. Immune responses during dengue virus infection

Virus can either directly or indirectly cause illness. In direct mechanism, cytopathic effect cause by the virus infection of the cells leads to organ malfunctions and illness[50]. In contrast, in indirect mechanism, organs malfunctions and illness may have been caused by immune responses to the virus[50]. The innate responses and adaptive cellular immune responses facilitate recovery from virus infection and confer protection against homologous infection. However, immune response could also cause disease (immunopathogenesis)[50,51].

1.8.1. Humoral immunity

Humoral immune response is mediated by antibody molecules that are secreted by plasma cells[52]. In humoral immunity, virus or virus-infected cell stimulated B lymphocytes to induce antibody. When the antigen binds to the immunoglobulin receptor at cell surface, B cell will interact with macrophages and helper T lymphocytes, and B cell will then differentiate into clones of antibody-secreting plasma cells. This cells than secreting antigen-specific immunoglobulin (IgG) of one five major classes: IgA, IgD, IgE, IgM, and IgG antibody. The IgA, IgM, and IgG antibody have antiviral properties[53]. IgG antibody is responsible for the most antiviral activity in serum. Interestingly, IgG antibody also plays an important role in disease enhancement[10].

1.8.2. Cell-mediated immunity

Cell-mediated immunity refers to identification and/or elimination of virus or virus-infected cell by leukocytes, and production of different soluble factors (cytokines) by cells when induced by virus or virus-infected cell[53]. Cell-mediated immunity involves T lymphocytes, antibody-dependent cell-mediated cytotoxicity (ADCC), macrophages, natural killer (NK) cells, lymphokines, and monokines to respond for viral infection.

1.8.3. Innate responses

Innate responses refer to those that neither require previous antigenic exposure nor show enhanced response during subsequent exposure (memory). Innate responses provide the host the time that needed to induce adaptive immunity. Innate immune responses including interferons (IFNs) and NK cells that are rapidly activated after viral infection. Dendritic cells (DC), NK cells, and natural killer T (NKT) cells participate in innate immune responses during DENV infection.

1.8.4. Adaptive responses

In contrast, adaptive immune responses are develop upon exposure to pathogens and respond in an antigen-specific manner upon further exposure; these include T lymphocytes, and B lymphocytes and their associated antibodies[37,54]. During primary infection, the adaptive immune responses follow the innate immune responses in time. The naïve T lymphocytes activation require antigen that are expressed by the specialized antigen-presenting cells including macrophages, B lymphocytes, or dendritic cells. After the elimination, T lymphocytes are converted into a "memory" phenotypes for more rapid activation and response upon re-exposure[54]. During

secondary infection, T-cell responses are rapidly induced, and antibodies levels are increased earlier following infection. Although, adaptive immune responses are important to prevent re-infect, these responses are also the risk factors for disease severity because of the enhancement properties and the strong association between severe DEN case and preexisting neutralizing antibodies[39]. In addition of that, severe DEN case was proposed to be caused by immune-mediated than viral mediated because severe DEN was developed after the clearance of viremia.[55].

1.8.5. Antibody response

Antibody is the products of adaptive humoral immune response[56]. During primary infection, Immunoglobulin M (IgM) antibody responses earlier to DEN antigen followed by IgG antibody. IgM antibody is detectable on 3rd afebrile day after defervescence in clinically apparent case while IgG antibody is detectable during convalescent phase. IgM antibody persists for 1-2 months after the infection[57]. In contrast, during secondary infection, IgG antibody respond earlier than IgM antibody. In DENV infection, IgM antibody predominantly respond to E protein, but response to NS proteins are also detected[57]. In convalescent phase during primary infection, IgG antibody predominantly respond to prM and E proteins. However, following primary and secondary heterologous infection, IgG antibody response to C, NS1, ND3, and NS5 proteins are also detected[58–60].

1.8.6. Neutralizing antibodies

Neutralizing antibodies play a central role in protection against DENV infection and disease pathogenesis. Neutralizing antibodies neutralized the virus either by blocking the attachment of the virus to the cells or blocking a post-attachment step in

the entry process[53]. Virus neutralization occurs when antibody binds to the complementary epitopes on the virus surface, and the numbers of antibody bound to the virions exceed critical threshold preventing the virus binding to target cells [56–58]. E and pre-M proteins are the principal target of antibody responses[2]. A majority of neutralizing antibodies response against E protein are thought to be cross-reactive among serotypes because DENV serotypes are antigenically related[2,63]. Nonetheless, serotype-specific neutralizing antibodies are also induced after DENV infection, and these antibodies were highly genotype-specific and antigen differences among genotypes and serotypes of DENV affected the abilities of neutralizing antibodies to offer protection[6]. The fundamental assumption that neutralizing antibodies induced during DENV infection confers protection against re-infection with homologous serotype of DENV were challenged when incidence of incomplete protection during heterologous genotype infection were reported[13,14,64,65]. Forshey et. al (2016) reported that during outbreak of DENV-2 Asian/American genotype in Peru, that neutralizing antibodies induced during previous outbreak of DENV-2 American genotype did not confer protection against infection with DENV-2 Asian/American genotype[13]. The incomplete protection against heterologous genotype may have been caused by the antigenic differences among genotypes. Antigenic differences cause the structural change on viral proteins that will modify the antibody binding site, and certain antibodies did not able to interact with epitopes in EDIII domain belonging to E protein of different genotypes within the same[3,12].

1.9. Dengue vaccine

The development of DEN vaccine has been hampered by antigenic and genetic differences of the virus, lack of cross-reactive immunity among genotypes/serotypes of DENV, and the host immune response[3]. In 2016, CYD-TVD become the first

commercially available DEN vaccine[66–69]. In addition of that, there are currently five candidate vaccines in human clinical trial phase including DENVax, TV003/TV005, TDENV PIV, V180 and D1ME10070.

CYD-TVD (Dengvaxia) is DEN vaccine that was developed by Sanofi Pasteur and was licensed to be use in 19 countries including DEN-endemic countries[66–69]. Dengvaxia is a tetravalent live-attenuated chimeric DENV vaccine based on the yellow fever (YF) 17D vaccine strain[70]. This tetravalent DEN vaccine was constructed by replacing the prM and E proteins of YF vaccine strain with those prM and E proteins of the four DENV serotypes[70,71]. Although Dengvaxia could reduce DEN-related hospital admission by 10-30% over the next 30 year, the efficacy of the vaccine against all DENV serotype was only 44.6-65.6% [67,72,73]. The efficacy against DENV-2 was only 33.6-47.1% and the lowest among other serotypes[72]. In 2016, WHO Strategic Advisory Group of Experts (SAGE) reported that Dengvaxia was safe to be used in DEN endemic regions and in targeted age group of people aged 9-45 years[33,66]. Nonetheless, Aguiar et. al (2016) suggested that Dengvaxia should only be given to DEN seropositive individual and immunological screening should be done prior to vaccination[67,68,74]. Several concerns were raised about the administration of Dengvaxia without the identification of the immune status at the time of vaccination as Dengvaxia increased the risk dengue-related admission in DEN seronegative individual[67,68,75]. In 2017, Sanofi acknowledged the differences in Dengvaxia performance based on prior DEN infection[69].

1.10. Animal model of dengue virus infection

Animal model of DENV infection was used in dengue tropism and pathogenesis studies, dengue immunopathogenesis studies, development of the immune response studies, therapeutics testing and vaccine evaluation studies[36,76,77]. Development of reliable animal model that can better reflect the DENV infection in human will allow a better understanding on the DENV pathogenesis topics. A good animal model of DENV infection should be able to demonstrate high sensitivity to DENV, induce high levels of DENV replication and exhibit disease sign upon DENV inoculation. However, due to the complexity of DENV, it was difficult to find a good animal models of DENV infection that better reflect and mimicking DENV infection in humans.

1.10.1. Mouse model

The wild-type mice, human tissue engrafted-SCID, interferon alpha, beta, gamma deficient AG129, RAG-hu, and the NOD/SCID/IL-2Rγ/human CD34 transplant or immunized mice are the breeds that had been used as animal model of DENV infection[55,76]. Initial mouse models of DEN demonstrated low-levels of virus replication upon inoculation with clinically isolated DENV. The neurotropic disease and splenomegaly symptoms that were observed in AG129 mouse and paralysis symptom that were observed in engrafted-SCID mouse did not faithfully reflect the clinical signs of DENV infection in human[55,76]. Nonetheless, the AG129 mice was a reliable model to study the role of NS1 antigen and anti-NSI antibody due to high levels of circulating NS1 antigen and anti-NS1 antibody during DENV infection[76]. In addition of that, AG129 mouse model had been utilized in therapeutics testing because these mice developed disease features resembling human dengue disease[76].

1.10.2. Non-human primate model

Non-human primate (NHP) is reliable animal model of DENV infection because of the similarities in genetic proximity between the NHP and human. NHP had been used as animal model in dengue tropism and pathogenesis studies, immunopathogenesis studies, and vaccine development studies [78–82]. Detection of DENV antibodies in sera of wild NHP indicated that NHP were involved in DEN transmission in sylvatic cycle[77]. NHP was able to sustain viral replication after inoculation with 10^4-10^6 PFU of DENV (mimic the inoculum in a mosquito bite) via subcutaneous inoculation[76]. NHP also developed antibody responses similar to those in humans[78]. Nonetheless, the viral replication in some NHPs including pigtail macaques, rhesus macaques, and owl monkeys were lower than those in humans[76,77,83–86]. Some rhesus macaques displayed low platelet count and lack of clinical disease signs[36,76,80]. In recent years, rhesus macaques demonstrated hemorrhagic manifestations when inoculated with higher dose of DENV via intravenous route[80].

1.10.3. Common marmoset

Common marmoset (*Callithrix jacchus)* is small New World monkey[36]. Common marmoset inhabits mainly in South America. Common marmoset was used as an experimental animal model in drug toxicology studies, neurosciences studies, autoimmune diseases studies, and infectious diseases studies[87]. Common marmoset was a good infection model for DENV because marmoset constantly developed high levels of viremia upon inoculation with clinically-isolated DENV[36,83]. Common marmoset also demonstrated antibody response patterns that were consistent with those in human DENV infections and candidate vaccines recipients[83,88].

1.11. Research purpose and objectives

DENV-2 is the most prevalent DENV serotype in worldwide epidemic. Neutralizing antibodies play an important role in protection against DENV infection. Current tetravalent DEN vaccine against all four serotypes of DENV was developed using only single genotype from each serotype of DENV, with assumption that infection with one genotype of a serotype will confer protection against all genotypes within the same serotype. It is widely believed that, despite the antigenic differences, strain variation within serotype does not affect the ability of neutralizing antibodies to offer protection. Nonetheless, the efficacy of DENV-2 vaccine was only 33.6-47.1% and the lowest among four serotypes. It was speculated that antigenic differences lead to structural change on viral protein that will modify the antibody binding site and affect the ability of neutralizing antibodies to neutralize heterologous genotype. Studies on the levels of neutralizing antibodies to different genotypes of DENV-2 have been limited. In addition of that, studies on correlation between antigenic differences in genotype and levels of viremia and cell-mediated immunity have been limited as well.

Thus, the present study aims to:

i. Determine levels of genotype-specific and genotype cross-reactive neutralizing antibody in response to DENV-2 in primary, secondary and tertiary DENV infections in common marmosets (*Callithrix jacchus)* model.
ii. Determine levels of viremia and immune-related gene in primary, secondary and tertiary DENV infections in common marmosets (*Callithrix jacchus)* model.

CHAPTER 2: MATERIALS AND METHODS

2.1. Virus

Three strains of DENV-1 were used in this study (Figure 1). Phylogenetic tree was constructed by using nucleotide sequence of the complete E protein region and obtained using Neighbor-joining (NJ) method in MEGA software[89]. DENV-1 Yoyogi-Tokyo strain (GenBank accession no. LC006123) was isolated from dengue fever (DF) case from Tokyo 2014 outbreak, and belongs to the genotype I (GI) genotype. DENV-1 16007 strain (GenBank accession no. AF180817) was isolated from dengue hemorrhagic fever (DHF) case, and belongs to the genotype II (GII) genotype. DENV-1 02-17 strain (GenBank accession no. AB111075) was isolated from imported DF case from Indonesia, and belongs to the genotype IV (GIV) genotype.

Four strains of DENV-2 were used in this study (Figure 2). Phylogenetic tree was constructed by using the nucleotide sequence of the complete E protein region and obtained using NJ method in MEGA software as well[89]. DENV-2 DHF0663 strain (GenBank accession no. AB189122) was isolated from imported DHF case from Indonesia, and belongs to the Cosmopolitan genotype. DENV-2 00-43 strain (GenBank accession no. AB111452) was isolated from imported DF case from Indonesia, and belongs to the Asian I genotype. DENV-2 16881 strain (GenBank accession no. M84727) was isolated from DHF case, and belongs to the Asian I genotype. DENV-2 08-77 strain (GenBank accession no. AB545874) was isolated from imported DF case from Maldives, and belongs to the Asian/American genotype.

All the DENV strains were first isolated using C6/36 cells, and passaged in baby hamster kidney (BHK) cells. Culture supernatants collected from infected BHK cells

were centrifuged at 3000 rpm for 5 minutes to remove cell debris. Virus stocks were used within four cell culture passages. Virus stock was stored at -80℃ before use.

2.2. Candidate vaccine strain

DENV-1 16007 PDK-13 vaccine strain is a monovalent live attenuated DENV-1 vaccine candidate that was attenuated by serial passage in primary dog kidney (PDK) cells (GenBank accession no. AF180818). DENV-2 16881 PDK-53 vaccine strain is a monovalent live attenuated DENV-2 vaccine candidate that was attenuated by serial passages in PDK cells (GenBank accession no. M84728). All the vaccine strains were prepared by Professor Sutee Yoksan at Institute of Molecular Biosciences, Mahidol University, Nakhon Pathom, Thailand, shipped in liquid form on dry ice to National Institute of Infectious Diseases, Tokyo, Japan, and stored at –80 °C before use.

2.3. Samples collected from marmosets

Common marmosets (*Callithrix jacchus)* were obtained from CLEA Japan, Inc. (Tokyo, Japan) and maintained in specific pathogen-free conditions at the National Institute of Infectious Diseases (Tokyo, Japan).

2.3.1 Study on the levels of genotype-specific and genotype cross-reactive neutralizing antibodies to DENV-2 genotype

A total of 59 blood samples were collected from 34 marmosets in the study. In groups 1-12, 43 plasma samples were collected from 26 marmosets with primary (N=28), secondary (N=12), and tertiary (N=3) DENV infection (Table 1). In group 1-12, the marmosets were inoculated subcutaneously with 10^6 plaque forming unit (pfu) $dose^{-1}$ of DENV. The interval between each of DENV inoculation was about 4-12

months (based on vaccine administration schedule). In groups 13-17, 16 blood samples were collected from eight marmosets that were inoculated with monovalent live-attenuated DEN vaccine candidates: DENV-1 16007 PDK-13 strain and DENV-2 16881 PDK-53 strain. In groups 15-16, marmosets received 10^4 pfu dose^{-1} of DENV-1 and DENV-2 vaccine strains, and about 6-9 months after the first administration, the marmosets were inoculated with 10^5 pfu dose^{-1} of DENV-1 or DENV-2. A total of 1 mL of whole blood was collected in EDTA tubes from each marmoset on day 0 (before the virus inoculation), and on days 4, 7, and 14 post-inoculation (p.i.). Next, the blood samples were centrifuged at 2000 rpm for 10 minutes at 4℃. Plasma samples were stored at -80℃ before use. We determined the presence of DENV-specific IgM antibody in all the marmosets in each group to indicate whether the DENV infection was established (Figure 3). Levels of DENV-specific IgM antibody in groups 1, 2, 3, 4, 5, 6, 11, 12, 13, 14, 15, 16, and 17 marmosets were above the baseline detection levels. Levels of DENV-specific IgM antibody in groups 7-10 marmosets were below the baseline detection levels.

2.3.2 Study on the levels of viremia and immune-related gene

A total of 19 plasma samples were collected from 7 groups of marmosets with primary (N=6), secondary (N=7), and tertiary (N=6) DENV infection (Table 2). There were 2 groups of primary infection marmosets, 3 groups of secondary infection marmosets, and 2 groups of tertiary infection marmosets. Group A consists of marmosets that were inoculated with DENV-2 Asian I genotype and group B consists of marmosets that were inoculated with DENV-2 Asian/American genotype. Group C and group D marmosets consist of marmosets that were inoculated with homologous serotype in secondary infection, while group E marmosets consists of marmosets that

were inoculated with heterologous serotype in secondary infection. Group F consists of marmoset that were inoculated with DENV-2 Asian I genotype in primary infection, DENV-2 Cosmopolitan genotype in secondary infection and DENV-1 GI genotype in tertiary infection, and group G consists of marmosets that were inoculated with DENV-2 Asian I genotype in primary infection, DENV-2 Cosmopolitan genotype in secondary infection and DENV-1 GIV genotype in tertiary infection. All the marmosets were inoculated with 10^6 pfu/DENV. The interval between each DENV inoculation was about 4-12 months. A total of 1 mL of whole blood was collected in EDTA tubes from each marmoset on day 0 (before the virus inoculation), and on days 2, 4, 7, 10, and 14 p.i. Next, the blood samples were centrifuge at 2000 rpm for 10 minutes at 4°C to collect plasma. Plasma samples were stored at -80°C before use.

2.4. Determination of neutralizing antibodies titers using plaque reduction neutralization test assay

Neutralizing antibodies titers to DENV-2 Cosmopolitan, Asian I, and Asian/American genotypes were determined using plaque reduction neutralization test assay (PRNT). BHK cells were seeded in 12-wells plate in Minimum Essential Medium (MEM) (Sigma Aldrich) containing 10% of heat-inactivated fetal bovine serum (Hi-FBS) (Gibco), and incubated at 37℃ overnight until 70% confluency was reached. Plasma samples were heat-inactivated at 56℃ for 30 minutes before use. Heat-inactivated plasma samples were serially diluted two-fold starting from 1:10 to 1:20480 in MEM containing 10% Hi-FBS. Virus-antibody complexes were prepared by mixing 25 µL of DENV at titers of 5000 pfu ml^{-1} with 25 µL serially diluted plasma samples to make final dilution series ranging from of 1:20 to 1:40960. Control samples were

prepared by mixing 25 µL of DENV at titers of 5000 pfu ml^{-1} with 25 µL MEM containing 10% Hi-FBS. Next, the virus-antibody complexes were incubated at 37℃ for 60 minutes. Next, a total of 50 µL of the virus-antibody complexes mixture were inoculated onto BHK cell monolayer in 12-wells plate. After incubation for 60 minutes, 1ml of maintenance medium containing MEM, 1% of methyl cellulose, and 2% of Hi-FBS was added. Plate was incubated at 37℃ in 5% CO_2 until visible plaques were observed (5-7 days of incubation). Plaques were fixed with 10% formaldehyde and stained with methylene blue, washed with water, and then counted. All tests were conducted in duplicate. Neutralizing antibodies titer was expressed as the maximum dilution of plasma sample that yielded a ≥50% plaque reduction in the virus inoculum compared with control virus sample. Results are shown as 50% $PRNT_{50}$ values, expressed as reciprocal of the highest plasma dilution (end-point titer) that results in ≤50% of the input plaque count.

2.5. Determination of DENV-specific IgM and IgG antibodies

Presence of DENV-specific IgM antibody was determined using Focus Dengue Fever IgM capture ELISA kit (Focus Diagnostic). Presence of DENV-specific IgG antibody was determined using Panbio Dengue IgG Indirect ELISA kit (Alere). Plasma samples from three DENV-naïve marmosets were used as negative control. Ratio positive (P): negative (N), P: N≥2 were considered positive. Ratio P: N was calculated using the formula: absorbance of the test sample/absorbance of the negative control. All ELISAs were conducted in duplicate.

2.6. Determination of NS1 antigen

Presence of NS1 antigen were determined using Platelia^{TM} Dengue NS1 Ag-ELISA kit (Biorad Laboratories). Plasma samples collected from 3 DENV-naïve marmosets were used as negative control. Ratio P: N≥2 were considered positive. All ELISAs were conducted in duplicate.

2.7. Isolation of viral RNA

Viral RNA (vRNA) of DENV was isolated from plasma by using High Pure Viral RNA kit (Roche Diagnostic) according to the manufacturer's instructions to a final volume of 50µl per sample.

2.8. Determination of viral RNA copy numbers

vRNA copy numbers were determined using quantitative Taqman real-time reverse transcriptase polymerase chain reaction (qRT-PCR) [90]. Result of vRNA copy numbers was expressed as log10 genome copies per milliliter.

2.9. Determination of DENV titers using plaque assay

BHK cells were seeded in 12-wells plate in MEM containing 10% of Hi-FBS, and incubated at 37°C overnight until 70% confluency was reached. Plasma samples were serially diluted 10-fold from 1:10 to 1:10^6. A total of 50 µL of the diluted plasma was inoculated onto BHK cells monolayer. After incubation for 60 minutes, 1 mL of maintenance medium containing MEM, 1% of methyl cellulose and 2% of Hi-FBS was added. Plate was incubated at 37°C in 5% CO_2 until visible plaques were observed (5-7 days of incubation). Plaques were fixed with 10% formaldehyde, stained with methylene blue, washed with water, and then counted. All tests were conducted in

duplicate. Viral titers were expressed as plaque forming unit per millimeter (pfu mL^{-1}) using the following formula: (average number of plaques x dilution)/inoculum volume.

2.10. Isolation of RNA from leukocytes

A total of 0.5 mL of whole blood was added into 10 mL of ammonium chloride (NH_4Cl) solution to lyse the red blood cells. Blood samples were centrifuged at 2700 rpm for 3 minutes at 4°C and supernatant was discarded. Pellet was washed with 10 mL of Hank's Balanced Salt Solution (HBSS), vortexed, and centrifuged at 2700 rpm for 3 minutes at 4°C, and supernatant was discarded. Next, pellet was dissolved by repeat pipetting, transferred into 1.5 mL of centrifuge tube, centrifuged at 4000 rpm for 1 min and supernatant was discarded. Leukocytes were lysed with QIAzol Lysis reagent (Qiagen, Germany). Total RNA was extracted using Direct-zol RNA MiniPrep kit (Zymo research, USA) according to manufacturer's instructions.

2.11. Determination of levels of immune-related gene

Levels of GAPDH, CD3α, CD4, CD8α, IFN-γ, IL-1β, IL-2, and IL-10 on days 0, 2, 4, 7, 10, and 14 p.i. were determined using qRT-PCR[87]. The first-strand of cDNA was synthesized using PrimeScript® RT reagent kit (Takara, Japan) with attached random hexamers and oligo (dT) primers. Reaction was incubated at 37ºC for 15 mins followed by 85ºC for 5 seconds according to the manufacturers' instructions. Levels of immune-related genes were quantitated by qRT-PCR using Applied Biosystems QuanStudio® 5 system. PCR reaction consisted of 10 µL of Thunderbird® SYBR® qPCR mix, 2µL of 5µM primer mix, 0.4µL of 50X ROX references dye in a total volume of 20µL. The cycling conditions were as follows: 30 secs at 95ºC followed by 40 rounds of 95ºC for 15 secs and 60ºC for 30 secs. Melting curve analysis to determine

the dissociation of PCR product was performed between 65ºC and 95ºC. The qRT-PCR data were analyzed using comparative Ct method and normalized using GAPDH level as a reference gene[91]. Data were expressed as mean values of $2^{-\Delta\Delta Ct}$.

2.12. Data analysis

Data were analyzed using the statistical analysis toolpack in Microsoft Excel 2016 (Microsoft Corporation) and GraphPad Prism 7 (GraphPad Software Inc.). Student's t-test analysis was used to determine the differences between neutralizing antibodies titers between genotypes and differences between levels of immune-related genes on days 2, 4, 7, 10, and 14 p.i. were determined and compared with those on day 0 as a reference. Probability values (p) less than 0.05 ($p<0.05$) were considered significant.

2.13. Ethics statement

These animal studies were conducted with accordance with the guidelines of the Institutional Animal Care and Use Committee of the National Institute of Infectious Diseases (NIID), Tokyo, Japan. This study was approved by the Institutional Animal Care and Use Committee of NIID (approval no.613006 and 516010). All animals and infection experiments were performed according to the NIID Institutional Guidelines, in additions to the guidelines of the Science Council, and local rules and regulations.

2.14. Tables and Figures

Table 1. List of marmosets that were used in study on levels of genotype specific and genotype cross-reactive neutralizing antibodies.

Type of infection	Primary infection	Secondary infection	Tertiary infection	Intervals between	Marmosets ID
	Serotype (Genotype)	Serotype (Genotype)	Serotype (Genotype)	DENV infection	
Primary infection					
Group 1	D1 (GI)				M1, M2
Group 2	D1 (GII)				M3-1, M4-1, M5-1, M6,
Group 3	D2 (Cosmopolitan)				M7-1, M8-1, M9-1, M10,
					M11, M12, M13, M14
Group 4	D2 (Asian I)				M15-1, M16-1, M17-1,
					M18-1, M19-1, M20-1
Group 5	D2 (Asian/American)				M21, M22, M23
Secondary infection					
Homologous genotype					
Group 6	D2 (Cosmopolitan)	D2 (Cosmopolitan)		6 months	M24, M25
Group 7	D2 (Asian I)	D2 (Asian)		4 months	M18-2, M19-2
Group 8	D2 (Asian American)	D2 (Asian/American)		4 months	M21-2, M22-2, M23-2
Heterologous genotype					
Group 9	D2 (Asian I)	D2 (Cosmopolitan)		6 months	M15-2, M16-2, M17-2, M26

Type of infection	Primary infection	Secondary infection	Tertiary infection	Intervals between	Marmosets ID
	Serotype (Genotype)	Serotype (Genotype)	Serotype (Genotype)	DENV infection	
Homologous serotype					
Group 10	D1 (GII)	D1 (GI)		7 months	M3-2, M4-2, M5-2
Heterologous serotype					
Group 11	D2 (Cosmopolitan)	D1 (GI)		12 months	M7-2, M8-2, M9-2
Tertiary infection					
Group 12	D2 16881 (Asian I)	D2 (Cosmopolitan)	D1 (GI)	12 months	M15-3, M16-3, M17-3
Primary infection with monovalent live-attenuated DEN vaccine candidate					
Group 13	D1 16007 PDK-13 (GII)				V1-1, V2-1, V3-1, V4-1
Group 14	D2 16881 PDK-53 (Asian I)				V5-1, V6-1
Secondary infection					
Group 15	D1 16007 PDK-13 (GII)	D1 (GI)		7 months	V1-2, V2-2, V3-2, V4-2
Group 16	D2 16881 PDK-53 (Asian I)	D2 (Cosmopolitan)		6 months	V5-2, V6-2, V7-1, V8
Tertiary infection					
Group 17	D2 16881 PDK-53 (Asian I)	D2 (Cosmopolitan)	D1 (GI)	12 months	V5-3, V7-2

D1=DENV-1, D2= DENV-2, D3= DENV-3, D4= DENV-4

Table 2. List of marmosets that were used in study on levels of viremia and immune-related genes.

Type of infection	Primary infection		Secondary infection		Tertiary infection		Marmoset ID
	Serotype	Genotype	Serotype	Genotype	Serotype	Genotype	
Primary infection							
Group A	D2	Asian I					A1, A2, A3
Group B		Asian/American					B1, B2, B3,
Secondary infection							
Homologous serotype							
Group C	D2	Asian I	D2	Asian I			C1, C2
Group D		Asian/American		Asian/American			D1, D2, D3
Heterologous serotype							
Group E	D2	Cosmopolitan	D1	GI			E1, E2
Tertiary infection							
Group F	D2	Asian I	D2	Cosmopolitan	D1	GI	F1, F2
Group G	D2	Asian I	D2	Cosmopolitan	D1	GIV	G1, G2, G3, G4

D1=DENV-1, D2= DENV-2, D3= DENV-3, D4= DENV-4

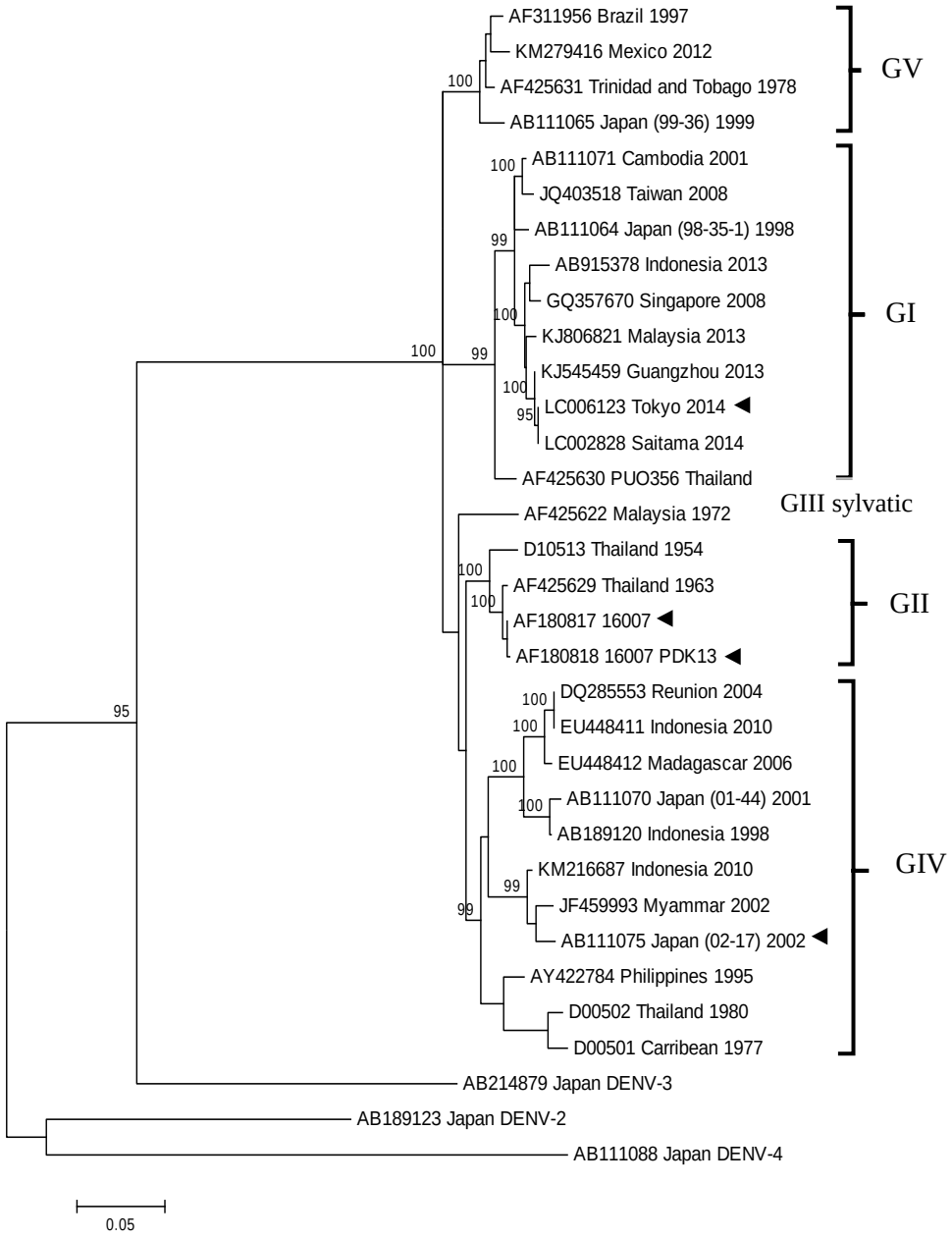

Figure 1. Phylogenetic tree of the DENV-1 strains that were used in this study. Closed arrowhead indicates virus strains that were used in this study, DENV-1 Tokyo-Yoyogi strain belongs to GI genotype, DENV-1 16007 strain and DENV-1 16007 PDK-13 vaccine strain belong to GII genotype, and DENV-1 02-17 strain belongs to GIV genotype.

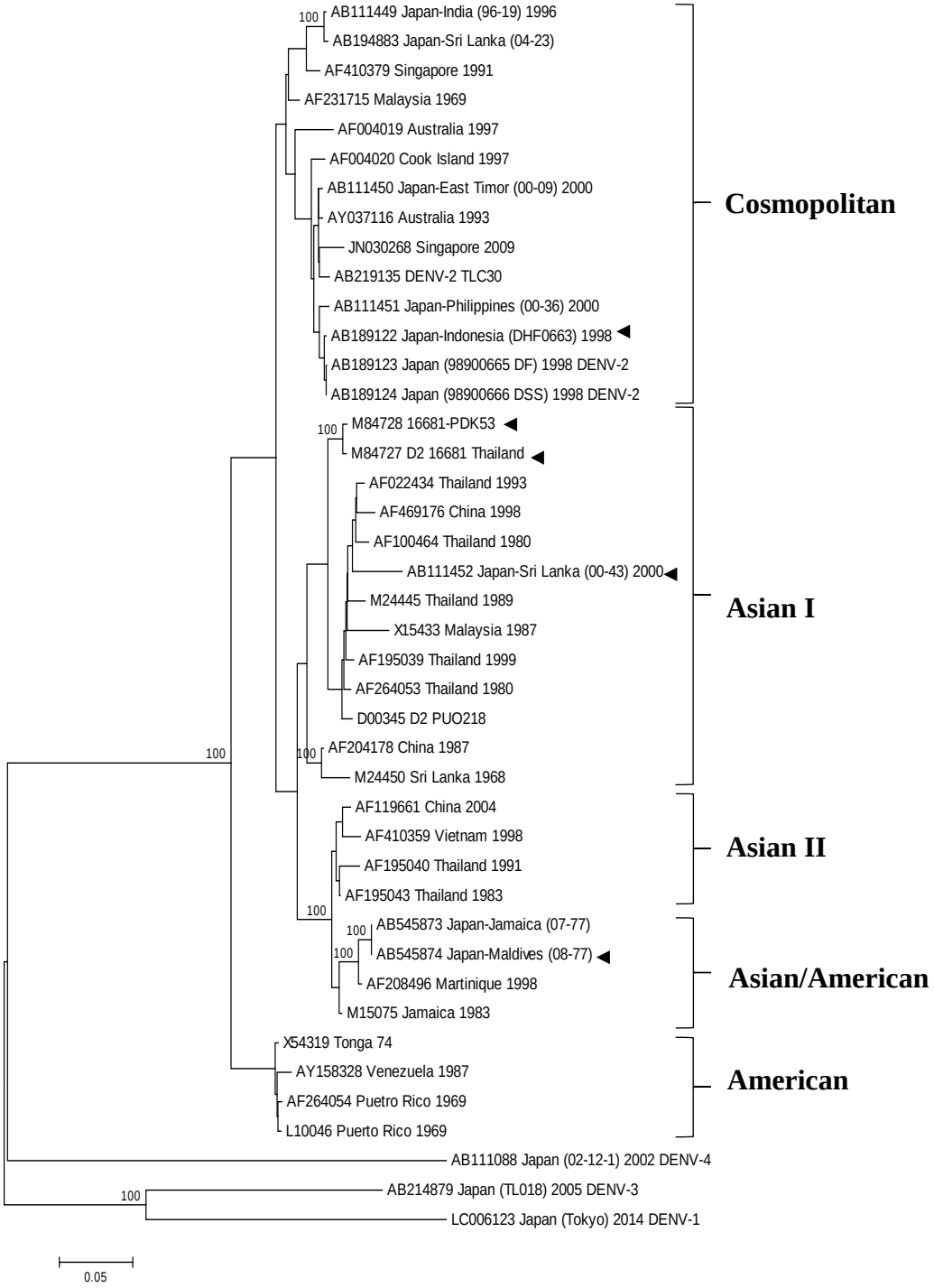

Figure 2. Phylogenetic tree of the DENV-2 strains that were used in this study. Closed arrowhead indicates virus strains that were used in this study. DENV-2 DHF0663 strain belongs to Cosmopolitan genotype. DENV-2 16881 strain, DENV-2 16881 PDK-53 vaccine strain, and DENV-2 00-43 strain belong to Asian I genotype. DENV-2 08-77 strain belongs to Asian/American genotype.

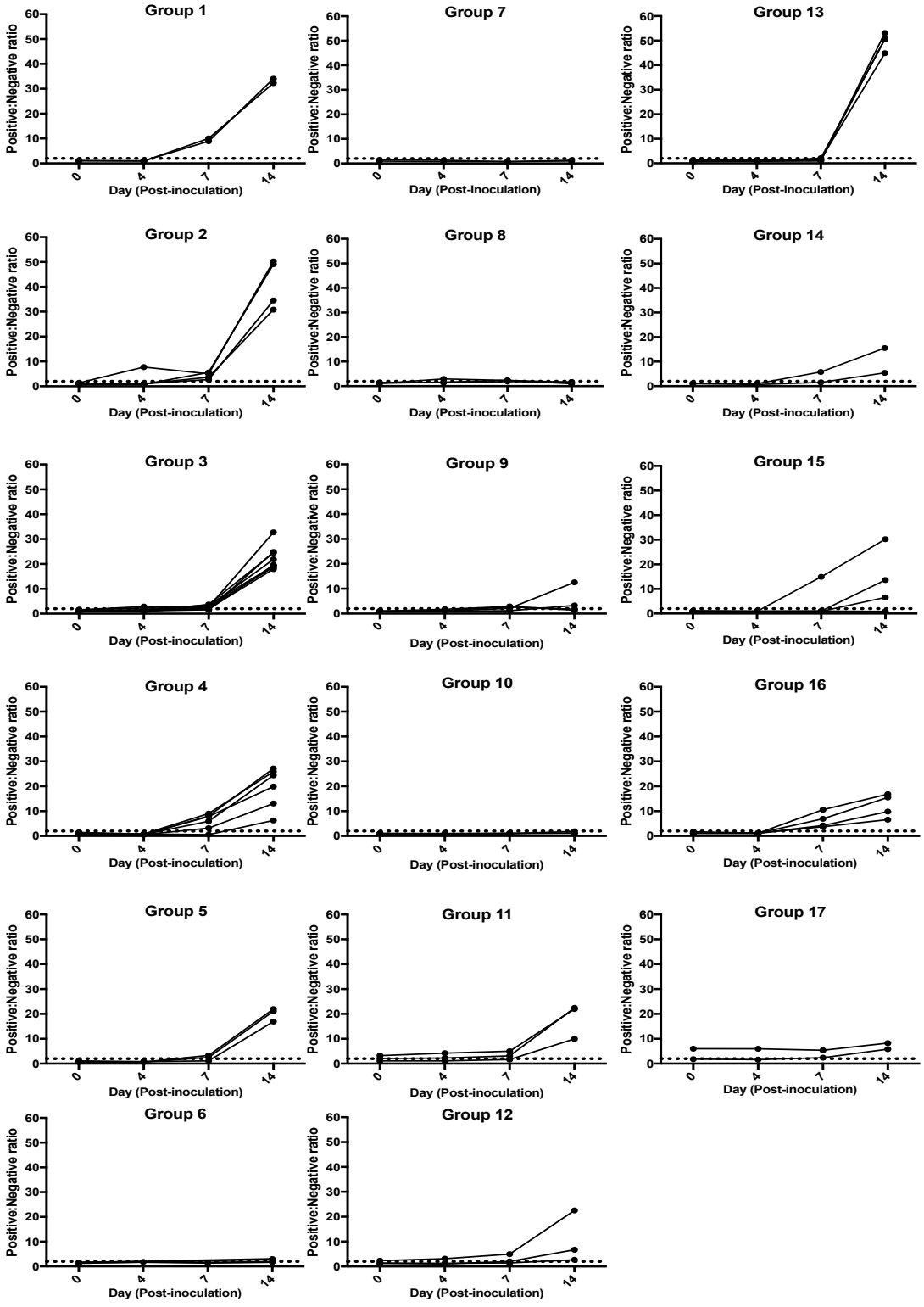

Figure 3. Levels of DENV-specific IgM antibody in plasma samples of group 1-17 marmosets on days 0, 4, 7, and 14 p.i. Dash line indicates baseline of the positive value for P:N ratio. Day 0 was defined as virus inoculation day.

CHAPTER 3: RESULTS

3.1. Levels of neutralizing antibodies

3.1.1 Levels of neutralizing antibodies to genotypes of DENV-2 following primary DENV-1 and DENV-2 infection

Five groups of marmosets were used in the study to determine levels of genotype-specific and genotype cross-reactive neutralizing antibodies to DENV-2 Cosmopolitan, Asian I, and Asian/American genotypes following primary DENV-1 and DENV-2 infections. Group 1 and group 2 consisted of marmosets that were inoculated with DENV-1 GI and DENV-1 GII genotypes, respectively. Group 3, group 4, and group 5 consisted of marmosets that were inoculated with DENV-2 Cosmopolitan, DENV-2 Asian I, and DENV-2 Asian/American genotypes, respectively.

Following primary DENV-2 infection, neutralizing antibodies to all three genotypes of DENV-2 (Cosmopolitan, Asian I and Asian/America genotypes) were absent in group 3, group 4, and group 5 marmosets from days 0-7 p.i. (Figure 4a-c). Neutralizing antibodies to Cosmopolitan, Asian I, and Asian/American genotypes were detected in group 3, group 4, and group 5 marmosets on day 14 p.i. (Figure 4a-c). In Group 3 marmosets, the levels of genotype cross-reactive neutralizing antibodies to Asian I genotype were significantly higher than the levels of genotype-specific neutralizing antibodies to Cosmopolitan genotype ($p=0.007$) on day 14 p.i. (Figure 4a). These results suggested that infection with the Cosmopolitan genotype induced higher levels of neutralizing antibodies to the Asian I genotype. In group 4 and group 5

marmosets, the levels of neutralizing antibodies to all three genotypes of DENV-2 did not differ on day 14 p.i. (Figure 4b-c).

In contrast, following primary DENV-1 infection, neutralizing antibodies to all three genotypes of DENV-2 (Cosmopolitan, Asian I, and Asian/American genotypes) were absent in group 1 and group 2 marmosets on day 0 (prior to virus inoculation), as well as on days 4, 7, and 14 p.i. (Figure 4d-e). These results confirmed that neutralizing antibodies induced during primary DENV-1 infection did not neutralize heterologous serotype (DENV-2).

3.1.2 Levels of neutralizing antibodies to DENV-2 genotypes following secondary infection with homologous and heterologous DENV-2 genotype

Four groups of marmosets were used in the study to determine levels of genotype-specific and genotype cross-reactive neutralizing antibodies to DENV-2 Cosmopolitan, Asian I, and American/Asian genotypes following secondary infection with homologous and heterologous DENV-2 genotype. Group 6, group 7 and group 8 consisted of marmosets that were inoculated with homologous DENV-2 genotype in secondary infection. Group 9 consisted of marmosets that were inoculated with heterologous DENV-2 genotype in secondary infection.

Following secondary infection with homologous genotype, in group 6 and group 7 marmosets, neutralizing antibodies to three genotypes of DENV-2 (Cosmopolitan, Asian I, and Asian/American genotypes) were absence or at low levels prior to virus inoculation (day 0), but the neutralizing antibodies levels were rapidly

increased from day 7 p.i. (Figure 5a-b). The levels of neutralizing antibodies to three genotypes of DENV-2 did not differ between genotypes from days 0-14 p.i. Interestingly, in group 8 marmosets, neutralizing antibodies to three genotypes of DENV-2 were detected prior to virus inoculation (day 0), and the levels increased from day 7 p.i. (Figure 5c). Levels of genotype-specific neutralizing antibodies to the Asian/American genotype were significantly higher than the levels of genotype cross-reactive neutralizing antibodies to the Cosmopolitan and Asian I genotypes on days 0, 4, 7, and 14 p.i. (p_{day0}=0.047, p_{day4}=0.048, p_{day7}=0.042, and p_{day14}=0.027 for the Cosmopolitan genotype, and p_{day0}=0.044, p_{day4}=0.044, and p_{day14}=0.027 for the Asian I genotype).

Following secondary infection with heterologous genotype (primary DENV-2 Asian I genotype, secondary DENV-2 Cosmopolitan genotype), the levels of neutralizing antibodies in group 9 marmosets rapidly increased from day 7 p.i. (Figure 5d). Levels of genotype cross-reactive neutralizing antibodies to the Asian/American genotype were significantly higher than those to the Cosmopolitan and Asian I genotypes on day 7 p.i (p=0.029 and p=0.020, respectively). Levels of genotype cross-reactive neutralizing antibodies to the Asian I genotype were significantly higher than levels of genotype-specific neutralizing antibodies to the Cosmopolitan genotype on day 7 p.i. as well (p=0.034). Similarly, levels of genotype cross-reactive neutralizing antibodies to the Asian/American genotype were significantly higher than those to the Cosmopolitan and Asian I genotypes on day 14 p.i. (p=0.01 and p=0.009, respectively). The results suggested that antibodies possessed higher neutralization activities towards the Asian/American genotype, which were not used in the primary and secondary infection, than towards the Cosmopolitan and Asian I genotypes. After day 365 p.i.,

neutralizing antibodies to Cosmopolitan, Asian I and Asian/American genotypes were still detectable but the levels of neutralizing antibodies between genotypes did not differ.

3.1.3 Levels of neutralizing antibodies to DENV-1 and DENV-2 following secondary infection with homologous and heterologous serotype

Two groups of marmosets were used in the study to determine levels of neutralizing antibodies to DENV-1 and DENV-2 following secondary infection with homologous or heterologous serotype. Group 10 consisted of marmosets that were inoculated with DENV-1 GII genotype in primary infection and DENV-1 GI genotype in secondary infection. Group 11 consisted of marmosets that were inoculated with DENV-2 Cosmopolitan genotype in primary infection and DENV-1 GI genotype in secondary infection.

In group 10 marmosets, serotype cross-reactive neutralizing antibodies to three genotypes of DENV-2 (Cosmopolitan, Asian I and Asian/American genotypes) were absent on days 0, 4, and 7 p.i. (Figure 6a). Low levels of serotype cross-reactive neutralizing antibodies to DENV-2 Asian I genotype were detected on day 7 p.i. The marmosets demonstrated neutralizing activities to three genotypes of DENV-2 on day 14 p.i. and the levels of neutralizing antibodies between genotypes did not differ. High levels of serotype-specific neutralizing antibodies to DENV-1 were detected in all the marmosets on day 14 p.i. (Figure 6b). These results indicate that two inoculation with DENV-1 induced serotype cross-reactive neutralizing antibodies to three genotypes of DENV-2 (Cosmopolitan, Asian I, and Asian/American genotypes).

In group 11 marmoset, levels of neutralizing antibodies to three genotypes of DENV-2 (Cosmopolitan, Asian I, and Asian/American genotypes) were low prior to virus inoculation (day 0) (Figure 6c). The levels of serotype cross-reactive neutralizing antibodies to DENV-2 increased from day 7 p.i. The levels of serotype cross-reactive neutralizing antibodies to three genotypes of DENV-2 did not differ on day 14 p.i. (Figure 6c). Serotype-specific neutralizing antibodies to DENV-1 were detected on day 7 p.i. (Figure 6d). The levels of neutralizing antibodies to the previously infecting serotypes (DENV-2) were, however, higher than those to the currently infecting serotype (DENV-1) (Figure 6c and 6d). This antibody response pattern demonstrates the antigenic sin phenomenon.

3.1.4 Levels of neutralizing antibodies to DENV-2 genotypes in marmosets that were inoculated with candidate DENV-1 and DENV-2 vaccines

Live-attenuated vaccine candidates and four groups of marmosets were used in the study to determine the levels of neutralizing antibodies to DENV-2 genotypes following inoculation with candidate DENV-1 and DENV-2 vaccines. Group 13 consisted of marmosets that were immunized with DENV-1 16007 PDK-13 (GII genotype) vaccine strain and group 14 consisted of marmosets that were immunized with DENV-2 16881 PDK-53 (Asian I genotype) vaccine strain. Group 15 and group 16 consisted of marmosets that were immunized with candidate DENV-1 or DENV-2 vaccine strain in primary infection and then inoculated with homologous serotype in secondary infection.

In group 13 marmosets that were inoculated with DENV-1 16007 PDK-13 (DENV-1 GII genotype) vaccine strain, levels of serotype cross-reactive neutralizing antibody to three genotypes of DENV-2 (Cosmopolitan, Asian I, and Asian/American genotypes) were absent from days 0-14 p.i. (Figure 7a). In group 14 marmosets, one marmoset that was inoculated with DENV-2 16881 PDK-53 (DENV-2 Asian I genotype) vaccine strain, however, demonstrated neutralizing antibodies to DENV-2 Cosmopolitan, Asian I and Asian/American genotypes on day 14 p.i. (Figure 7b).

In group 15 marmosets, following inoculation with DENV-1 GI genotype in secondary infection, serotype cross-reactive neutralizing antibodies to three genotypes of DENV-2 (Cosmopolitan, Asian I, and Asian/American genotypes) were detected on day 14 p.i. (Figure 7c). These results were consistent with those results shown in Figure 3a, and indicated that two inoculation with DENV-1 induced serotype cross-reactive neutralizing antibodies which neutralized heterologous serotype, DENV-2. In group 16 marmosets, following inoculation with DENV-2 Cosmopolitan genotype in secondary infection, neutralizing antibodies to three genotypes of DENV-2 were detected from day 7 (Figure 7d). The levels of genotype cross-reactive neutralizing antibodies to Asian/American genotype were significantly higher than those to Cosmopolitan and Asian I genotypes on day 14 p.i., $p=0.005$ and $p=0.003$, respectively.

3.1.5 Levels of neutralizing antibodies to DENV-1, DENV-2, DENV-3, and DENV-4 following tertiary DENV infection

Two groups of marmosets were used in the study to determine the neutralizing antibodies patterns between marmosets that were inoculated with clinically isolated DENV strain (parent strain) and those that were inoculated with candidate vaccine strain following tertiary infection. Group 12 consisted of marmosets that were first inoculated with DENV-2 16881 strain (Asian I genotype) in primary infection, and then inoculated with DENV-2 Cosmopolitan genotype in secondary infection, and then inoculated with DENV-1 GI genotype in tertiary infection. Group 17 consisted of marmosets that were first inoculated with DENV-2 16881 PDK-53 vaccine strain (Asian I genotype) in primary infection, and then inoculated with DENV-2 Cosmopolitan genotype in secondary infection, and then inoculated with DENV-1 GI genotype in tertiary infection.

In group 12 marmosets (inoculated with the parent strain), neutralizing antibodies to DENV-2 Cosmopolitan, Asian I, and Asian/American genotypes were detected prior to virus inoculation (on day 0) and the levels increased from day 4 p.i. (Figure 8a). Levels of neutralizing antibodies to the Asian/American genotype were significantly higher than those to the Cosmopolitan and Asian I genotypes on days 4, 7 and 14 p.i. (p_{day4}=0.029, p_{day7}=0.017, and p_{day14}=0.0013 for the Cosmopolitan genotype, and p_{day4}=0.029, p_{day7}=0.034, and p_{day14}=0.0013 for the Asian I genotype). Serotypspecific neutralizing antibodies to DENV-1 were detected on day 14 p.i. (Figure 8b). Serotype cross-reactive neutralizing to non-infecting serotypes (DENV-3 and DENV-4) were detected on day 14 p.i. (Figure 8c).

In group 17 marmosets (inoculated with the candidate DENV-2 vaccine strain), neutralizing antibodies to DENV-2 Cosmopolitan, Asian I, and Asian/American genotypes were detected prior to virus inoculation (on day 0), and the levels increased from day 7 p.i. (Figure 8d). Neutralizing antibodies to three genotypes of DENV-2 were at similar levels among genotypes from days 0-14 p.i. Serotype-specific neutralizing antibodies to DENV-1 were detected in one of the marmosets on day 14 p.i. (Figure 8e). Interestingly, cross-reactive neutralizing antibodies to DENV-3, non-infecting serotype, were detected in one of the marmoset on day 14 p.i., but not to DENV-4 (Figure 8f).

3.2. DENV specific IgM and IgG antibodies responses

Presence of DENV-specific IgM and IgG antibodies were determined in groups A, B, C, D, E, F, and G marmosets on days 0, 2, 4, 7, 10, and 14 p.i. (Figure 9).

In primary infection, presence of DENV-specific IgM antibodies in group A and group B marmosets were detected from days 4-14 p.i. (Figure 9a-b). In secondary infection with homologous serotype, levels of DENV-specific IgM antibodies in group C and group D marmosets were below the detection baseline from days 0-14 p.i. (Figure 9c-d). In secondary infection with heterologous infection, presence of DENV-specific IgM antibodies in group E marmosets were detected from days 7-14 p.i. (Figure 9e). In tertiary infection, one marmoset in group F marmoset demonstrated DENV-specific IgM antibodies from days 0-14 p.i, but the other marmoset demonstrated DENV-specific IgM antibodies from days 7-14 p.i., (Figure 9f). Presence of DENV-specific IgM antibodies in group G marmosets were detected from days 4-14 p.i. (Figure 9g).

In primary infection, presence of DENV-specific IgG antibodies in group A and group B marmosets were detected from days 10-14 p.i. (Figure 9h-i). Presence of DENV-specific IgG antibodies in secondary infection with homologous serotype (group C and D marmosets), in secondary infection with heterologous (group E marmosets), and in tertiary infection (group F and group G marmosets) were detected from days 0-14 p.i. (Figure 9j-n).

3.3. Presence of NS1 antigen

Presence of NS1 antigen was determined in groups A, B, C, D, E, F, and G marmosets on days 0, 2, 4, 7, 10, and 14 p.i. (Figure 10). In primary infection (group A and group B marmosets), NS1 antigen was detected from days 2-10 p.i. (Figure 10a-b). In secondary infection with homologous serotype (group C and group D marmosets), NS1 antigen was absent from days 0-14 p.i. (Figure 10c-d). Interestingly, in secondary infection with heterologous serotype (group E marmosets), and in tertiary infection (group F and group G marmosets), NS1 antigen was detected from days 2-10 p.i. (Figure 10e-g).

3.4. Levels of viremia

Viral RNA (vRNA) copy numbers and infectious virus titers were determined in groups A, B, C, D, E, F, and G marmosets on days 0, 2, 4, 7, 10, and 14 p.i. (Figure 11). In primary infection, vRNA copy numbers were detected in group A and group B marmosets from days 2-10 p.i., and peaked on day 4 p.i. (Figure 11a-b). In group A marmosets, infectious virus titers were detected from days 2-7 p.i. (Figure 11f). In group B marmosets, infectious virus titers were detected from days 4-7 p.i. (Figure 11g).

In secondary infection with homologous serotype, vRNA copy numbers and infectious viruses were not detected in group C and group D marmosets. However, in secondary infection with heterologous serotype, vRNA copy numbers and infectious virus were detected in group E marmoset from days 2-7 p.i. (Figure 11c and 11h). In tertiary infection, vRNA copy numbers and infectious virus were detected in group F marmoset from days 2-7 p.i. and days 4-7 p.i., respectively (Figure 11d and 11i). In group G marmosets, vRNA copy numbers and infectious virus were detected from days 2-10 p.i. (Figure 11e and 11j).

3.5. Levels of immune-related genes

3.5.1 CD3ε levels in primary, secondary, and tertiary DENV infections

Levels of CD3ε were determined in groups A, B, C, D, E, F, and G marmosets on days 0, 2, 4, 7, 10, and 14 p.i. (Figure 12). Differences in the levels of CD3ε on days 2, 4, 7, 10, and 14 p.i. were determined and compared with those on day 0 as a reference. In primary infection, the levels of CD3ε did not differ in group A marmosets from days 0-14 p.i. (Figure 12a). Interestingly, in group B marmosets, the levels of CD3ε were significantly increased on days 2 and 7 p.i. ($p=0.0087$ and $p=0.0061$, respectively) (Figure 12b). In secondary infection with homologous serotype, the levels of CD3ε did not differ in group C marmosets from days 0-14 p.i. (Figure 12c). In group D marmosets, the levels of CD3ε significantly increased on day 2 p.i., $p=0.0276$ (Figure 12d). In secondary infection with heterologous serotype, the levels of CD3ε did not differ in group E marmosets from days 0-14 p.i. (Figure 12e). In tertiary infection, the levels of CD3ε significantly increased in group F marmosets on day 14 p.i., ($p=0.0018$), but the levels did not differ in group G marmosets from days 0-14 p.i. (Figure 12f-g).

3.5.2 CD4 levels in primary, secondary, and tertiary DENV infection

Levels of CD4 were determined in groups A, B, C, D, E, F, and G marmosets on days 0, 2, 4, 7, 10, and 14 p.i. (Figure 13). Differences in the levels of CD4 on days 2, 4, 7, 10, and 14 p.i. were determined and compared with those on day 0 as a reference. In primary infection, the levels of CD4 did not differ in group A marmosets from days 0-14 p.i. but the levels significantly increased in group B marmosets on day 7 p.i., p=0.0307 (Figure 13a-b). In secondary infection with homologous serotype, the levels of CD4 did not differ in group C and group D marmosets from days 0-14 p.i. (Figure 13c-d). In secondary infection with heterologous serotype, the levels of CD4 significantly decreased in group E marmosets on day 4 p.i., p=0.0274 (Figure 13e). In tertiary infection, the levels of CD4 did not diffed in group F marmosets from days 0-14 p.i., but the levels significantly decreased in group G marmosets on day 14 p.i. (Figure 13f-g).

3.5.3 CD8α levels in primary, secondary, and tertiary DENV infection

Levels of CD8α were determined in groups A, B, C, D, E, F, and G marmosets on days 0, 2, 4, 7, 10, and 14 p.i. (Figure 14). Differences in the levels of CD8α on days 2, 4, 7, 10, and 14 p.i. were determined and compared with those on day 0 as a reference. In primary infection, the levels of CD8α significantly decreased in group A marmosets on day 4 p.i. (p=0.0070), but the levels did not differ in group B marmosets from days 0-14 p.i. (Figure 14a-b). In secondary infection with homologous serotype, the levels of CD8α did not differ in group C and group D marmosets from days 0-14 p.i. (Figure 14c-d). In secondary infection with heterologous serotype, the levels of CD8α significantly decreased in group E marmosets on day 14 p.i., p=0.0069 (Figure 14e). In

tertiary infection, the levels of CD8α did not differ in group F marmosets from days 0-14 p.i., but the levels significantly decreased on day 2 p.i. ($p=0.0060$) and significantly increased on day 7 p.i. ($p=0.0099$) in group F marmosets (Figure 14f-g).

3.5.4 IFNγ levels in primary, secondary, and tertiary DENV infection

Levels of IFNγ were determined in groups A, B, C, D, E, F, and G marmosets on days 0, 2, 4, 7, 10, and 14 p.i. (Figure 15). Differences in the levels of IFNγ on days 2, 4, 7, 10, and 14 p.i. were determined and compared with those on day 0 as a reference. In primary infection, the levels of IFNγ significantly decreased in group A marmosets on days 2, 4, and 7 p.i. ($p_{day2}=0.0104$, $p_{day4}=0.0012$, $p_{day7}=0.0240$), but the levels did not differ in group B marmosets from days 0-14 p.i. (Figure 15a-b). In secondary infection with homologous serotype, the levels of IFNγ did not differ in group C and group D marmosets from days 0-14 p.i. (Figure 15c-d). In secondary infection with heterologous serotype, the levels of IFNγ did not differ in group E marmosets from days 0-14 p.i. (Figure 15e). In tertiary infection, the levels of IFNγ did not differ in group F marmosets from day 0-14 p.i. but the levels significantly decreased in group G marmosets on day 2 p.i. ($p=0.0354$) (Figure 15f-g).

3.5.5 IL-1β levels in primary, secondary, and tertiary DENV infection

Levels of IL-1β were determined in groups A, B, C, D, E, F, and G marmosets on days 0, 2, 4, 7, 10, and 14 p.i. (Figure 16). Differences in the levels of IL-1β on days 2, 4, 7, 10, and 14 p.i. were determined and compared with those on day 0 as a reference. In primary infection, the levels of IL-1β significantly decreased in group A marmosets

on days 4, 7, and 14 p.i. and in group B marmosets on days 7 and 14 p.i. (p_{day4}=0.0108, p_{day7}=0.0246, p_{day14}=0.0030 for group A marmosets, p_{day7}=0.0051, p_{day14}=0.0194 for group B marmosets) (Figure 16a-b). In secondary infection with homologous serotype, the levels of IL-1β did not differ in group C and group D marmosets from days 0-14 p.i. (Figure 16c-d). In secondary infection with heterologous serotype, levels of IL-1β significantly decreased in group E marmosets on days 7 and 10 p.i. (p_{day7}=0.0383, p_{day10}=0.0475) (Figure 16e). In tertiary infection, the levels of IL-1β significantly decreased in group F and group G marmosets on days 7, 10, and 14 p.i. (p_{day7}=0.0321, p_{day10}=0.0473, p_{day14}=0.0256 for group F, and p_{day7}=0.0014 p_{day10}=0.0447, p_{day14}=0.0020 for group G) (Figure 16f-g).

3.5.6 IL-2 levels in primary, secondary, and tertiary DENV infection

Levels of IL-2 were determined in groups A, B, C, D, E, F, and G marmosets on days 0, 2, 4, 7, 10, and 14 p.i. (Figure 17). Differences in the levels of IL-2 on days 2, 4, 7, 10, and 14 p.i. were determined and compared with those on day 0 as a reference. In primary infection, the levels of IL-2 did not differ in group A and group B marmosets from days 0-14 p.i. (Figure 17a-b). In secondary infection with homologous serotype, the levels of IL-2 did not differ in group C and group D marmosets from days 0-14 p.i. (Figure 17c-d). In secondary infection with heterologous serotype, the levels of IL-2 significantly decreased in group E marmosets on day 7 p.i., p=0.0140 (Figure 17e). In tertiary infection, the levels of IL-2 significantly decreased in group F marmosets on day 14 p.i. (p=0.0377) but the levels did not differ in group G marmosets from days 0-14 p.i. (Figure 17f-g).

3.5.7 IL-10 levels in primary, secondary, and tertiary DENV infection

Levels of IL-10 were determined in groups A, B, C, D, E, F, and G marmosets on days 0, 2, 4, 7, 10, and 14 p.i. (Figure 18). Differences in the levels of IL-10 on days 2, 4, 7, 10, and 14 p.i. were determined and compared with those day 0 as a reference. In primary infection, the levels of IL-10 did not differ in group A and group B marmosets from days 0-14 p.i. (Figure 18a-b). In secondary infection with homologous serotype, levels of IL-10 significantly decreased in group C marmoset on days 7, 10, and 14 p.i. (p_{day7}=0.0391, p_{day10}=0.0242, p_{day14}=0.0084), but the levels did not differ in group D marmosets from days 0-14 p.i. (Figure 18c-d). In secondary infection with heterologous serotype, the levels of IL-10 did not differ in group E marmosets from days 0-14 p.i. (Figure 18e). In tertiary infection, the levels of IL-10 did not differ in group F and group G marmosets from days 0-14 p.i (Figure 18f-g).

3.5.8 Relationship between levels of CD8α and levels of viremia

Levels of CD8α were compared side-by-side with levels of viremia (viral RNA copies number and infectious virus titers) in group A marmosets (primary infection), group E marmosets (secondary infection with heterologous serotype), and group G marmosets (tertiary infection) (Figure 19). In group A marmosets, the levels of CD8α decreased on day 4 p.i when the levels of viremia increased (Figure 19a-c). In group E marmosets, the levels of CD8α increased on day 14 p.i. when viremia was cleared (Figure 19d-f). In group G marmosets, the levels of CD8α decreased on day 2 p.i when viremia levels increased, and the levels of CD8α increased on day 7 p.i. when viremia levels decreased (Figure 19g-i).

3.6. Figures

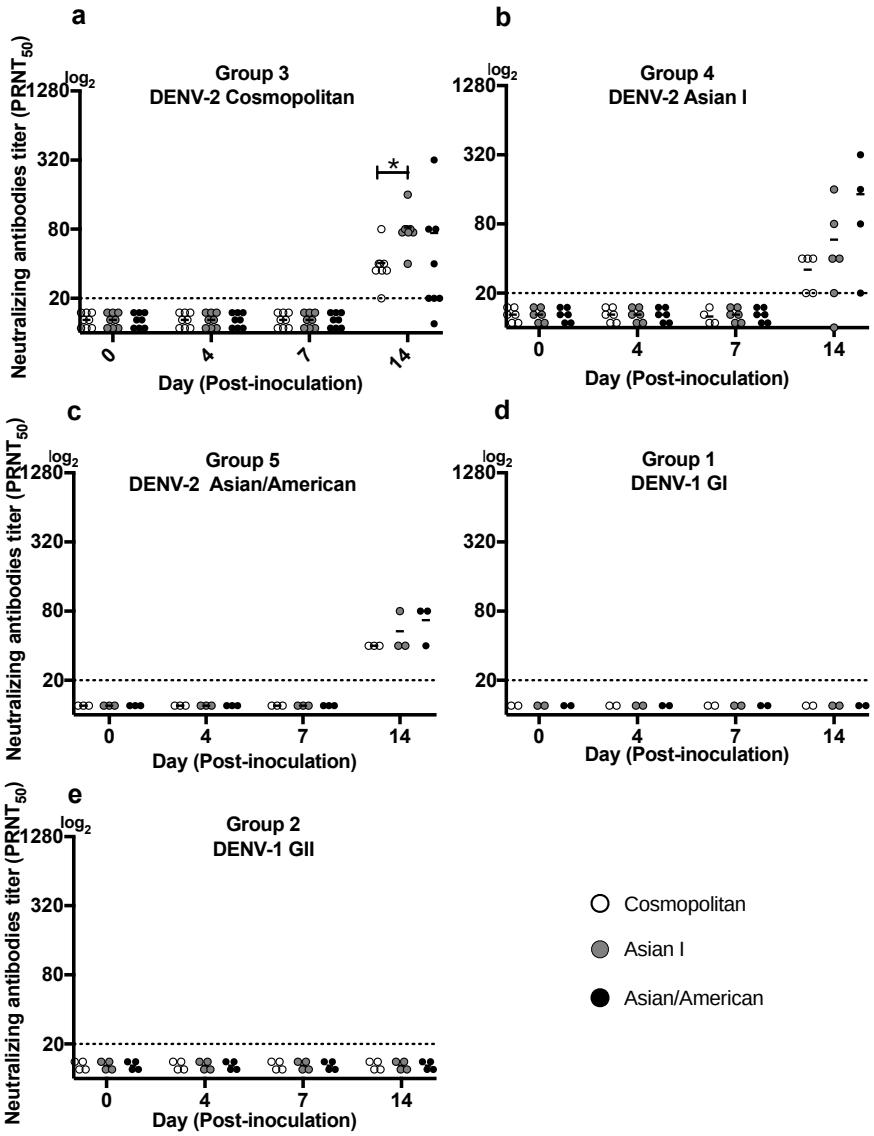

Figure 4. Levels of neutralizing antibodies to DENV-2 Cosmopolitan, Asian I and Asian/American genotypes following primary DENV-1 and DENV-2 infection on days 0, 4, 7, and 14 p.i. Neutralizing antibodies titers of marmosets that were inoculated with: (a) DENV-2 Cosmopolitan genotype, (b) DENV-2 Asian I genotype, (c) DENV-2 Asian/American genotype, (d) DENV-1 GI genotype, (e) DENV-1 GII genotype. Asterisks (*) indicates significant differences ($p<0.05$) in titers of neutralizing antibodies between genotypes.

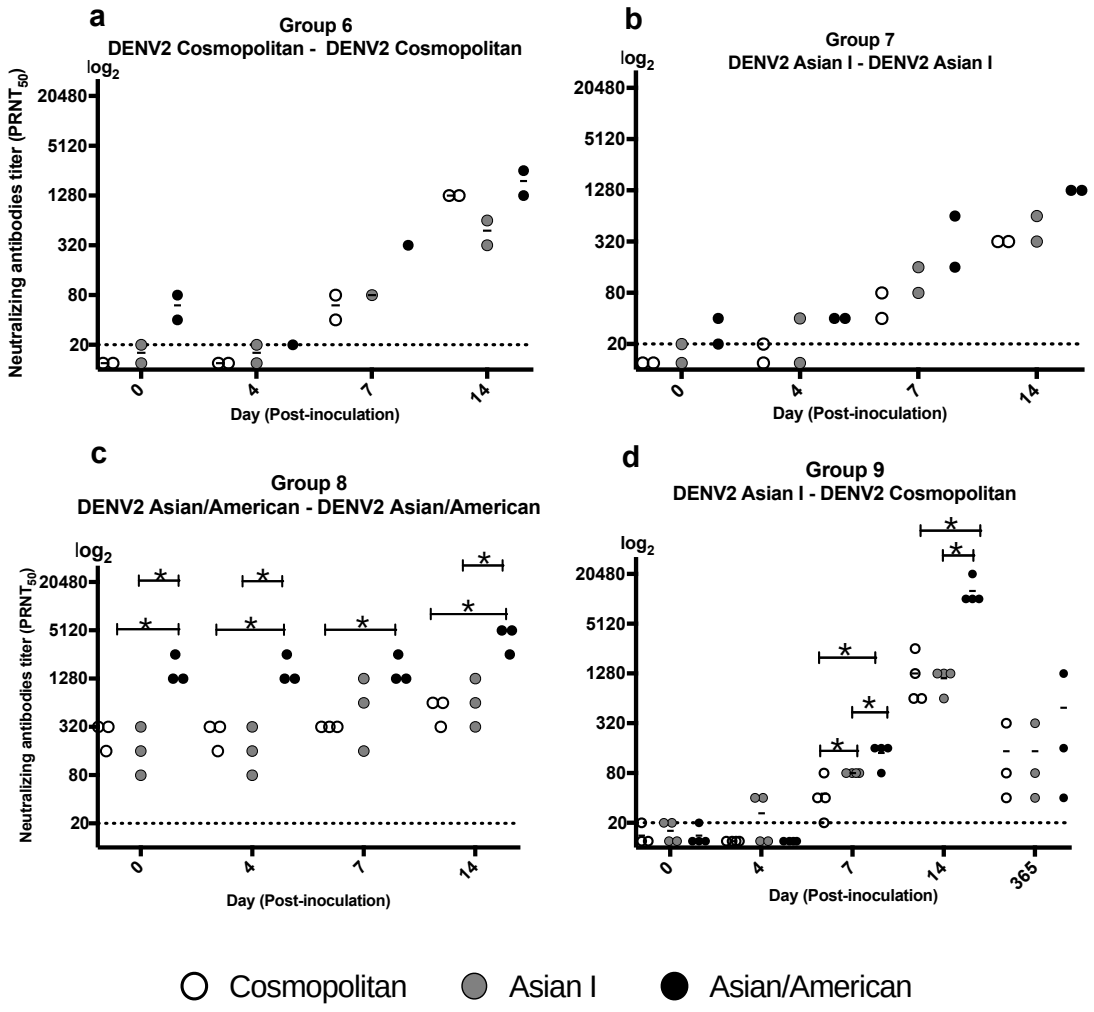

Figure 5. Levels of neutralizing antibodies to DENV-2 Cosmopolitan, Asian I and Asian/American genotypes following secondary infection with homologous and heterologous genotype of DENV-2 on days 0, 4, 7, and 14 p.i. Neutralizing antibodies titers of marmosets with: (a) Homologous DENV-2 Cosmopolitan genotype infection, (b) Homologous DENV-2 Asian I genotype infection, (c) Homologous DENV-2 Asian/American genotype infection, (d) Heterologous genotype DENV-2 infection. Asterisks (*) indicates significant differences ($p<0.05$) in titers of neutralizing antibodies between genotypes.

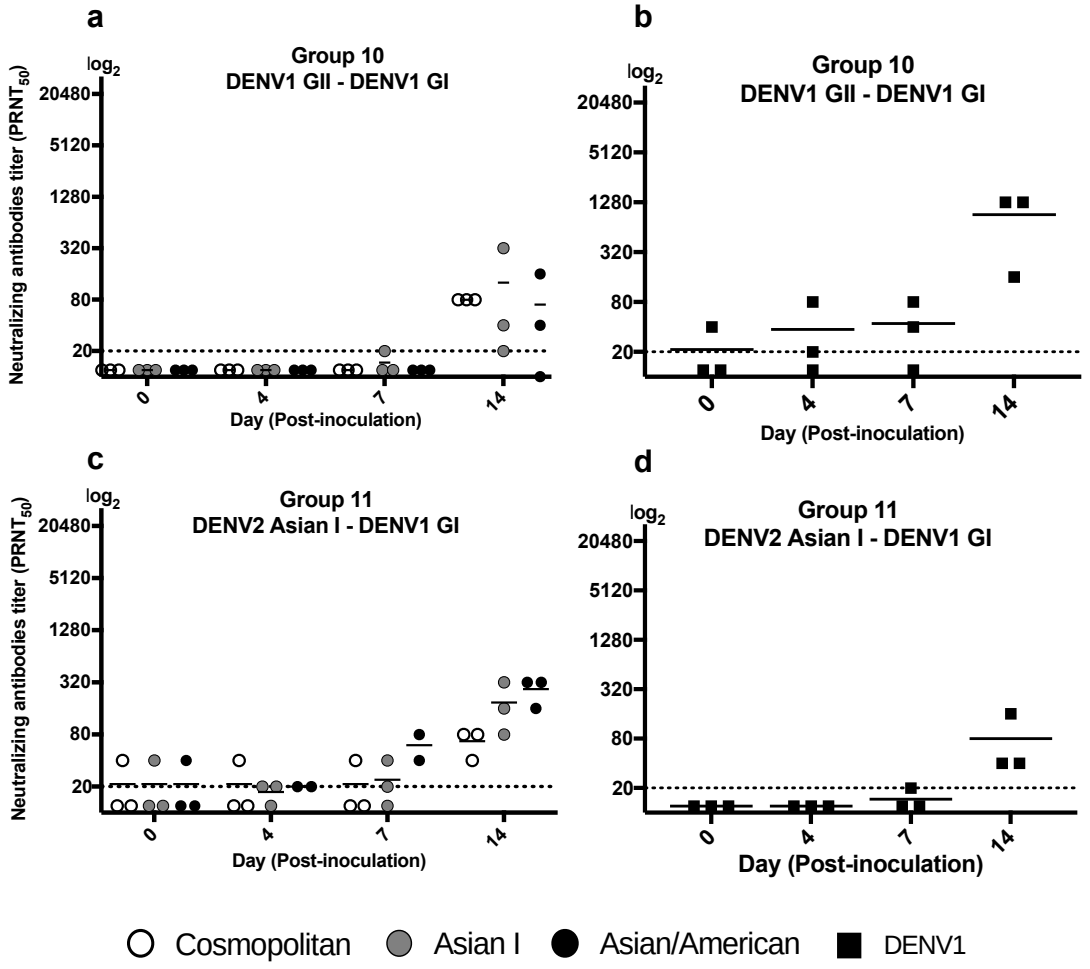

Figure 6. Levels of neutralizing antibodies to DENV-1 and DENV-2 Cosmopolitan, Asian I and Asian/American genotypes following secondary infection with homologous and heterologous serotype of DENV on days 0, 4, 7, and 14 p.i. Neutralizing antibodies titers of marmosets with: (a) Homologous serotype DENV-1 infection, (b) Neutralizing antibodies titers to DENV-1 during homologous serotypes DENV-1 infection, (c) Heterologous serotype DENV-1 infection, (d) Neutralizing antibodies titers to DENV-1 during heterologous serotypes DENV-1 infection.

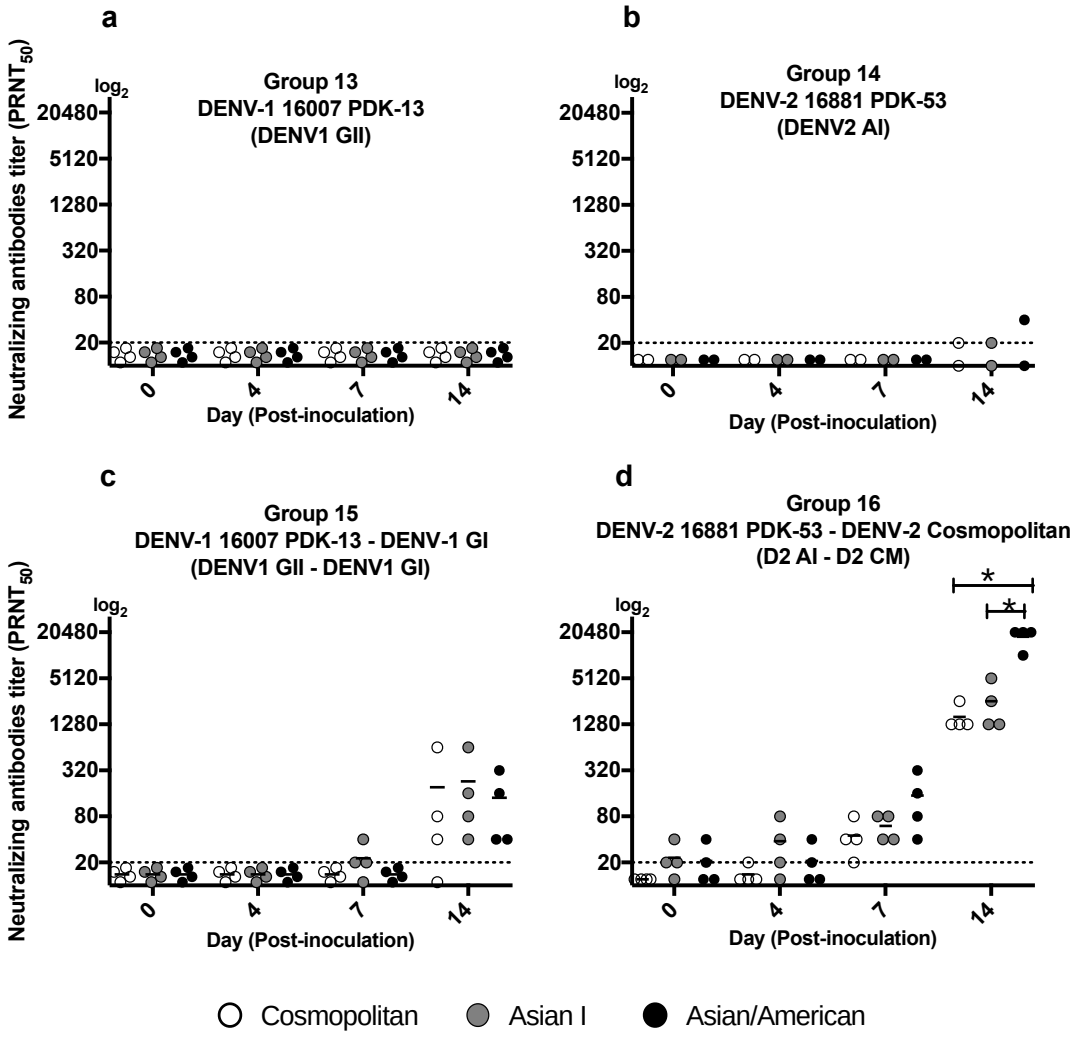

Figure 7. Levels of neutralizing antibodies to DENV-2 Cosmopolitan, Asian I and Asian/American genotypes in marmosets that were inoculated with candidate DENV-1 and DENV-2 vaccine strain on days 0, 4, 7, and 14 p.i. Neutralizing antibodies titers in marmosets inoculated with: (a) DENV-1 16007 PDK-13 vaccine strain, (b) DENV-2 16881 PDK-53 vaccine strain, (c) DENV-1 16007 PDK-13 vaccine strain in primary infection and DENV-1 GI genotype in secondary infection, (d) DENV-2 16881 PDK-53 vaccine strain in primary infection and DENV-2 Cosmopolitan genotype in secondary infection. Asterisks (*) indicates significant differences ($p<0.05$) in neutralizing antibodies titers between genotypes.

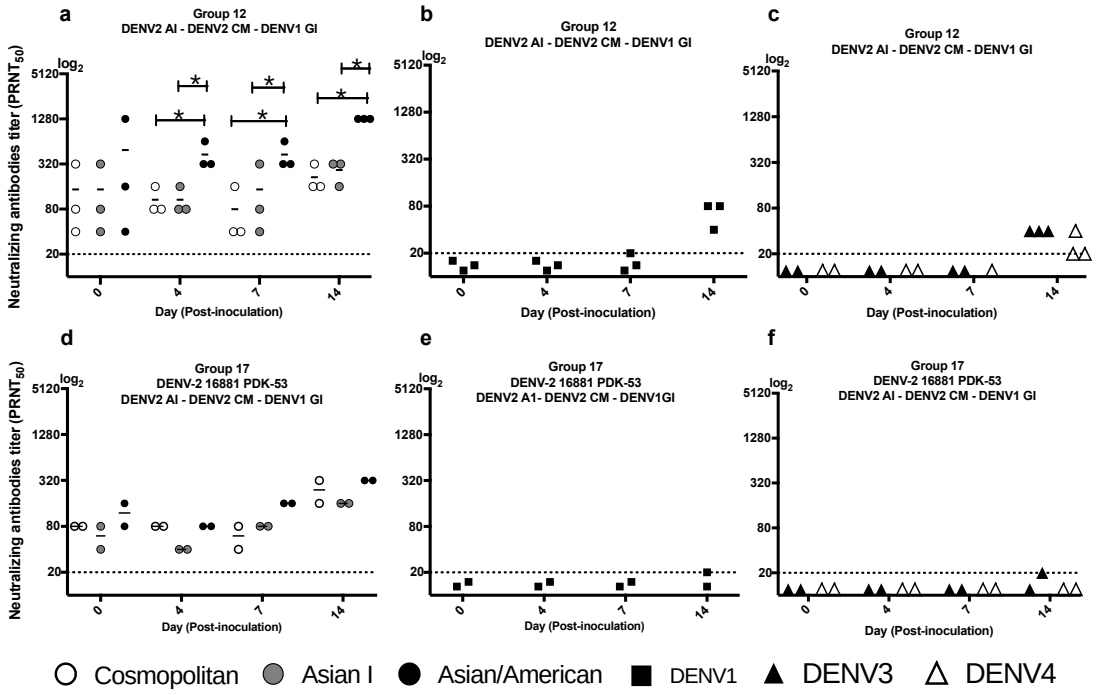

Figure 8. Levels of neutralizing antibodies to DENV-2 Cosmopolitan, Asian I, and Asian/American genotypes, DENV-1, DENV-3, and DENV-4 following tertiary infection on days 0, 4, 7, and 14 p.i. (a) Marmosets that were inoculated with DENV-2 16881 strain (Asian I genotype) in primary infection, inoculated with the DENV-2 Cosmopolitan genotype in secondary infection, and inoculated with DENV-1 GI genotype in tertiary infection. (b) Neutralizing antibodies titers to DENV-1 in the same marmosets in (a). (c) Neutralizing antibodies titers to DENV-3 and DENV-4 in the same marmosets in (a). (d) Marmosets that were inoculated with DENV-2 16881 PDK-53 vaccine strain (Asian I genotype) in primary infection, inoculated with DENV-2 Cosmopolitan genotype in secondary infection, and inoculated with DENV-1 GI genotype in tertiary infection. (e) Neutralizing antibodies titers to DENV-1 in the same marmosets in (d). (f) Neutralizing antibodies titers to DENV-3 and DENV-4 in the same marmosets in (d). Asterisks (*) indicates significant differences ($p<0.05$) in neutralizing antibodies titers between genotypes. AI indicates DENV-2 Asian I genotype. CM indicates DENV-2 Cosmopolitan genotype.

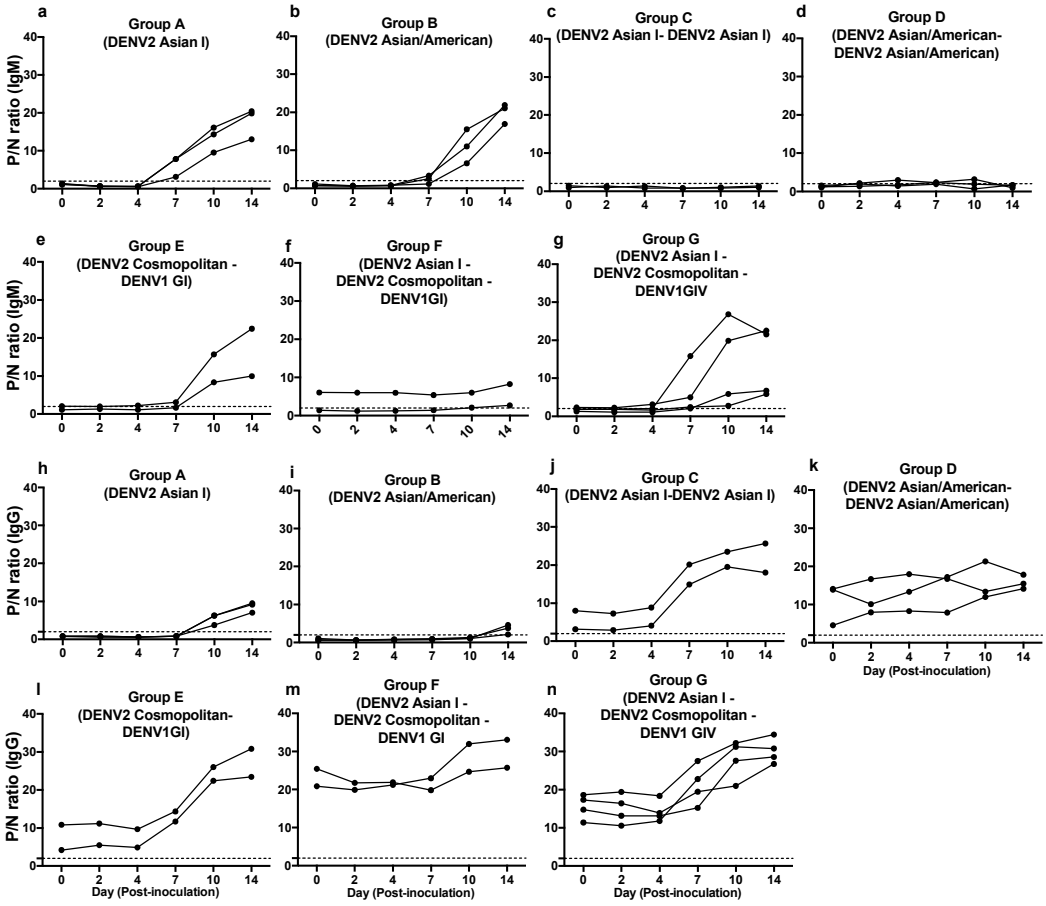

Figure 9. **Levels of DENV-specific IgM and IgG antibody in plasma samples of groups A, B, C, D, E, F, and G marmosets on days 0, 2, 4, 7, 10, and 14 p.i.** (a-g) Positive negative (P/N) ratio of DENV-specific IgM antibody in groups A, B, C, D, E, F, and G marmosets, respectively. (h-n) P/N ratio of DENV-specific IgG antibody in groups A, B, C, D, E, F, and G marmosets, respectively. Dash line indicates the baseline of positive value for P/N ratio. Day 0 was defined as day of virus inoculation.

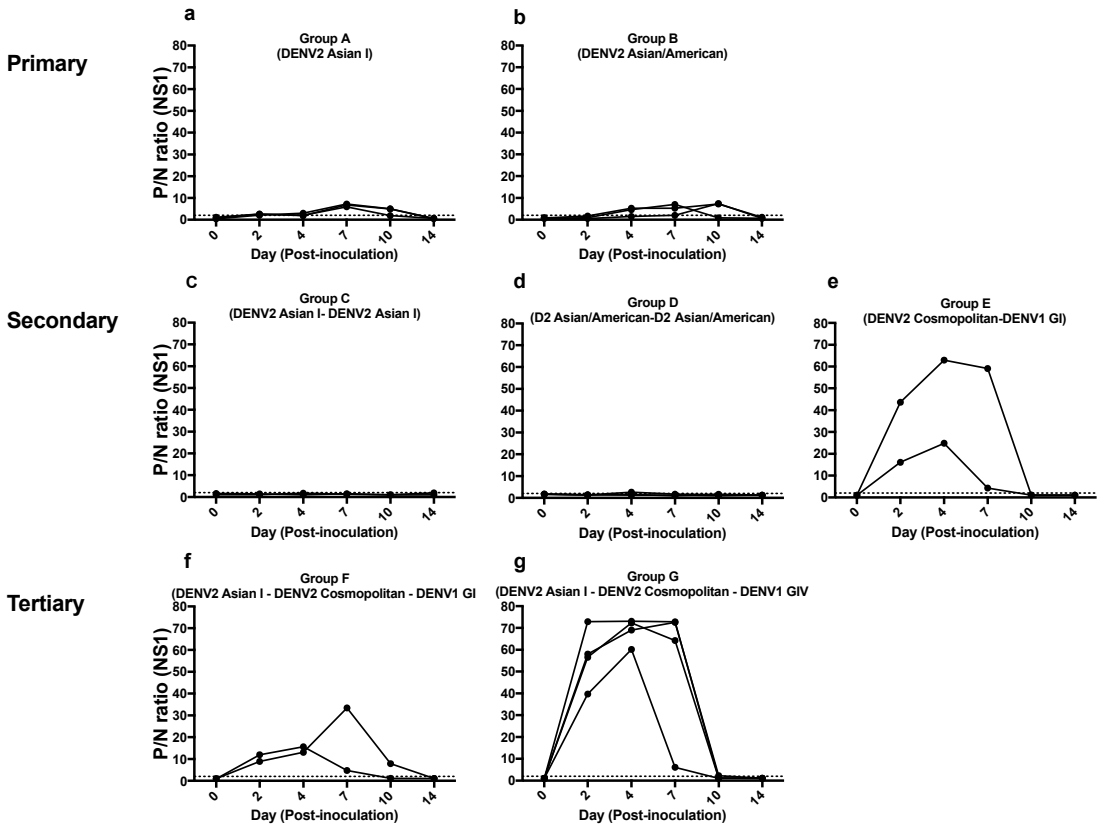

Figure 10. Levels of NS1 antigen in plasma samples of groups A, B, C, D, E, F, and G marmosets on days 0, 2, 4, 7, 10, and 14 p.i. Levels of NS1 antigen in marmosets that were inoculated with: (a) DENV-2 Asian I genotype, (b) DENV-2 Asian/American genotype, (c) Secondary infection with homologous serotype, DENV-2 Asian I genotype, (d) Secondary infection with homologous serotype, DENV-2 Asian/American genotype (e) Secondary infection with heterologous serotype, DENV-1 GI genotype, (f) Tertiary infection with DENV-1 GI genotype, (g) Tertiary infection with DENV-1 GI genotype. Dash line indicates the baseline of positive value for P/N ratio. Day 0 defined as day of virus inoculation.

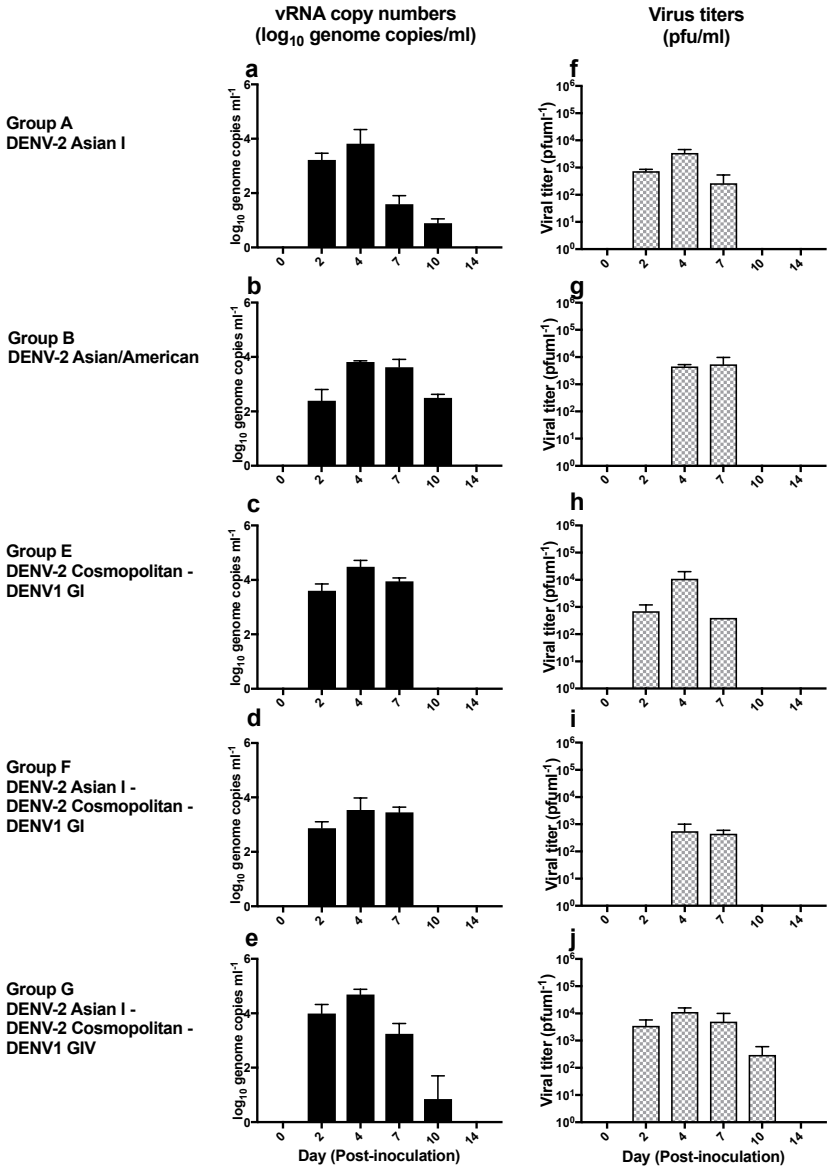

Figure 11. Levels of viral RNA copy numbers and infectious virus titers in plasma samples of groups A, B, E, F, and G marmosets on days 0, 2, 4, 7, 10, and 14 p.i. Levels of vRNA copy numbers in marmosets that were inoculated with: (a) Primary DENV-2 Asian I genotype, (b) Primary DENV-2 Asian/American genotype, (c) Secondary infection with heterologous DENV-1 serotype, (d) Tertiary DENV-1 GI genotype, (e) Tertiary DENV-1 GIV genotype; Levels of infectious virus titers in marmosets that were inoculated with : (f) Primary DENV-2 Asian I genotype, (g) Primary DENV-2 Asian/American genotype, (h) Secondary infection with heterologous DENV-1 serotype, (i) Tertiary DENV-1 GI genotype, (j) Tertiary DENV-1 GIV genotype. Filled bar indicates levels of viral RNA copy numbers, log_{10} genome copies/ml. Pattern bar indicates titer of DENV, pfu/ml.

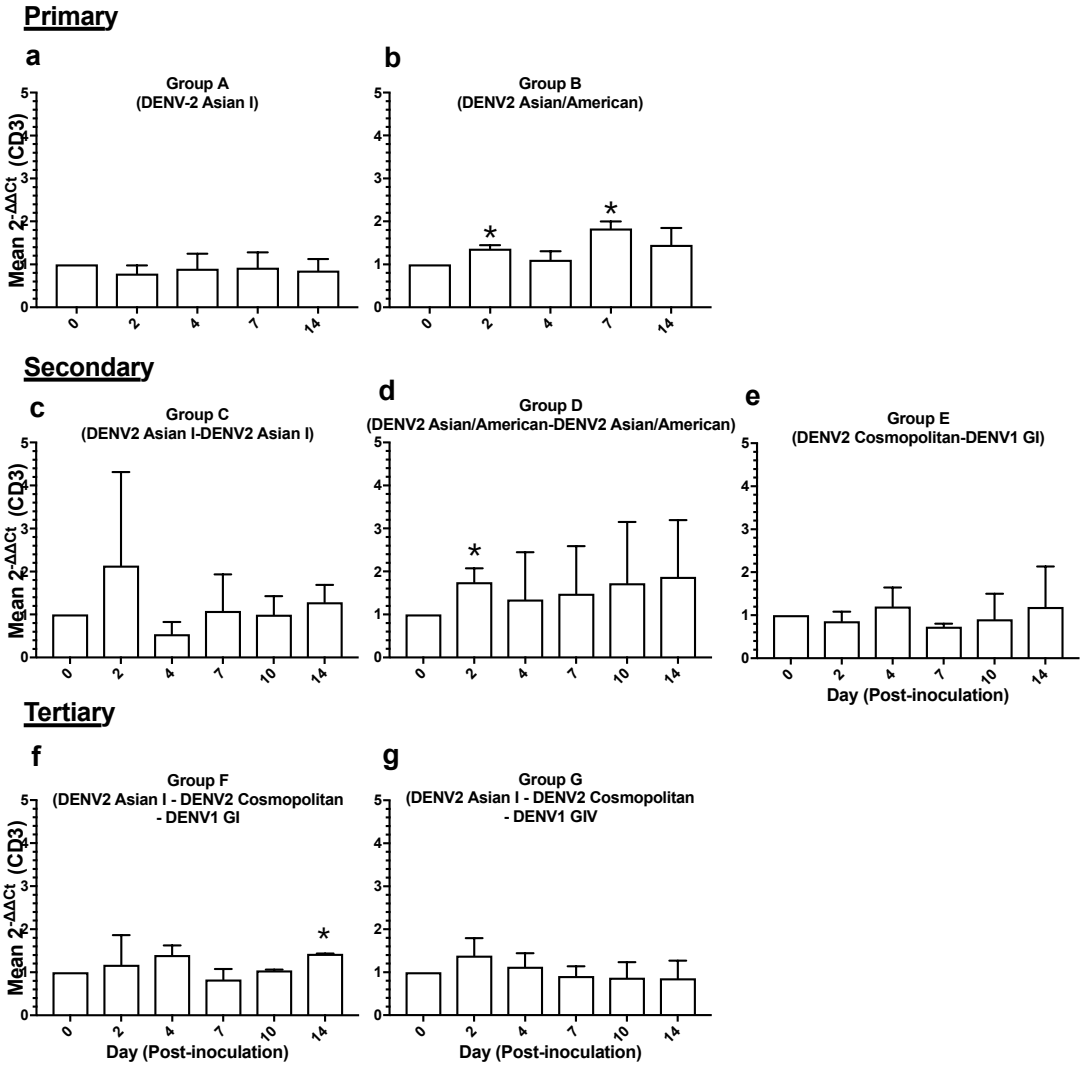

Figure 12. Levels of CD3ε in PBMC samples of groups A, B, C, D, E, F, and G marmosets on days 0, 2, 4, 7, 10, and 14 p.i. Levels of CD3ε in marmosets that were inoculated with: (a) Primary DENV-2 Asian I genotype, (b) Primary DENV-2 Asian/American genotype, (c) Secondary infection with homologous serotype, DENV-2 Asian I genotype, (d) Secondary infection with homologous serotype, DENV-2 Asian/American genotype, (e) Secondary infection with heterologous serotype, DENV-1 GI genotype, (f) Tertiary DENV-1 GI genotype, (g) Tertiary DENV-1 GIV genotype. Bar represents mean expression levels + standard deviation (SD). Asterisk (*) indicates significant differences ($p<0.05$) in levels of CD3ε, compared with day 0 as a reference.

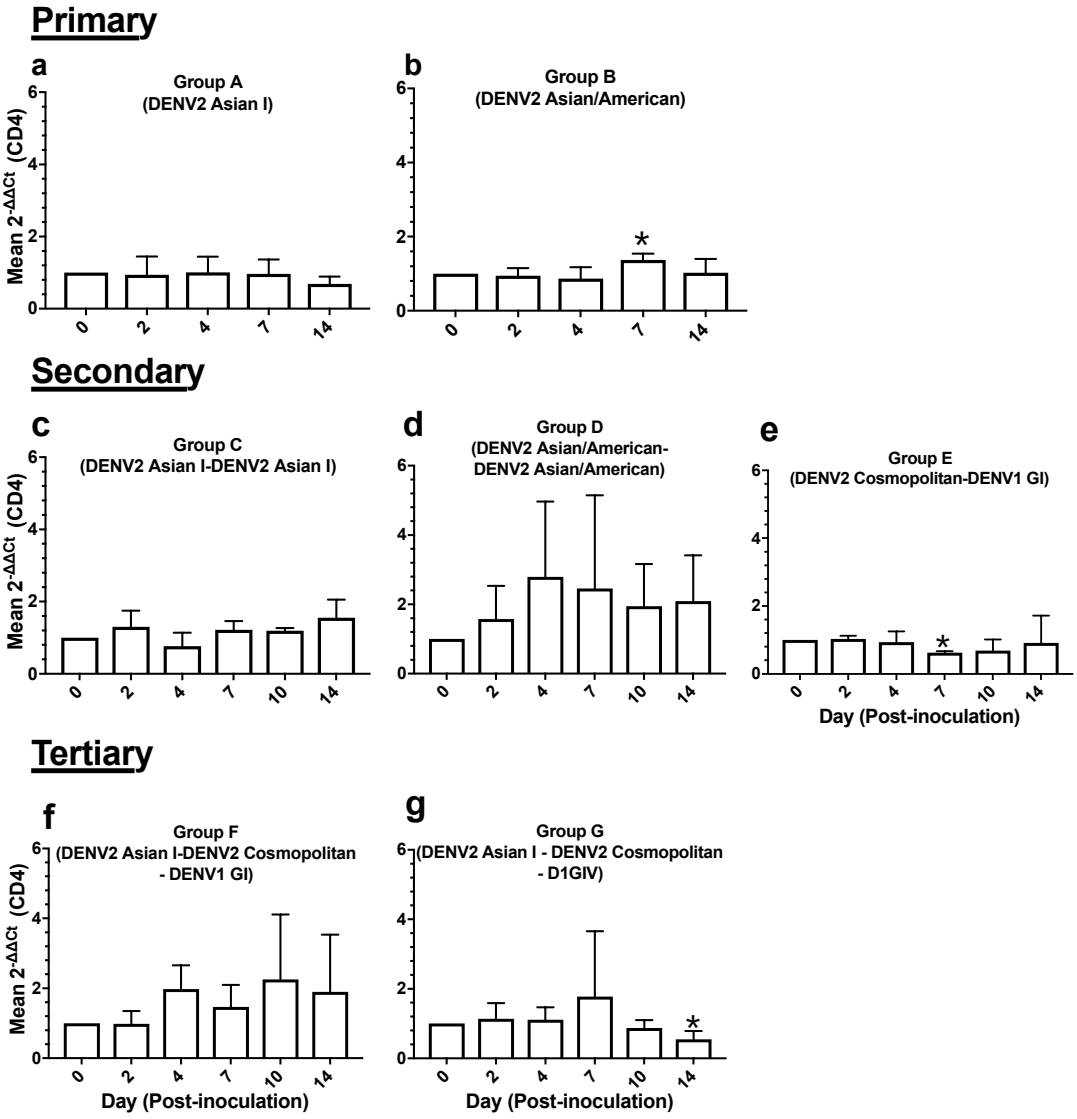

Figure 13. Levels of CD4 in PBMC samples of groups A, B, C, D, E, F, and G marmosets on days 0, 2 4, 7, 10, and 14 p.i. Levels of CD4 in marmosets that were inoculated with: (a) Primary DENV-2 Asian I genotype, (b) Primary DENV-2 Asian/American genotype, (c) Secondary infection with homologous serotype, DENV-2 Asian I genotype, (d) Secondary infection with homologous serotype, DENV-2 Asian/American genotype, (e) Secondary infection with heterologous serotype, DENV-1 GI serotype, (f) Tertiary DENV-1 GI genotype, (g) Tertiary DENV-1 GII genotype. Bar represents mean expression levels + SD. Asterisk (*) indicates significant differences ($p<0.05$) in levels of CD4, compared with day 0 as a reference.

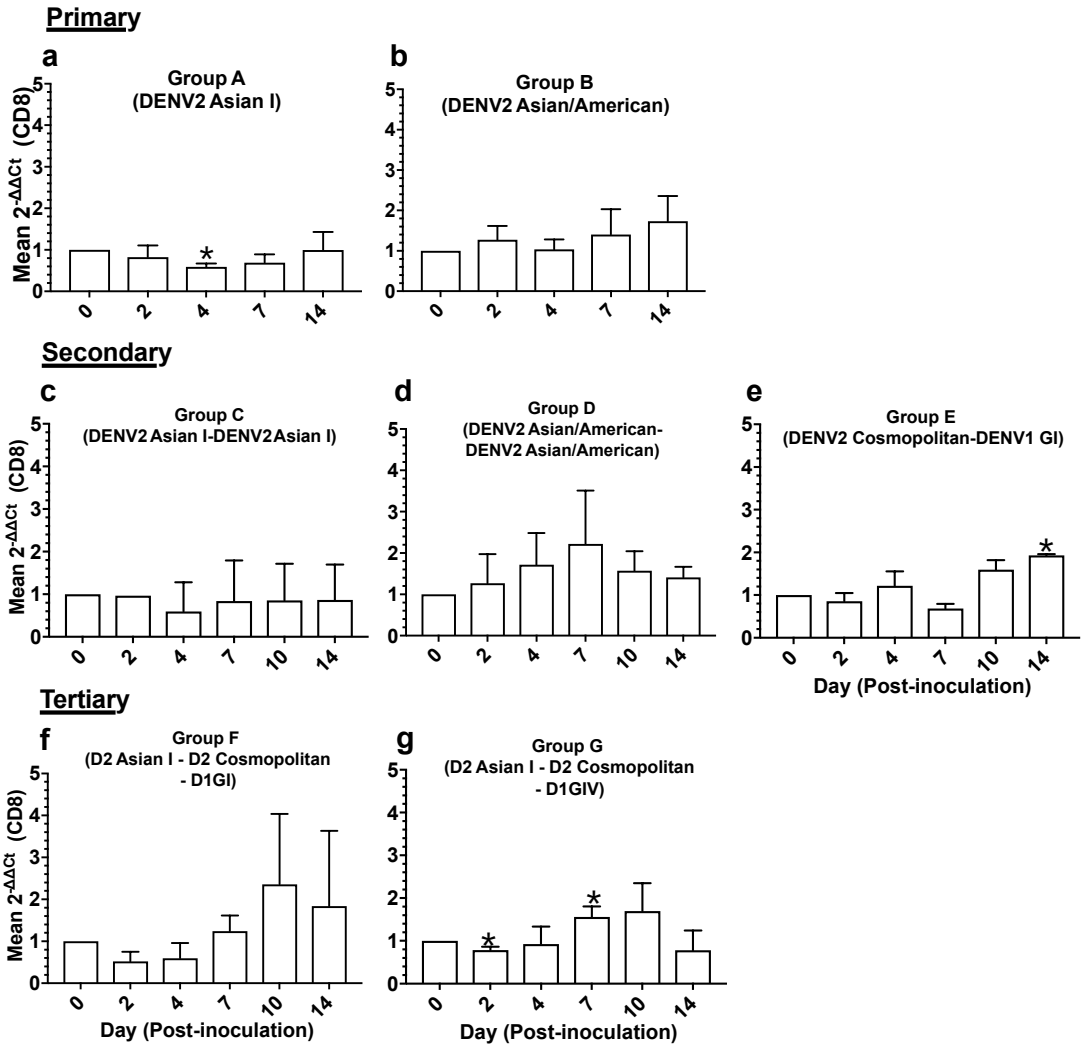

Figure 14. Levels of CD8α in PBMC samples of groups A, B, C, D, E, F, and G marmosets on days 0, 2, 4, 7, 10, and 14 p.i. Levels of CD8α in marmosets that were inoculated with: (a) Primary DENV-2 Asian I genotype, (b) Primary DENV-2 Asian/American genotype, (c) Secondary infection with homologous serotype, DENV-2 Asian I genotype (d) Secondary infection with homologous serotype, DENV-2 Asian/American genotype, (e) Secondary infection with heterologous serotype, DENV-1 GI genotype (f) Tertiary DENV-1 GI genotype, (g) Tertiary DENV-1 GIV genotype. Bar represents mean expression levels +SD. Asterisk (*) indicates significant differences ($p<0.05$) in levels of CD8α, compared with day 0 as a reference.

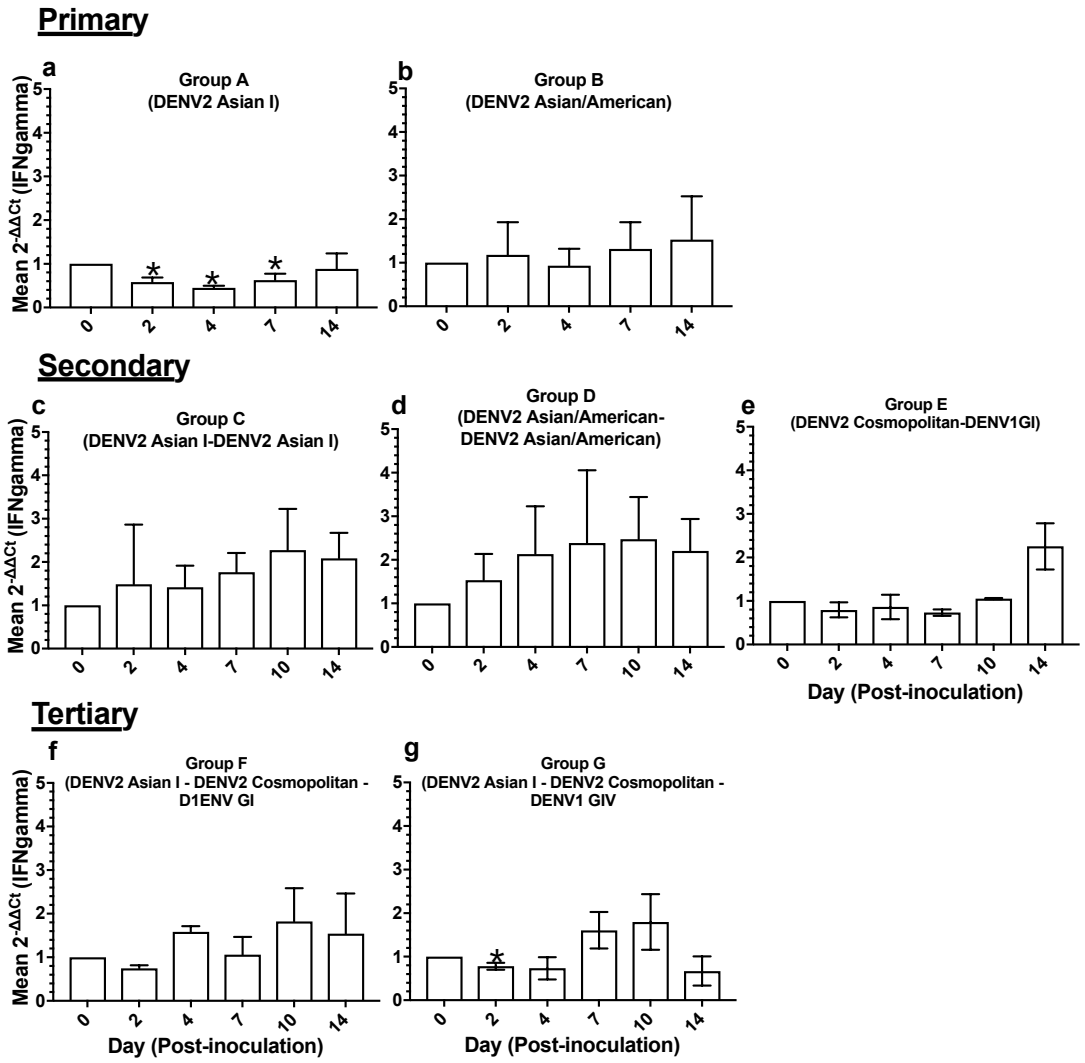

Figure 15. Levels of IFNγ in PBMC samples of groups A, B, C, D, E, F, and G marmosets on days 0, 2, 4, 7, 10, and 14 p.i. Levels of IFNγ in marmosets that were inoculated with: (a) Primary DENV-2 Asian I genotype, (b) Primary DENV-2 Asian/American genotype, (c) Secondary infection with homologous serotype, DENV-2 Asian I genotype, (d) Secondary infection with homologous serotype, DENV-1 Asian/American genotype, (e) Secondary infection with heterologous serotype, DENV-1 GI genotype, (f) Tertiary DENV-1 GI genotype, (g) Tertiary DENV-1 GIV genotype. Bar represents mean expression levels +SD. Asterisk (*) indicates significant differences ($p<0.05$) in levels of CD8α, compared with day 0 as a reference.

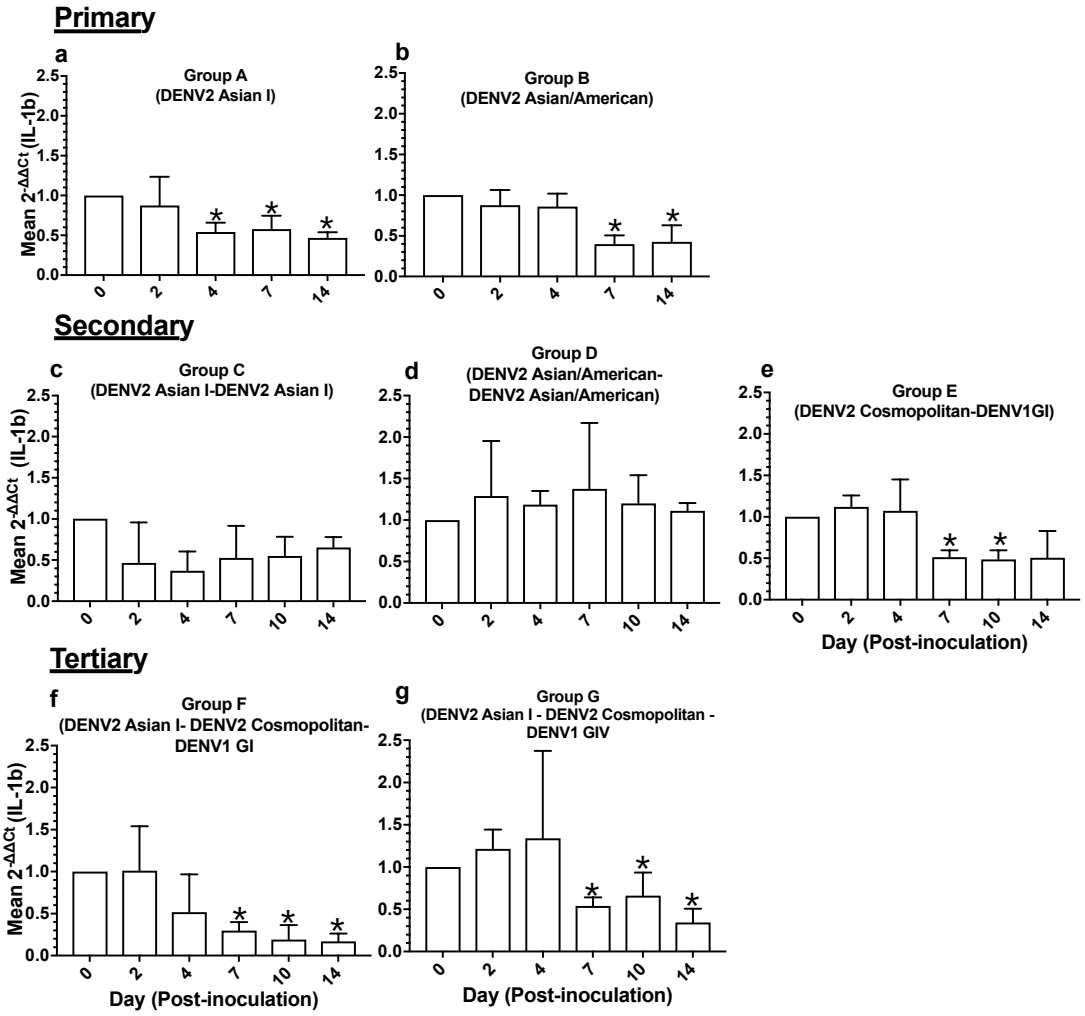

Figure 16. Levels of IL-1β in PBMC samples of groups A, B, C, D, E, F, and G marmosets on days 0, 2, 4, 7, 10, and 14 p.i. Levels of IL-1β in marmosets that were inoculated with: (a) Primary DENV-2 Asian I genotype, (b) Primary DENV-2 Asian/American genotype, (c) Secondary infection with homologous serotype, DENV-2 Asian I genotype, (d) Secondary infection with homologous serotype, DENV-2 Asian/American genotype, (e) Secondary infection with heterologous serotype, DENV-1 GI genotype, (f) Tertiary DENV-1 GI genotype, (g) Tertiary DENV-1 GIV genotype. Bar represents mean expression levels +SD. Asterisk (*) indicates significant differences ($p<0.05$) in levels of IL-1β, compared with day 0 as a reference.

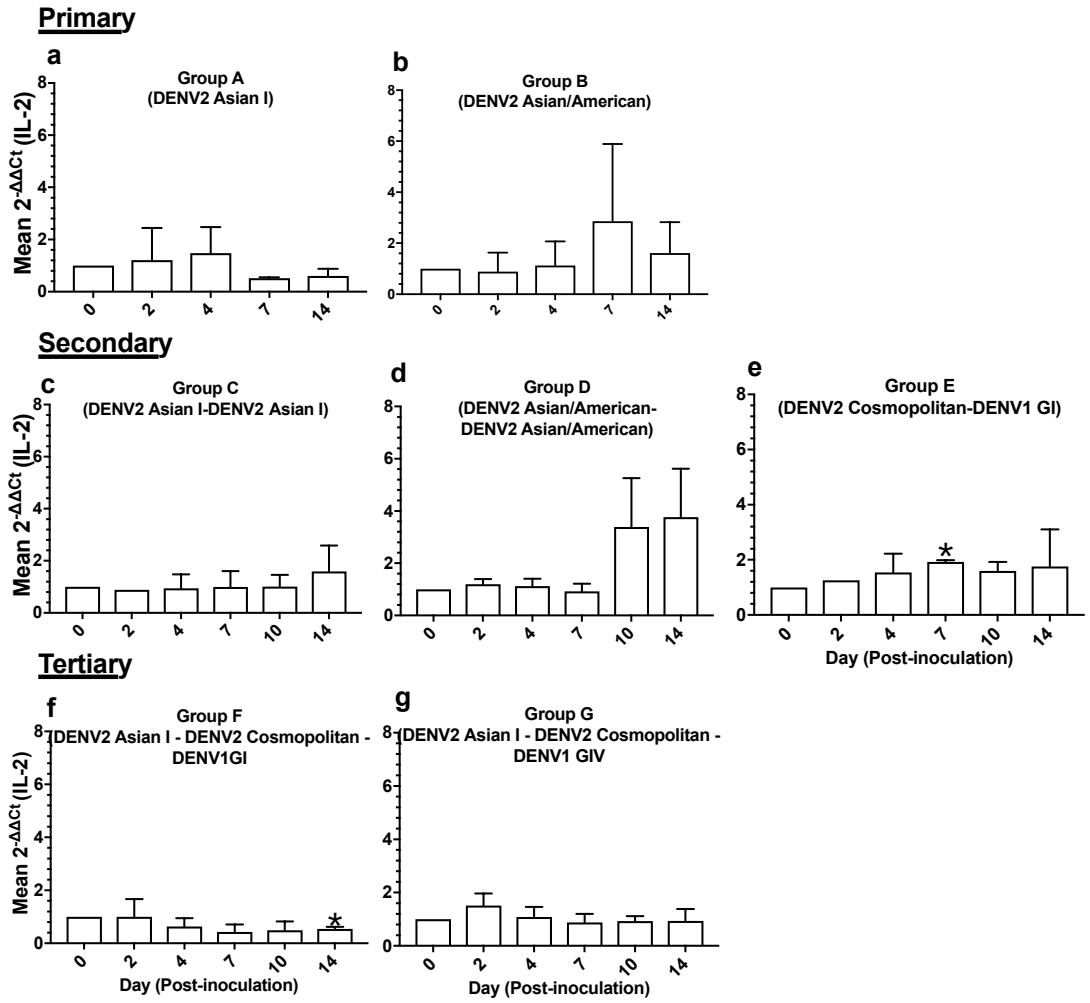

Figure 17. Levels of IL-2 in PBMC samples of groups A, B, C, D, E, F, and G marmosets on days 0, 2, 4, 7, 10, and 14 p.i. Levels of IL-2 in marmosets that were inoculated with: (a) Primary DENV-2 Asian I genotype, (b) Primary DENV-2 Asian/American genotype, (c) Secondary infection with homologous serotype, DENV-2 Asian I genotype, (d) Secondary infection with homologous serotype, DENV-2 Asian/American genotype, (e) Secondary infection with heterologous serotype, DENV-1 GI genotype, (f) Tertiary DENV-1 GI genotype, (g) Tertiary DENV-1 GIV genotype. Bar represents mean expression levels +SD. Asterisk (*) indicates significant differences ($p<0.05$) in levels of IL-2, compared with day 0 as a reference.

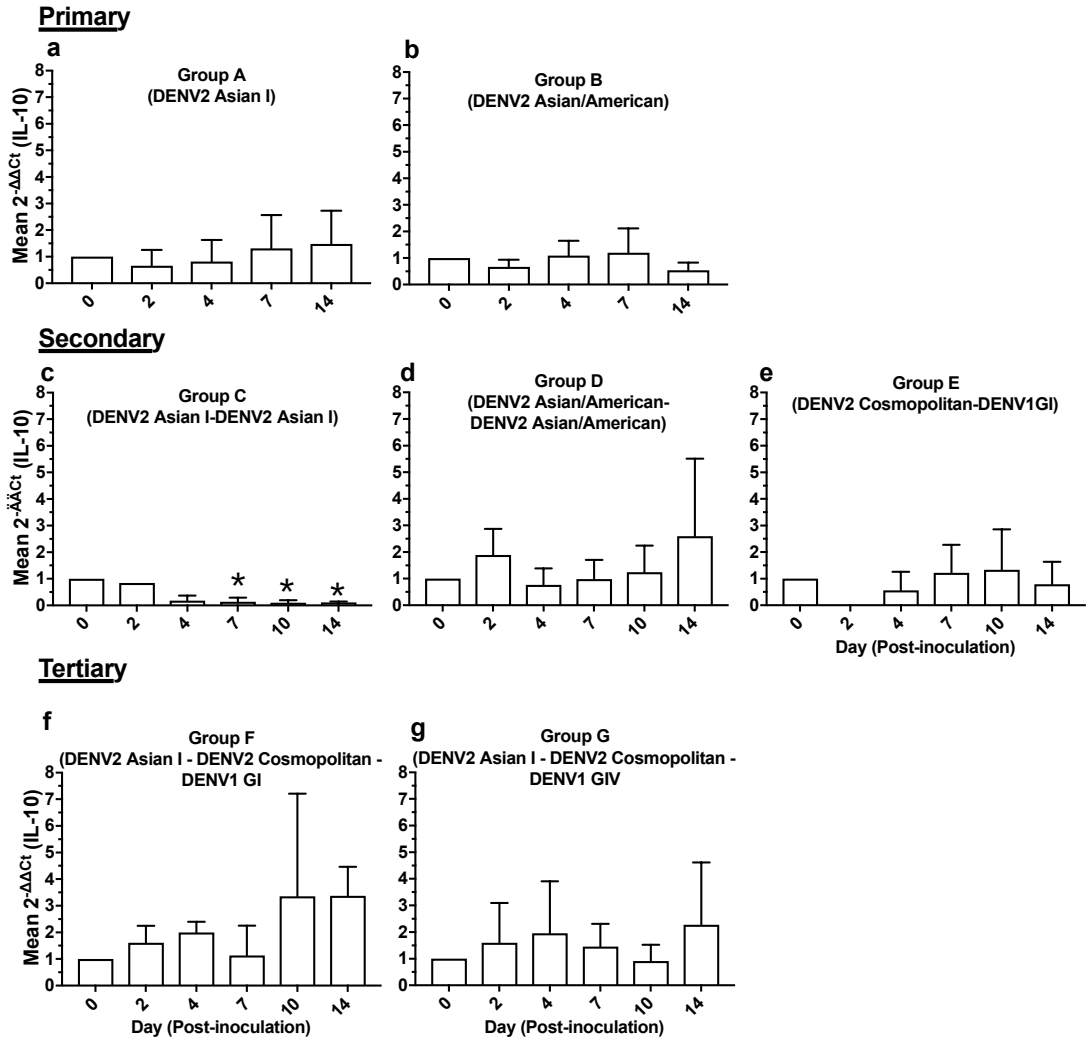

Figure 18. Levels of IL-10 in PBMC samples of groups A, B, C, D, E, F, and G marmosets on days 0, 2 4, 7, 10, and 14 p.i. Levels of IL-10 in marmosets that were inoculated with: (a) Primary DENV-2 Asian I genotype, (b) Primary DENV-2 Asian/American genotype, (c) Secondary infection with homologous serotype, DENV-2 Asian I genotype, (d) Secondary infection with homologous serotype, DENV-2 Asian/American genotype, (e) Secondary infection with heterologous serotype, DENV-1 GI genotype, (f) Tertiary DENV-1 GI genotype, (g) Tertiary DENV-1 GIV genotype. Bar represents mean expression levels +SD. Asterisk (*) indicates significant differences ($p<0.05$) in levels of IL-10, compared with day 0 as a reference.

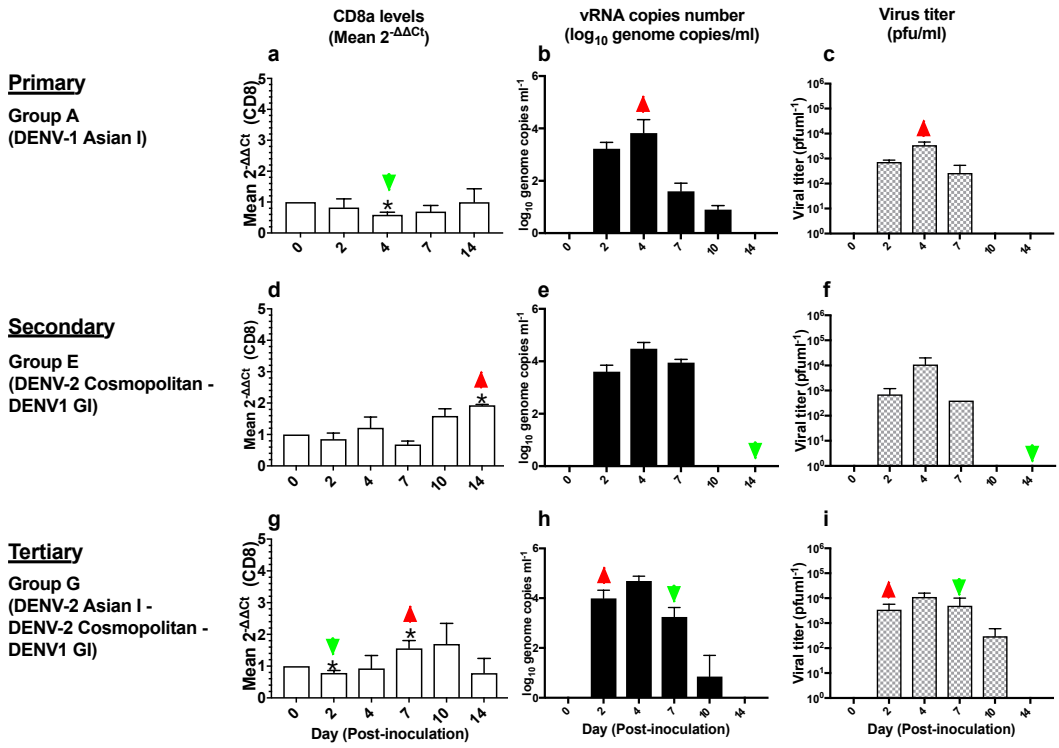

Figure 19. Side by side comparison between levels of CD8α and levels of viremia of group A, E, and G marmosets on days 0, 2, 4, 7, 10, and 14 p.i. Group A of primary marmosets: (a) Levels of CD8α, (b) Viral RNA copy numbers, (c) Infectious virus titers. Group E of secondary infection with heterologous serotype (d) Levels of CD8α, (e) Viral RNA copy numbers, (f) Infectious virus titers. Group G of tertiary infection marmosets (g) Levels of CD8α, (h) Viral RNA copy numbers (i) Infectious virus titers. Open bar indicates CD8α levels. Filled bar indicates viral RNA copy numbers. Pattern bar indicates infectious virus titers. Green arrowhead indicates decreased in expression levels and red arrowhead indicates increased in expression levels.

CHAPTER 4: DISCUSSION

In this present study, levels of genotype-specific and genotype cross-reactive neutralizing antibodies to DENV-2 were analyzed in primary, secondary, and tertiary DENV infection in common marmosets. Neutralizing antibodies that were induced during primary DENV-2 infection with a single genotype neutralized multiple genotypes within DENV-2. In marmosets that were inoculated with Asian I and Asian/American genotypes, levels of neutralizing antibodies to three genotypes of DENV-2 were at similar. Levels of neutralizing antibodies to heterologous Asian I genotype appeared to be higher than those to homologous Cosmopolitan genotype in marmosets that were inoculated with Cosmopolitan genotype.

In secondary infection with homologous genotype, genotype-specific and genotype cross-reactive neutralizing antibodies to DENV-2 Cosmopolitan, Asian I, and Asian/American genotypes were at similar levels. In case of secondary infection with homologous Asian/American genotype, levels of neutralizing antibodies to homologous genotype were higher than those to heterologous genotypes. Interestingly, in secondary infection with heterologous genotype, levels of neutralizing antibodies to non-infecting genotype (Asian/American genotype) were higher than those to infecting genotype (Cosmopolitan and Asian I genotypes). These results were consistent with those obtained in secondary infection using candidate vaccine strains (Figure 7d) and in tertiary infection (Figure 8a). Differences in levels of neutralizing antibodies against three genotypes were most prominent when levels of the neutralizing antibody were high ($PRNT_{50}$=640-20480). The results suggest that heterogeneity in antigenic

molecules between genotypes may lead to induction of higher levels of neutralizing antibodies to the epitope of the third genotype (Asian/American genotype in this study), and synergize the neutralization of the third virus. However, elevated levels of neutralizing antibody to the Asian/American genotype were not observed one year after secondary infection, when the levels of neutralizing antibodies already decreased. These data suggest that subset of antibodies that neutralized the Asian/American genotype better than those to the Cosmopolitan and Asian genotypes were diluted during these period, and that neutralization activities of these subsets of antibodies were only observed when the overall neutralizing antibodies activities were at high levels.

Following primary DENV-1 infection, neutralizing antibodies did not neutralize heterologous serotype, DENV-2. However, following secondary DENV-1 infection, neutralizing antibodies that were induced were serotype cross-reactive and able to neutralize DENV-2. Nonetheless, levels of neutralizing antibodies to homologous serotype (DENV-1) were higher compared to heterologous serotype (DENV-2). It has been reported that following DENV infection, even though neutralizing antibodies that were induced are serotype cross-reactive, levels of neutralizing antibodies against the infecting serotype were remained the highest[92–95]. These results suggest that serotype cross-reactive neutralizing antibodies are induced at some level, and that a robust secondary homologous immune response (mean secondary homologous DENV-1 $PRNT_{50}$=907; primary DENV-1 $PRNT_{50}$=60) may cause serotype cross-reactive neutralizing antibodies response more apparent. While infection with a single serotype typically leads to high levels of serotype-specific antibodies, it is likely that two infections with homologous serotype, serotype cross-reactive antibodies may be boosted to threshold levels and enable to cross-neutralize other serotypes. In this regard,

secondary homologous infection may partially contribute to serotype cross-protection in dengue hyper-endemic areas, due to higher risk of repeated exposure to the same serotype.

Sequential infection induced broad cross-reactive neutralizing antibodies that neutralized multiple serotypes including non-infecting serotypes in short period of time. Cross-reactive neutralizing antibodies induced during sequential infection generally possess broad cross-reactivity and able to neutralized multiple serotypes including non-infecting serotypes, as demonstrated in studies on DEN cohort patients [11,95–98] Notably, during secondary infection with heterologous serotype, levels of neutralizing antibodies against prior serotype were higher than those against the newly infected serotype. Although high levels of neutralizing antibodies to previously infecting serotypes persisted up to 9 months after sequential infection, the neutralizing antibodies level decreased over time[30]. Antibody decay and waning immunity induced susceptibility to infection with heterologous DENV serotype because of the partial protection and antibody-dependent enhancement of infection[8,48,99].

Levels of neutralizing antibodies to DENV-2 genotypes during homologous and heterologous infection differed. Homogeneity and heterogeneity of infecting genotype influence levels and cross-reactivity of neutralizing antibodies induced in subsequent infection because antigenic differences among genotypes lead to differences in antibody neutralization[6,11,45]. Antigenic differences may cause structural changes on viral protein that will modify the antibody binding site and affect the ability of neutralizing antibodies to confer protection against heterologous genotype[12,13]. The next step would be to expand the understanding on the spectrum of neutralizing

antibody patterns after homologous and heterologous infection, using combination of different genotype and serotype of DENV. While clinical trials of vaccine candidate demonstrated that vaccine is immunogenic in humans and animal models, there were limited data available on the variability of neutralizing activity against different genotypes within serotype. The variability in the levels of neutralizing antibodies between genotypes suggest that sequence heterogeneity between genotypes could result in complex cross-reactive immune responses; leading to distinct variable patterns of neutralizing antibodies among genotypes[11,18,100–102]. Similarly, marmosets inoculated with a candidate vaccine demonstrated variability in neutralizing antibodies patterns comparable to those inoculated with parental strain; suggesting that while attenuation leads to decreased pathogenicity, immunogenicity patterns between genotypes are retained.

Infection with different genotypes and serotypes of DENV induced different levels of viremia. Primary infection with DENV-2 Asian I genotype induced longer duration of viremia in marmosets than those with DENV-2 Asian/American genotype infection. Similar results were observed in tertiary infection marmosets. Infection with DENV-1 GIV genotype induced longer duration of viremia in marmosets than those with DENV-1 GI genotype. These results suggested that virus fitness and pathogenicity among genotypes differed. Studies on DEN patients reported that infection with Asian I genotype induced higher levels of viremia in dengue patients than those that were infected with Asian/American infection[16,19,20].

In secondary infection, viremia was detected in marmosets with heterologous serotype infection but not in marmosets with homologous serotype infection. The

results indicated that neutralizing antibodies are induced during primary infection to confer protection against homologous serotypes but did not protect against heterologous serotype. Results from secondary infection were in accordance with assumption that neutralizing antibodies induced after primary DENV infection will confers life-long protection against infection with homologous serotype but protection against heterologous serotype is short-lived. Nonetheless, since the secondary infections with homologous serotype were in fact homologous genotype infection, it is not clear whether neutralizing antibodies were induced during primary infection will offer complete protection against heterologous genotype. Incomplete protection against heterologous genotype incidence during DENV-2 outbreak were reported in Peru[13]. The next step would be to explore the ability of neutralizing antibodies to offer protection during heterologous genotype infection.

As most studies on cell-mediated immunity during DENV infection were performed by the comparison of data collected from health individual, acute DEN patient, and severe DEN patient, studies on correlation between antigenic differences and immune-related genes during DENV infection are still limited. The present study demonstrated that inoculation with different genotypes and serotypes of DENV induced different levels of CD3ε, CD4, CD8α, IFNγ, IL-2 and IL-10. Interestingly, the levels of IL-1β decreased in all marmosets that developed viremia following DENV inoculation. It has been reported that expression levels of immune-related genes were varied according to disease severity, rate of viral replication, and viral fitness[5,103].

Although relationship between antigenic differences and immune-related genes remain inconclusive, two genes, CD8α and IL-1β demonstrating interesting results. In

the present study, levels of CD8α decreased in early days after infection (days 2-4 p.i.) but then increased on later days after infection (days 7-14 p.i). It was reported that number of CD8+ T cells were low in days 1-4 after onset of symptoms, but then increased at late phase of disease in acute dengue patients[104]. Further comparison suggested that levels of CD8α were inversely correlated with levels of viremia. Depletion of CD8+ T cells in mouse model resulted in increased DENV infection[105]. These results suggested that the CD8+ T cells have protective roles in the host defense during DENV infection, and that CD8+ cells may have been activated to control the viral replication [104–106].

Levels of IL-1β is reduced in the marmosets that developed viremia following DENV inoculation. While one study reported that IL-1β did not play an important role in the dengue pathogenesis, other studies reported levels of IL-1β were elevated in dengue patients than those in healthy individuals[107–110]. The antibody-dependent enhancement were reported involved in facilitate the elevation of the IL-1β levels[109]. Increased levels of IL-1β were associated with vascular permeability, hypotension, and hemoconcentration in DEN patients[110]. In the present study, no marmosets that developed viremia showed vascular permeability symptoms. Decreased levels of IL-1β in marmosets maybe associated in the absence of vascular permeability symptoms.

This study highlighted that infection with different genotype and serotype of DENV induce different levels of neutralizing antibodies, viremia and immune related genes in marmosets. Differences in neutralizing antibodies pattern, viremia levels, and immune responses may have been caused by antigenic differences in DENV genotypes and serotypes. Antigenic differences in DENV likely plays an important role in disease severity, viral fitness, and ability to change the viral protein that affect the neutralizing activities of antibodies[12,14].

CHAPTER 5: CONCLUSION AND FUTURE RESEARCH

Levels and patterns of genotype-specificity and genotype cross-reactivity in neutralizing antibodies response to genotypes of DENV-2 are different in primary, secondary, and tertiary infection. These differences in induced neutralizing antibodies are caused by the antigenic differences of infected DENV. Antigenic differences among genotypes affect the induction of neutralizing antibodies, and eventually levels of protection against heterologous genotypes and serotypes of DENV. In addition, antigenic differences may contribute to disease severity, epidemic cycling and viral evolution. To maximize the efficacy of DEN vaccine, it is important to further understand the spectrum of neutralizing antibodies in relation to protection capabilities against different genotypes and serotypes. Furthermore, understanding of antigenic differences among genotypes within each of 4 serotypes is also important, since different genotypes induce different levels of neutralizing antibodies. Based on this understanding, identification of DENV strains that able to induce similar levels of neutralizing antibodies against homologous or heterologous genotypes should be addressed in DENV vaccine development study.

The relationship between antigenic differences and immune responses are remain inconclusive. Nonetheless, it is worth addressing the possibilities of the use of T-cell markers and cytokines as biomarkers in predicting disease severity. Research on the identification of biomarkers for this purpose is also important. The biomarkers will be able to evaluate the prognosis, and improved the treatment and management of DEN patients.

REFERENCES

1. Rigau-Pérez JG, Rigau-Perez JG, Rigau-Pérez JG, Rigau-Perez JG. The early use of break-bone fever (Quebranta huesos, 1771) and dengue (1801) in Spanish. *Am J Trop Med Hyg*. 1998;59(2):272-274. doi:10.4269/ajtmh.1998.59.272.
2. Rothman AL. Immunity to dengue virus: a tale of original antigenic sin and tropical cytokine storms. *Nat Rev Immunol*. 2011;11(8):532-543. http://dx.doi.org/10.1038/nri3014.
3. Usme-Ciro JA, Méndez JA, Laiton KD, Páez A. The relevance of dengue virus genotypes surveillance at country level before vaccine approval. *Hum Vaccin Immunother*. 2014;10(9):2674-2678. doi:10.4161/hv.29563.
4. Drumond BP, Mondini A, Schmidt DJ, Bronzoni RV de M, Bosch I, Nogueira ML. Circulation of Different Lineages of Dengue Virus 2, Genotype American/Asian in Brazil: Dynamics and Molecular and Phylogenetic Characterization. *PLoS One*. 2013;8(3):e59422. https://doi.org/10.1371/journal.pone.0059422.
5. Cedillo-Barrón L, García-Cordero J, Bustos-Arriaga J, León-Juárez M, Gutiérrez-Castañeda B. Antibody response to dengue virus. *Microbes Infect*. 2014;16(9):711-720. doi:http://dx.doi.org/10.1016/j.micinf.2014.07.011.
6. Brien JD, Austin SK, Sukupolvi-Petty S, et al. Genotype-Specific Neutralization and Protection by Antibodies against Dengue Virus Type 3. *J Virol*. 2010;84(20):10630-10643.
7. Mathew A, Rothman AL. Understanding the contribution of cellular immunity to dengue disease pathogenesis. *Immunol Rev*. 2008;225(1):300-313. doi:10.1111/j.1600-065X.2008.00678.x.
8. Moi ML, Takasaki T, Kurane I. Human antibody response to dengue virus: implications for dengue vaccine design. *Trop Med Health*. 2016;44(1):1. doi:10.1186/s41182-016-0004-y.
9. Weaver SC, Vasilakis N. Molecular evolution of dengue viruses: Contributions of phylogenetics to understanding the history and epidemiology of the preeminent arboviral disease. *Infect Genet Evol*. 2009;9(4):523-540. doi:http://dx.doi.org/10.1016/j.meegid.2009.02.003.
10. Srikiatkhachorn A, Yoon I-K. Immune correlates for dengue vaccine development. *Expert Rev Vaccines*. 2016;15(4):455-465. doi:10.1586/14760584.2016.1116949.
11. Wahala WMPB, De Silva AM. The Human Antibody Response to Dengue Virus Infection. *Viruses*. 2011;3:2374-2395. doi:10.3390/v3122374.
12. Katzelnick LC, Fonville JM, Gromowski GD, et al. Dengue viruses cluster antigenically but not as discrete serotypes. *Science (80-)*. 2015;349(6254):1338-1343. doi:10.1126/science.aac5017.
13. Forshey BM, Reiner RC, Olkowski S, et al. Incomplete Protection against Dengue Virus Type 2 Re-infection in Peru. *PLoS Negl Trop Dis*. 2016;10(2):1-17. doi:10.1371/journal.pntd.0004398.
14. Guzman MG, Harris E. Dengue. *Lancet*. 2015;385(9966):453-465.
15. Waman VP, Kolekar P, Ramtirthkar MR, Kale MM, Kulkarni-Kale U. Analysis of genotype diversity and evolution of Dengue virus serotype 2 using complete genomes. *PeerJ*. 2016;4:e2326. doi:10.7717/peerj.2326.
16. Twiddy SS, Farrar JJ, Vinh Chau N, et al. Phylogenetic relationships and differential selection pressures among genotypes of dengue-2 virus. *Virology*.

2002;298(1):63-72. http://www.ncbi.nlm.nih.gov/pubmed/12093174. Accessed May 17, 2017.
17. Salazar MI, Pino MAL, Ale JAF, Olson KE, Beaty BJ. American and American/Asian genotypes of dengue virus differ in mosquito infection efficiency: candidate molecular determinants of productive vector infection. *Rev biomédica*. 2010;21(3):121-135.
18. Leitmeyer KC, Vaughn DW, Watts DM, et al. Dengue virus structural differences that correlate with pathogenesis. *J Virol*. 1999;73(6):4738-4747. http://www.ncbi.nlm.nih.gov/pubmed/10233934. Accessed May 22, 2017.
19. Ty Hang VT, Holmes EC, Veasna D, et al. Emergence of the Asian 1 Genotype of Dengue Virus Serotype 2 in Viet Nam: In Vivo Fitness Advantage and Lineage Replacement in South-East Asia. Rico-Hesse R, ed. *PLoS Negl Trop Dis*. 2010;4(7):e757. doi:10.1371/journal.pntd.0000757.
20. Wang C, Katzelnick LC, Montoya M, Hue KDT, Simmons CP, Harris E. Evolutionarily successful Asian 1 dengue virus 2 lineages contain one substitution in envelope that increases sensitivity to polyclonal antibody neutralization. *J Infect Dis*. 2015;213(6):975-984.
21. Thomas SJ, Nisalak A, Anderson KB, et al. Dengue Plaque Reduction Neutralization Test (PRNT) in Primary and Secondary Dengue Virus Infections: How Alterations in Assay Conditions Impact Performance. *Am J Trop Med Hyg*. 2009;81(5):825-833. doi:10.4269/ajtmh.2009.08-0625.
22. Hotta S. Experimental Studies on Dengue: I. Isolation, Identification and Modification of the Virus. *J Infect Dis*. 1952;90(1):1-9. http://www.jstor.org/stable/30093992.
23. Halstead S. *Dengue*.; 2008.
24. Guzman MC, Halstead SB, Artsob H, et al. Dengue: a continuing global threat. *Nat Rev Microbiol*. 2010:S7-S16.
25. Gould EA, Gallian P, De Lamballerie X, Charrel RN. First cases of autochthonous dengue fever and chikungunya fever in France: from bad dream to reality! *Clin Microbiol Infect*. 2010;16:1702-1704. doi:10.1111/j.1469-0691.2010.03386.x.
26. Gjenero-Margan I, Aleraj B, Krajcar D, et al. Autochthonous dengue fever in Croatia, August- September 2010. *Eurosurveillance*. 2011. doi:19805 [pii].
27. WHO. Dengue: guidelines for diagnosis, treatment, prevention and control. 2009.
28. Lee K-SS, Lo S, Tan SS-YY, et al. Dengue virus surveillance in Singapore reveals high viral diversity through multiple introductions and in situ evolution. *Infect Genet Evol*. 2012;12(1):77-85. doi:http://dx.doi.org/10.1016/j.meegid.2011.10.012.
29. Satoshi K, Yasuyuki K, Meng Ling M, et al. Autochthonous Dengue Fever, Tokyo, Japan, 2014. *Emerg Infect Dis J*. 2015;21(3):517. doi:10.3201/eid2103.141662.
30. Katzelnick LC, Montoya M, Gresh L, Balmaseda A, Harris E. Neutralizing antibody titers against dengue virus correlate with protection from symptomatic infection in a longitudinal cohort. *Proc Natl Acad Sci* . 2016;113(3):728-733. doi:10.1073/pnas.1522136113.
31. Bhatt S, Gething PW, Brady OJ, et al. The global distribution and burden of dengue. *Nature*. 2013;496(7446):504-507. doi:10.1038/nature12060http://www.nature.com/nature/journal/v496/n7446/abs/nature12060.html#supplementary-information.

32. Muhammad Azami NA, Salleh S, Neoh H, Syed Zakaria S, Jamal R. Dengue epidemic in Malaysia: Not a predominantly urban disease anymore. *BMC Res Notes*. 2011;4(1):216. http://www.biomedcentral.com/1756-0500/4/216.
33. World Health Organization. Dengue vaccine: WHO position paper, July 2016 – recommendations. *Vaccine*. 2017;35(9):1200-1201. doi:https://doi.org/10.1016/j.vaccine.2016.10.070.
34. Hadinegoro SRS. The revised WHO dengue case classification: does the system need to be modified? *Paediatr Int Child Health*. 2012;32(s1):33-38. doi:10.1179/2046904712Z.00000000052.
35. Simmons CP, Farrar JJ, van Vinh Chau N, Wills B. Dengue. *N Engl J Med*. 2012;366(15):1423-1432. doi:doi:10.1056/NEJMra1110265.
36. Omatsu T, Moi ML, Hirayama T, et al. Common marmoset (Callithrix jacchus) as a primate model of dengue virus infection: development of high levels of viraemia and demonstration of protective immunity. *J Gen Virol*. 2011;92(10):2272-2280. doi:10.1099/vir.0.031229-0.
37. Martina BEE, Koraka P, Osterhaus ADME. Dengue Virus Pathogenesis: an Integrated View. *Clin Microbiol Rev*. 2009;22(4):564-581. doi:10.1128/cmr.00035-09.
38. Gunther VJ, Putnak R, Eckels KH, et al. A human challenge model for dengue infection reveals a possible protective role for sustained interferon gamma levels during the acute phase of illness. *Vaccine*. 2011;29(22):3895-3904. doi:https://doi.org/10.1016/j.vaccine.2011.03.038.
39. Yacoub S, Mongkolsapaya J, Screaton G. The pathogenesis of dengue. *Curr Opin Infect Dis*. 2013;26(3):284-289. doi:10.1097/QCO.0b013e32835fb938.
40. Azami NAM, Moi ML, Takasaki T. Neutralization Assay for Chikungunya Virus Infection: Plaque Reduction Neutralization Test BT - Chikungunya Virus: Methods and Protocols. In: Chu JJH, Ang SK, eds. New York, NY: Springer New York; 2016:273-282. doi:10.1007/978-1-4939-3618-2_25.
41. Roehrig JT, Hombach J, Barrett AD. Guidelines for plaque-reduction neutralization testing of human antibodies to dengue viruses. *Virol Immunol*. 2008;21:123-132.
42. Tsai T-T, Chuang Y-J, Lin Y-S, Wan S-W, Chen C-L, Lin C-F. An emerging role for the anti-inflammatory cytokine interleukin-10 in dengue virus infection. *J Biomed Sci*. 2013;20(1):40. doi:10.1186/1423-0127-20-40.
43. Rico-Hesse R, Harrison LM, Salas RA, et al. Origins of Dengue Type 2 Viruses Associated with Increased Pathogenicity in the Americas. *Virology*. 1997;230(2):244-251. doi:10.1006/viro.1997.8504.
44. Yung C-F, Lee K-S, Thein T-L, et al. Dengue Serotype-Specific Differences in Clinical Manifestation, Laboratory Parameters and Risk of Severe Disease in Adults, Singapore. *Am J Trop Med Hyg*. 2015;92(5):999-1005. doi:10.4269/ajtmh.14-0628.
45. Endy TP, Nisalak A, Chunsuttitwat S, et al. Relationship of Preexisting Dengue Virus (DV) Neutralizing Antibody Levels to Viremia and Severity of Disease in a Prospective Cohort Study of DV Infection in Thailand. *J Infect Dis*. 2004;189(6):990-1000. doi:10.1086/382280.
46. Thomas L, Najioullah F, Besnier F, et al. Clinical Presentation of Dengue by Serotype and Year of Epidemic in Martinique. *Am J Trop Med Hyg*. 2014;91(1):138-145. doi:10.4269/ajtmh.13-0595.
47. Burke DS, Kliks S. Antibody-dependent enhancement in dengue virus infections.

J Infect Dis. 2006;193(4):601-603.
48. Nimmanitya S, Kliks SC, Burke DS, Nisalak A. Evidence That Maternal Dengue Antibodies Are Important in the Development of Dengue Hemorrhagic Fever in Infants. *Am J Trop Med Hyg*. 1988;38(2):411-419. doi:10.4269/ajtmh.1988.38.411.
49. Zompi S, Harris E. Original antigenic sin in dengue revisited. *Proc Natl Acad Sci* . 2013;110(22):8761-8762. doi:10.1073/pnas.1306333110.
50. Kurane I, Ennis FA. Immunopathogenesis of dengue virus infection. In: *Dengue and Dengue Hemorrhagic Fever*. ; 1997:273-290.
51. Rothman AL. Viral pathogenesis of dengue infections. In: *Dengue and Dengue Hemorrhagic Fever*. ; 1997:245-271.
52. Janeway Jr CA, Travers P, Walport M, Shlomchik MJ. The humoral immune response. 2001.
53. Klimpel GR. Immune defenses. 1996.
54. Rothman AL, Ennis FA. Elimination of Infection. In: Halstead SB, ed. *Dengue*. Imperial College Press; 2008:257-284.
55. Clark KB, Onlamoon N, Hsiao H-M, et al. Can non-human primates serve as models for investigating dengue disease pathogenesis? *Front Microbiol* . 2013;4:305. doi:10.3389/fmicb.2013.00305.
56. Klasse PJ. Neutralization of virus infectivity by antibodies: old problems in new perspectives. *Adv Biol*. 2014;2014.
57. Vaughn DW, Scherer LJM, Sun W. Resistance to Infection. In: *Dengue*. Imperial College Press; 2008:123-170.
58. Churdboonchart V, Bhamarapravati N, Peampramprecha S, Sirinavin S. Antibodies against dengue viral proteins in primary and secondary dengue hemorrhagic fever. *Am J Trop Med Hyg*. 1991;44(5):481-493.
59. Valdés K, Alvarez M, Pupo M, Vázquez S, Rodríguez R, Guzmán MG. Human dengue antibodies against structural and nonstructural proteins. *Clin Diagn Lab Immunol*. 2000;7(5):856-857.
60. Se-Thoe SY, Ng MML, Ling A-E. Retrospective study of Western blot profiles in immune sera of natural dengue virus infections. *J Med Virol*. 1999;57(3):322-330.
61. Dubovi EJBT-FVV (Fifth E, ed. Chapter 4 - Antiviral Immunity and Virus Vaccines A2 - MacLachlan, N. James. 2017:79-104. doi:https://doi.org/10.1016/B978-0-12-800946-8.00004-0.
62. Puschnik A, Lau L, Cromwell EA, Balmaseda A, Zompi S, Harris E. Correlation between Dengue-Specific Neutralizing Antibodies and Serum Avidity in Primary and Secondary Dengue Virus 3 Natural Infections in Humans. Guzman MG, ed. *PLoS Negl Trop Dis*. 2013;7(6):e2274. doi:10.1371/journal.pntd.0002274.
63. Schieffelin JS, Costin JM, Nicholson CO, et al. Neutralizing and non-neutralizing monoclonal antibodies against dengue virus E protein derived from a naturally infected patient. *Virol J*. 2010;7(1):28. doi:10.1186/1743-422X-7-28.
64. Gallichotte EN, Baric TJ, Yount Jr BL, et al. Human dengue virus serotype 2 neutralizing antibodies target two distinct quaternary epitopes. *PLOS Pathog*. 2018;14(2):e1006934. https://doi.org/10.1371/journal.ppat.1006934.
65. Halstead SB. Dengue. *Lancet*. 2007;370(9599):1644-1652.
66. Pang T. SAGE committee advice on dengue vaccine. *Lancet Infect Dis*. 2018;16(8):880-882. doi:10.1016/S1473-3099(16)30167-0.

67. Aguiar M, Stollenwerk N, Halstead SB. The risks behind Dengvaxia recommendation. *Lancet Infect Dis*. 2018;16(8):882-883. doi:10.1016/S1473-3099(16)30168-2.
68. Aguiar M, Stollenwerk N. Dengvaxia: age as surrogate for serostatus. *Lancet Infect Dis*. 2018;18(3):245. doi:https://doi.org/10.1016/S1473-3099(17)30752-1.
69. Sanofi. Press release: Sanofi updates information on dengue vaccine. http://mediaroom.sanofi.com/sanofi-updates-information-on-dengue-vaccine/. Published 2017. Accessed April 14, 2018.
70. Guy B, Briand O, Lang J, Saville M, Jackson N. Development of the Sanofi Pasteur tetravalent dengue vaccine: One more step forward. *Vaccine*. 2015;33(50):7100-7111. doi:http://dx.doi.org/10.1016/j.vaccine.2015.09.108.
71. Guy B, Saville M, Lang J. Development of Sanofi Pasteur tetravalent dengue vaccine. *Hum Vaccin*. 2010;6(9):696-705.
72. Hadinegoro SR, Arredondo-García JL, Capeding MR, et al. Efficacy and Long-Term Safety of a Dengue Vaccine in Regions of Endemic Disease. *N Engl J Med*. 2015;373(13):1195-1206. doi:10.1056/NEJMoa1506223.
73. Pang T, Cardosa MJ, Guzman MG. Of cascades and perfect storms: the immunopathogenesis of dengue haemorrhagic fever-dengue shock syndrome (DHF//DSS). *Immunol Cell Biol*. 2006;85(1):43-45. http://dx.doi.org/10.1038/sj.icb.7100008.
74. Aguiar M, Stollenwerk N, Halstead SB. The Impact of the Newly Licensed Dengue Vaccine in Endemic Countries. *PLoS Negl Trop Dis*. 2016;10(12):e0005179. https://doi.org/10.1371/journal.pntd.0005179.
75. Halstead SB. Critique of World Health Organization Recommendation of a Dengue Vaccine. *J Infect Dis*. 2016;214(12):1793-1795. http://dx.doi.org/10.1093/infdis/jiw340.
76. Zompi S, Harris E. Animal models of dengue virus infection. *Viruses*. 2012;4(1):62-82. doi:10.3390/v4010062.
77. Bente DA, Rico-Hesse R. Models of dengue virus infection. *Drug Discov Today Dis Models*. 2006;3(1):97-103. doi:10.1016/j.ddmod.2006.03.014.
78. Marchette NJ, Halstead SB, Falkler WA, Stenhouse A, Nash D. Studies on the Pathogenesis of Dengue Infection in Monkeys. III. Sequential Distribution of Virus in Primary and Heterologous Infections. *J Infect Dis*. 1973;128(1):23-30. doi:10.1093/infdis/128.1.23.
79. Halstead SB, Shotwell H, Casals J. Studies on the pathogenesis of dengue infection in monkeys. II. Clinical laboratory responses to heterologous infection. *J Infect Dis*. 1973;128(1):15-22.
80. Onlamoon N, Noisakran S, Hsiao H-M, et al. Dengue virus–induced hemorrhage in a nonhuman primate model. *Blood*. 2010;115(9):1823-1834. doi:10.1182/blood.
81. Kochel TJ, Watts DM, Gozalo AS, Ewing DF, Porter KR, Russell KL. Cross-Serotype Neutralization of Dengue Virus in *Aotus nancymae* Monkeys. *J Infect Dis*. 2005;191(6):1000-1004. doi:10.1086/427511.
82. Osorio JE, Brewoo JN, Silengo SJ, et al. Efficacy of a Tetravalent Chimeric Dengue Vaccine (DENVax) in Cynomolgus Macaques. *Am J Trop Med Hyg*. 2011;84(6):978-987. doi:10.4269/ajtmh.2011.10-0592.
83. Moi MLL, Takasaki T, Omatsu T, et al. Demonstration of marmosets (Callithrix jacchus) as a non-human primate model for secondary dengue virus infection: High levels of viraemia and serotype cross-reactive antibody responses

consistent with secondary infection of humans. *J Gen Virol*. 2014;95(PART3):591-600. doi:10.1099/vir.0.060384-0.

84. Widjaja S, Winoto I, Sturgis J, et al. Macaca nemestrina and dengue virus infectivity: a potential model for evaluating dengue vaccine candidates. *Microbiol Indones*. 2010;4(2):1.
85. Halstead SB. Dengue haemorrhagic fever--a public health problem and a field for research. *Bull World Heal Organ*. 1980;58(1):1-21. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=6966540.
86. Freire MS, Marchevsky RS, Almeida LFC, et al. Wild dengue virus types 1, 2 and 3 viremia in rhesus monkeys. *MemÃ\textthreesuperiorrias do Inst Oswaldo Cruz*. 2007;102:203-208. http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0074-02762007000200013&nrm=iso.
87. Fujii Y, Kitaura K, Matsutani T, et al. Immune-Related Gene Expression Profile in Laboratory Common Marmosets Assessed by an Accurate Quantitative Real-Time PCR Using Selected Reference Genes. *PLoS One*. 2013;8(2):e56296. https://doi.org/10.1371/journal.pone.0056296.
88. Moi ML, Ami Y, Muhammad Azami NA, et al. Marmosets (Callithrix jacchus) as a non-human primate model for evaluation of candidate dengue vaccines: induction and maintenance of specific protective immunity against challenges with clinical isolates. *J Gen Virol*. 2017. http://jgv.microbiologyresearch.org/content/journal/jgv/10.1099/jgv.0.000913.v1.
89. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. *Mol Biol Evol*. 2013;30(12):2725-2729. doi:10.1093/molbev/mst197.
90. Ito M, Takasaki T, Yamada K, Nerome R, Tajima S, Kurane I. Development and evaluation of fluorogenic TaqMan reverse transcriptase PCR assays for detection of dengue virus types 1 to 4. *J Clin Microbiol*. 2004;42(12):5935-5937. doi:10.1128/JCM.42.12.5935-5937.2004.
91. Schmittgen TD, Livak KJ. Analyzing real-time PCR data by the comparative CT method. *Nat Protoc*. 2008;3:1101. http://dx.doi.org/10.1038/nprot.2008.73.
92. Lai C-Y, Tsai W-Y, Lin S-R, et al. Antibodies to envelope glycoprotein of dengue virus during the natural course of infection are predominantly cross-reactive and recognize epitopes containing highly conserved residues at the fusion loop of domain II. *J Virol*. 2008;82(13):6631-6643. doi:10.1128/JVI.00316-08.
93. de Alwis R, Beltramello M, Messer WB, et al. In-Depth Analysis of the Antibody Response of Individuals Exposed to Primary Dengue Virus Infection. Rico-Hesse R, ed. *PLoS Negl Trop Dis*. 2011;5(6):e1188. doi:10.1371/journal.pntd.0001188.
94. Rothman AL, Innis B, Houghten R, Henchal L, Muller-Eberhard H. Dengue: defining protective versus pathologic immunity. *J Clin Invest*. 2004;113(7):946-951. doi:10.1172/JCI21512.
95. Mathew A, West K, Kalayanarooj S, et al. B-Cell Responses During Primary and Secondary Dengue Virus Infections in Humans. *J Infect Dis*. 2011;204(10):1514-1522. doi:10.1093/infdis/jir607.
96. Mathew A, Kurane I, Green S, et al. Predominance of HLA-restricted cytotoxic

T-lymphocyte responses to serotype-cross-reactive epitopes on nonstructural proteins following natural secondary dengue virus infection. *J Virol*. 1998;72(5):3999-4004. http://www.ncbi.nlm.nih.gov/pubmed/9557687. Accessed May 23, 2017.

97. Kosasih H, Yusuf H, Sudjana P, et al. Report of Four Volunteers with Primary, Secondary and Tertiary Dengue Infections during a Prospective Cohort Study. *Dengue Bull* –. 2006;30. http://apps.who.int/iris/bitstream/10665/170253/1/db2006v30p87.pdf. Accessed May 23, 2017.
98. Corbett KS, Katzelnick L, Tissera H, Amerasinghe A, de Silva ADAMAD, de Silva ADAMAD. Preexisting Neutralizing Antibody Responses Distinguish Clinically Inapparent and Apparent Dengue Virus Infections in a Sri Lankan Pediatric Cohort. *J Infect Dis*. 2015;211(4):590-599. doi:10.1093/infdis/jiu481.
99. Guzman MG, Vazquez S. The complexity of antibody-dependent enhancement of dengue virus infection. *Viruses*. 2010;2(12):2649-2662. doi:10.3390/v2122649.
100. Chen Y, Maguire T, Marks RM. Demonstration of Binding of Dengue Virus Envelope Protein to Target Cells. *J Virol*. 1996;70(12):8765-8772. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC190973/pdf/708765.pdf. Accessed May 22, 2017.
101. Zulueta A, Martín J, Hermida L, et al. Amino acid changes in the recombinant Dengue 3 Envelope domain III determine its antigenicity and immunogenicity in mice. *Virus Res*. 2006;121(1):65-73. doi:10.1016/j.virusres.2006.04.003.
102. Sánchez MD, Pierson TC, McAllister D, et al. Characterization of neutralizing antibodies to West Nile virus. *Virology*. 2005;336(1):70-82. doi:10.1016/j.virol.2005.02.020.
103. Holmes EC. RNA virus genomics: a world of possibilities. *J Clin Invest*. 2009;119(9):2488-2495. doi:10.1172/JCI38050.
104. de Matos AM, Carvalho KI, Rosa DS, et al. CD8+ T Lymphocyte Expansion, Proliferation and Activation in Dengue Fever. *PLoS Negl Trop Dis*. 2015;9(2):e0003520. https://doi.org/10.1371/journal.pntd.0003520.
105. Yauch LE, Zellweger RM, Kotturi MF, et al. A Protective Role for Dengue Virus-Specific CD8(+) T Cells. *J Immunol*. 2009;182(8):4865-4873. doi:10.4049/jimmunol.0801974.
106. Dung NTP, Duyen HT Le, Thuy NT Van, et al. Timing of CD8⁺ T Cell Responses in Relation to Commencement of Capillary Leakage in Children with Dengue. *J Immunol*. 2010;184(12):7281 LP-7287. http://www.jimmunol.org/content/184/12/7281.abstract.
107. Levy A, Valero N, Espina LM, Añez G, Arias J, Mosquera J. Increment of interleukin 6, tumour necrosis factor alpha, nitric oxide, C-reactive protein and apoptosis in dengue. *Trans R Soc Trop Med Hyg*. 2010;104(1):16-23. http://dx.doi.org/10.1016/j.trstmh.2009.06.013.
108. Arias J, Valero N, Mosquera J, et al. Increased expression of cytokines, soluble cytokine receptors, soluble apoptosis ligand and apoptosis in dengue. *Virology*. 2014;452-453:42-51. doi:https://doi.org/10.1016/j.virol.2013.12.027.
109. Callaway JB, Smith SA, McKinnon KP, De Silva AM, Crowe JE, Ting JP-Y. Spleen tyrosine kinase (Syk) mediates IL-1β induction by primary human monocytes during antibody-enhanced dengue virus infection. *J Biol Chem*. 2015;290(28):17306-17320.

110. Hottz ED, Medeiros-de-Moraes IM, Vieira-de-Abreu A, et al. Platelet Activation and Apoptosis Modulate Monocyte Inflammatory Responses in Dengue. *J Immunol*. July 2014. http://www.jimmunol.org/content/early/2014/07/11/jimmunol.1400091.abstract.

参　考　論　文

Reference journal

REFERENCE JOURNAL

1. Azami, Nor Azila Muhammad, Meng Ling Moi, Yasushi Ami, Yuriko Suzaki, Chang-Kweng Lim, Satoshi Taniguchi, Masayuki Saijo, Tomohiko Takasaki, and Ichiro Kurane. "Genotype-specific and cross-reactive neutralizing antibodies induced by dengue virus infection: detection of antibodies with different levels of neutralizing activities against homologous and heterologous genotypes of dengue virus type 2 in common marmosets (Callithrix jacchus)." *Virology journal* 15, no. 1 (2018): 51.