New antiviral therapies to treat zika infections : neutralizing monoclonal antibodies and inhibitors of interactions between non-structural proteins

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**NEW ANTIVIRAL THERAPIES TO TREAT ZIKA
INFECTIONS: NEUTRALIZING MONOCLONAL
ANTIBODIES AND INHIBITORS OF
INTERACTIONS BETWEEN NON-STRUCTURAL
PROTEINS**

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SCHOOL OF BIOLOGICAL SCIENCES

2019

**New Antiviral Therapies to Treat Zika Infections:
Neutralizing Monoclonal Antibodies and Inhibitors of
Interactions Between Non-Structural Proteins**

Jia Huan

SCHOOL OF BIOLOGICAL SCIENCES

A thesis submitted to the Nanyang
Technological University in partial fulfilment of
the requirement for the degree of Doctor of
Philosophy

2019

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research done by me except where otherwise stated in this thesis. The thesis work has not been submitted for a degree or professional qualification to any other university or institution. I declare that this thesis is written by myself and is free of plagiarism and of sufficient grammatical clarity to be examined. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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16-Aug-2019

Date

Prof. Julien Lescar

Authorship Attribution Statement

This thesis contains material from 1 paper published in the following peer-reviewed journal where I was the co-first author.

Chapter III Section 3.3 is published as Tharakaraman, K.*, Watanabe, S.*, Chan, K. R.*, Huan, J.*, Subramanian, V., Chionh, Y. H., ... & Gan, E. S. (2018). Rational Engineering and Characterization of an mAb that Neutralizes Zika Virus by Targeting a Mutationally Constrained Quaternary Epitope. *Cell host & microbe*, 23(5), 618-627. DOI: <https://doi.org/10.1016/j.chom.2018.04.004>.

The contributions of the co-authors are as follows:

- Dr. Kannan Tharakaraman, Dr. Satoru Watanabe, Dr. Kuan Rong Chan, Prof. Eng Eong Ooi and Prof. Ram Sasisekharan conceived and designed the study experiments.
- Dr. Kannan Tharakaraman, Dr. Devin Quinlan, Dr. Aditya Raguram and Prof. Ram Sasisekharan conducted computational analysis including epitope prediction and antibody engineering.
- Dr. Kuan Rong Chan, Dr. Vidya Subramanian, Dr. Yok Hian Chionh, Dr. Megan McBee, Dr. Eugenia Z. Ong, Dr. Esther S. Gan, Dr. Hwee Cheng Tan and Prof. Eng Eong Ooi performed the biochemical, *in vitro*, and regular A129 *in vivo* experiments.
- Dr. Watoru Watanabe, Dr. Kuan Rong Chan, Dr. Eugenia Z. Ong, Dr. Esther S. Gan, Dr. Hwee Cheng Tan and Prof. Subhash G. Vasudevan designed and conducted the pregnant A129 mice experiments.

- I co-designed the structural studies with Prof. Julien Lescar and Prof. Shashi Bhushan. And I carried out all the experiments for immune complex preparation as well as the initial data collection.
- Dr. Anu Tyagi, Prof. Shashi Bhushan, Prof. Julien Lescar and I solved the Cryo-EM structure of ZIKV-Fab ZAb_FLEP complex and completed the structural analysis.
- I assisted in writing the manuscript.

Chapter IV contains work with manuscript in preparation as Huan Jia, Pasunooti, K., Yee Hwa Wong, Aicha Gharbi Ayachi, Abbas El Sahili, Jian Qing Lin & Julien Lescar (2019). Interactions Between NS3 Helicase and NS4B of Zika Virus and Its Inhibition.

The contributions of the co-authors are as follows:

- I designed the main experiments with help from Prof. Julien Lescar and Dr. Abbas El Sahili. I performed all biochemical experiments and conducted data analysis
- Dr. Kalyan Kumar Pasunooti synthesized the fluorescein peptide and prepared SMA polymer.
- Yee Hwa Wong provided the purified peptide ligase (*OaAEP*). Jian Qing Lin prepared the plasmids for ZIKV NS4B protein expression.
- Dr. Aicha Gharbi Ayachi helped in optimizing experiments.
- I wrote the drafts of the manuscript. The manuscript is under revising together with Dr. Aicha Gharbi Ayachi and Prof Julien Lescar.

16-Aug-2019

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Jia Huan

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Abbreviations

Abbreviation	Full Name
BVDV	Bovine Viral Diarrhea Virus
DENV	Dengue Virus
HCV	Hepatitis C Virus
HIV	Human Immunodeficiency Virus
WNV	West Nile Virus
YFV	Yellow Fever Virus
ZIKV	Zika Virus
(+) ss RNA	Positive-sense single-stand RNA
(-) ss RNA	Negative-sense single-stand RNA
AEPs	Asparaginyl Endopeptidases
BAb	Binding Antibody
BIDMC	Beth Israel Deaconess Medical Center
BSA	Bovine Serum Albumin
cDNA	Chromosomal DNA
Cryo-EM	Cryogenic Electron Microscopy
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
E Protein	Envelope Protein
ED ₅₀	50% Effective Doses

EDTA	Ethylenediaminetetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
ER	Endoplasmic Reticulum
Fab	Antigen-binding Fragment
FBS	Fetal Bovine Serum
Fc	Fragment crystallizable region
FDA	Food and Drug Administration
FSC	Fourier Shell Correlation
Fv	Variable Fragment
GSH	L-Glutathione Reduced
GSSG	Glutathione Disulfide
GST	Glutathione S-transferase
His ₆	6 Consecutive Histidine
IMAC	Immobilized Metal Affinity Chromatography
IPTG	Isopropyl β -D-1-thiogalactopyranoside
ITC	Isothermal Titration Calorimetry
IWTO	Inactivated Whole Target Organism
kDa	Kilodaltons
M Protein	Membrane Protein
MTase	Methyl transferase
NAb	Neutralizing Antibody
NIAID	National Institute of Allergy and Infectious Diseases
NIH	National Institutes of Health

NS Protein	Non-Structural Protein
ORF	Open Reading Frame
PBS	Phosphate Buffered Saline
PDB	Protein Data Bank
PHEIC	Public Health Emergency of International Concern
prM Protein	Precursor-Membrane Protein
RdRp	RNA dependent RNA polymerase
RNA	Ribonucleic Acid
scFv	Single-chain Variable Fragment
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis
SEC	Size Exclusion Chromatography
SMA	Styrene Maleic Acid
SMALPs	SMA Lipid Particles
SPR	Surface Plasmon Resonance
TBST	Tris-buffered Saline with Tween 20
TEM	Transmission Electron Microscopy
UA	Uranyl Acetate
UTRs	Untranslated Regions
WHO	World Health Organization

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Abstract

Zika Virus (ZIKV) was discovered in 1947 in Zika forest of Uganda, hence the name. It belongs to the *Flaviviridae* family along with Yellow Fever Virus, Dengue Virus, West Nile Virus and more than 80 identified viruses. ZIKV contains a single stranded, positive sense RNA with size of 11 kb as its genomic material. ZIKV was not considered as a threaten before outbreaks in the 2000s. Infections are often asymptomatic, and common symptoms include fever, rashes and joint pain. Not much attention had been devoted to ZIKV until the epidemic outbreaks in French Polynesia in 2013 and in Brazil in 2015. From this point, the connection between ZIKV infection and severe neurological complications such as Guillain-Barré and microcephaly were intensively studied. It has also been discovered that transmission pathway for ZIKV was not limited to mosquito as the vector but also sexual and mother-fetus transmission, which made the control of ZIKV infection harder. In February 2016, WHO declared ZIKV a public health emergency. Though the declaration was withdrawn in November 2016, there is still no antiviral drug or vaccine available for ZIKV and more research must be devoted to find therapies against ZIKV infection. For my PhD studies, I addressed the research gaps in therapies for ZIKV infection. I focused on two aspects, firstly neutralizing antibodies against ZIKV that can stop viral infection at its entry stage; and secondly the interactions between non-structural proteins that are important for the replication of viral RNA.

Chapter I: Literature Review

1.1 Molecular Biology of ZIKA Virus

1.1.1 Zika Virus and its origin

Zika virus (ZIKV), an arbovirus, was first isolated from infected rhesus monkeys in 1947 in Zika Forrest of Uganda (Dick 1952, Ioos, Mallet et al. 2014, Wahid, Ali et al. 2016). It belongs to the viral family of *Flaviviridae* along with Dengue Virus (DENV), Yellow Fever Virus (YFV) and West Nile Virus (WNV). The first case of human infection by ZIKV was later reported in Uganda in 1964, but it had yet to catch public health attention (Simpson 1964, Wikan and Smith 2017). In year 2007, first outbreak of ZIKV was documented with around 73% population infected in Yap Island of the Federated States of Micronesia (Qadir, Riaz et al. 2018). This was followed by an outbreak in French Polynesia in 2013 (Cao-Lormeau, Blake et al. 2016). The most recent and severe epidemic outbreak was reported in Brazil in early 2015, where ZIKV infection was associated with microcephaly (Campos, Bandeira et al. 2015, Zanluca, Melo et al. 2015). On February 2016, World Health Organization (WHO) declared a Public Health Emergency of International Concern (PHEIC) against ZIKV until November of the same year. Until now, it was the fourth PHEIC that WHO has declared since 2005 and it was the first PHEIC against a virus from *Flaviviridae* family (S., & H, L., 2016). Spread of ZIKV has affected Southern and Northern America as well as countries in the Pacific area including Singapore (Dyer 2016) (**Figure 1-1 A**).

Currently, there is no vaccine nor antiviral drug available in the market to treat ZIKV infection. It is therefore important to perform more research to

understand the biology of ZIKV and to find novel therapies against ZIKV infection.

Figure 1-1. Distribution map and transmission pathways of ZIKV

A. Spreading map of ZIKV infection by ECDC. (Adapted and modified from https://ecdc.europa.eu/sites/portal/files/images/ZikaMap_OutbreakClassification_World%20wide_4.png). **B. Transmission pathways of ZIKV.** (Adapted and modified from <https://www.vectorstock.com/royalty-free-vector/zika-virus-infographic-transmission-vector-9529470>).

1.1.2 Diseases caused by ZIKV infection

Zika fever describes the major disease caused by ZIKV infection, with symptoms including fever, rashes, red eyes and joint pain (Dick, Kitchen et al. 1952, Ioos, Mallet et al. 2014). The symptoms of zika fever are highly similar to those of dengue fever (Gubler 2002). In addition, ZIKV infection can cause Guillain-Barré syndrome, a neural-related muscle weakness (Oehler, Watrin et al. 2014, Cao-Lormeau, Blake et al. 2016). During the outbreak in French Polynesia, the positive correlation between ZIKV infection and Guillain-Barré syndrome was reported (Oehler, Watrin et al. 2014, Cao-Lormeau, Blake et al. 2016)

It has also been reported that ZIKV infection of pregnant women could be passed to infant and could lead to severe neurological defects like microcephaly in new-borns (Cauchemez, Besnard et al. 2016, Heymann, Hodgson et al. 2016, Mlakar, Korva et al. 2016, Oliveira Melo, Malinger et al. 2016, Ventura, Maia et al. 2016). Around 8.3% fatality rate for ZIKV infection associated microcephaly was reported in Brazil in year 2017 (da Cunha, de Magalhães-Barbosa et al. 2017, Qadir, Riaz et al. 2018). ZIKV has been found in fetal brain tissue upon autopsy of a terminated pregnancy due to ZIKV infection to mother during the first trimester of pregnancy (Mlakar, Korva et al. 2016). ZIKV infection could disrupt neural progenitor development in mice as well, which explains the development of microcephaly in new-borns due to ZIKV infection (Li, Xu et al. 2016). Moreover, ZIKV infection was reported to affect male reproductive tract negatively (Puggioni, Pintus et al. 2018).

These severe complications had established ZIKV as a rather serious threat for public health. Besides, ZIKV has spread extensively during past epidemic outbreaks that infected more than 100,000 people in Brazil and around 400,000 people around the world (Nunes, Carlini et al. 2016, Ventura, Maia et al. 2016). Currently, all the formerly infected areas including America and tropical countries still have high risk for ZIKV infection (Bogoch, Brady et al. 2016).

1.1.3 The transmission of ZIKV

ZIKV was initially thought to be transmitted by mosquito bites only. The major vectors for transmission are day-time active *Aedes aegypti* and *Aedes albopictus* mosquitos (Dick, Kitchen et al. 1952, Malone, Homan et al. 2016, Wahid, Ali et al. 2016). It has been reported that ZIKV can be detected in patients' blood as well as seminal fluid, thus ZIKV infection can also be transmitted through blood transfusion or sexual contacts (Musso, Nhan et al. 2014, Musso, Roche et al. 2015, Tesla, Demakovsky et al. 2018). Prenatal transmission is another non-vector transmission pathway adopted by ZIKV, which is the main reason for its association to microcephaly (Besnard, Lastere et al. 2014). **(Figure 1-1 B)** The presence of ZIKV in a wide range of body fluids, the detection of ZIKV in patients that are asymptomatic, and the acquisition of multiple transmission pathways have made the prevention of ZIKV infection much more difficult. A treatment of ZIKV infection is needed.

1.1.4 Current treatments

Due to the limited attention caught by ZIKV for the past decades, both fundamental understandings and therapeutic studies are limited (Dick, Kitchen et al. 1952, Shan, Xie et al. 2016, Wahid, Ali et al. 2016). To date, there is neither a drug nor a vaccine developed targeting ZIKV (Dawes, Smalley et al. 2016, Malone, Homan et al. 2016, Shan, Xie et al. 2016). According to the site *ClinicalTrials.gov*, 22 clinical trials are being carrying out or have been completed for vaccines and antiviral drug interventions (**Figure 1-2**). Currently, ZIKV infection can only be contained by controlling the transmission vector and by alerting infected patients of possible sexual transmission. Symptoms of Zika fever can be treated with general medicines.

Figure 1-2. Map of clinical trials against ZIKV infection worldwide

(Adapted and modified from: <https://ClinicalTrials.gov>).

1.1.5 Phylogeny of ZIKV

1.1.5.1 Flaviviridae

Flaviviridae represent a family of enveloped viruses with (+) sense single stranded RNA as its genome (Simmonds, Becher et al. 2017). *Flaviviridae* derive their name from a member, yellow fever virus (Westaway, Brinton et al. 1985, Lindenbach and Rice 2001, Thiel, Collett et al. 2005). Four genera of viruses belong to this family: Flavivirus (e.g. YFV, ZIKV, DENV and WNV), Hepacivirus (e.g. Hepatitis C Virus (HCV)), Pestivirus (e.g. Bovine Viral Diarrhea Virus (BVDV)) and the latest added Pegivirus (e.g. Pegivirus A – K) (Simmonds, Becher et al. 2017). The updated taxonomy of ZIKV in *Flaviviridae* family is shown in **Figure 1-3** (Simmonds, Becher et al. 2017). Recently, a newly described virus genera namely Jingmen viruses were debated to be added into *Flaviviridae* family as they share common genomic plans in NS3 and NS5 region (Shi, Lin et al. 2016). However, ICTV (International Committee on Taxonomy of Viruses) has decided to not update these viruses into the taxonomy of *Flaviviridae* as they are distinct from each other in virion structure and other viral structural and nonstructural genes, thus unlikely to be homologues (Simmonds, Becher et al. 2017). Viruses within family *Flaviviridae* are enveloped viruses with similar spherical virion structure ranging from 40 to 60 nm with a single core protein and 2 or 3 envelope glycoproteins (Thiel, Collett et al. 2005). They also have similar genome composition as single-stranded positive-sense ((+) SS) RNA genome of approximately 9,000 – 13,000 nucleotides in length. A total of 89 species of viruses have been catalogued under *Flaviviridae* family. Mammals and birds are the natural hosts for this family of viruses, and they are primarily spread through arthropod vectors such as ticks and mosquitos (Simmonds,

Becher et al. 2017). Interestingly, viruses within this family have quite diverse biological properties despite many shared similarities in virion structure and genome composition (Kuno, Chang et al. 1998). The differences in biological properties make it hard for scientists to adapt findings from one virus member to another.

Figure 1-3. Taxonomy of *Flaviviridae* (Simmonds, Becher et al. 2017)

1.1.5.2 Flaviviruses

Flavivirus is a genus under family *Flaviviridae*. DENV, YFV, WNV and ZIKV all belong to this genus. Flavivirus is a large genus that contain close to 80 viruses and most of the viruses are human or animal pathogens (Kuno, Chang et al. 1998). Flaviviruses are widely distributed around the world and have caused high level of public health threat (Barzon, Trevisan et al. 2016, Prevcention 2016). A widely spread virus member within flaviviruses is DENV and much research has been done for DENV for vaccine and antiviral drug developments. In 2015, Dengvaxia® was registered in Mexico as the first vaccine for dengue virus, it was then commercially available in 11 countries in 2016 (Vannice, Wilder-Smith et al. 2018). However, in September 2018, WHO updated its recommendation on usage of Dengvaxia® for the excess risk of sever dengue within seronegative vaccine recipients (Aguiar, Stollenwerk et al. 2016, Aguiar, Halstead et al. 2017). Dengvaxia® is not known to be effective for preventing ZIKV infection as well (Shan, Xie et al. 2018).

1.1.5.3 Zika Virus

ZIKV is classified as a species under genus flavivirus. Currently, only one serotype of ZIKV has been documented. As shown in **Figure 1-4**, 3 genotypes of ZIKV have been recorded as Asian, East African and West African (Sun, Larsen et al. 2017).

Figure 1-4. Phylogeny tree of ZIKV

(Sun, Larsen et al. 2017). Open access © 2017 Sun et al.

1.1.6 Virion morphology and structure

Viruses in *Flaviviridae* family have a spherical virion structure. Using cryo-EM, ZIKV is also observed as spherical, with a diameter of 50 nm. The cryo-EM structure of mature zika virion has been solved at a resolution of 3.8 Å while the immature virion structure was solved at a resolution of 9 Å (Sirohi,

Chen et al. 2016, Prasad, Miller et al. 2017). ZIKV virion containing the viral genome RNA of 11 kb in complex with capsid protein, which is surrounded by a shell formed with 90 copies of envelope (E) glycoprotein dimer and membrane (M) protein. For the immature virion on the other hand, the shell is formed with E dimers and precursor membrane (prM) protein (**Figure 1-5 A**). In **Figure 1-5**, the cryo-EM structures of immature and mature virion are presented. It has also been reported that ZIKV virion is more thermally stable compared to other flaviviruses, which might explain the sexual and perinatal transmission of ZIKV (Kostyuchenko, Lim et al. 2016).

Figure 1-5. Virion structure of immature and mature ZIKV

(A). Cryo-EM reconstruction of immature ZIKV virion; (B). Immature Zika virion; (C). Micrographs of mature ZIKV particles under cryo-EM; (D). Cross-section of mature Zika virion, showing structural proteins and RNA genome. (Sirohi, Chen et al. 2016) Copyright obtained at RightsLink®.

1.1.7 Viral genome organization

ZIKV has a (+) ss RNA genome without segmentation. The genome size is 10,795 nt (Kuno and Chang 2007). Like the genome organization of other flavivirus, single stranded RNA genome of ZIKV contains 5' and 3' untranslated regions (UTRs) that flank a single long open reading frame (ORF). The single ORF is translated into a polypeptide, which undergoes co- and post-translational cleavages to form three structural and seven non-structural proteins (Wahid, Ali et al. 2016). The organization of the viral polyprotein is shown in **Figure 1-6**. During ZIKV replication, its genome plays three main roles. Firstly, it serves as mRNA for the translation of viral proteins that will then form replication complex. Secondly, it is the template for transcription of negative-sense RNA for genomic replication. Lastly, genomic RNA is packaged into viral particle as a complex with the capsid serve as infectious material.

Figure 1-6. Genomic organization of ZIKV

The translated viral proteins from ZIKV genomic RNA (Adapted from (Assenberg, Mastrangelo et al. 2009)). Copyright guaranteed by ASM.

1.1.8 ZIKV structural proteins

For ZIKV, there are four types of structural proteins including capsid (C), prM (Precursor-Membrane), M (Membrane) and E (Envelope) proteins, but only combination of three structural proteins appears concurrently in either immature viral particle or mature viral particle. Structural proteins are packaged with viral particle to serve its function in maintaining particle structure and in forming a shell to protect genomic RNA. At the immature stage, capsid, prM and E proteins are present. When immature viral particle passes through the Golgi apparatus, precursor of prM is cleaved by furin yielding M protein that is present in mature viral particles.

1.1.8.1 Capsid, prM and M proteins

Capsid locates at N-terminus of ZIKV polypeptide, it has a molecular mass of 11 kDa. The main function of capsid is to form a complex with genomic

RNA, which helps in genomic RNA packaging as well as in stabilizing genomic RNA (Barzon, Trevisan et al. 2016).

Both prM and M proteins can form heterodimer with E protein. When the viral particle is immature, the precursor of prM covers up the fusion loop of E dimer as a protection for the fusion loop. As the fusion loop was reported to be responsible for the pH-mediated endosomal fusion of viral particle, the immature viral particles with the fusion loop concealed are not infectious (Yu, Zhang et al. 2008, Sirohi, Chen et al. 2016). Upon cleavage of prM, M protein will be formed, the heterodimer formed by M and E proteins becomes the shell of mature viral particles.

1.1.8.2 Envelope (E) protein

Envelope protein as its name indicates, is located at the outmost position of viral particle. E protein is glycosylated with a molecular mass around 50 kDa. The main function of E protein is for viral entry into host cell through receptor attachment and membrane fusion. Glycosylated E dimer is responsible for the attachment of viral particles to host cell receptors leading viral particles to enter host cell through receptor-mediated endocytosis accompanied by membrane fusion (Kuhn, Zhang et al. 2002, Sirohi, Chen et al. 2016). The function of E protein makes it a major antiviral target since viral infection can be lowered or even eliminated at the cell entry stage by inhibiting the binding of E protein to host cells. It has been reported that antibodies neutralizing E protein of flavivirus would be highly potent immunotherapeutic agent (Crill and Roehrig 2001, Lok, Kostyuchenko et al. 2008, Schieffelin, Costin et al. 2010). To be able to find inhibitors or neutralizing antibodies against E protein, detailed understanding of

E protein structure is essential. In year 2016, two X-ray crystal structures of E protein have been reported. E protein has three domains, domain I is β -barrel shaped, domain II has a finger-like structure and domain III has an immunoglobulin-like C terminal structure. Three domains from two E protein monomers form a dimer in a head-to-tail manner with the help of domain II (**Figure 1-5**) (Dai, Song et al. 2016). In a recent paper, it was also mentioned that the glycosylation of E protein is important for the dimerization of E protein (Barba-Spaeth, Dejnirattisai et al. 2016).

Figure 1-7. Top view of structure of ZIKV E dimer

(Adapted from (Dai, Song et al. 2016)). Copyright obtained at RightsLink®.

pH-mediated membrane fusion is another function of ZIKV E protein for releasing of genomic materials into host cells. Fusion loop, a short ~12 amino acid peptide on ZIKV E protein subdomain II is in charge of the membrane fusion process (Sirohi, Chen et al. 2016). ZIKV fusion protein is classified structurally as class II fusion protein (Lescar, Roussel et al. 2001). The process of membrane fusion for flavivirus including ZIKV is shown in **Figure 1-8**. Briefly, when viral particle enters endosome, the low-pH environment leads to extensive

conformational changes of E protein dimers. The formation of E protein trimer exposes fusion loop peptide, which triggers membrane fusion with endosomal membrane and subsequent release of nucleocapsid into host cell cytoplasm (Stiasny and Heinz 2006, Smit, Moesker et al. 2011, Stiasny, Fritz et al. 2011).

Figure 1-8. Schematic of membrane fusion process for flavivirus

Color codes: viral membrane: yellow for out leaflet and blue for inner leaflet; target membrane: yellow for outer leaflet and red for inner leaflet; E protein: red for DI, yellow for DII, blue for DIII, orange for fusion loop peptide; light blue for helicase 1 and 2 in 'steam'; green for C-terminal transmembrane helicase. (A). E protein dimer on surface of a mature virion; (B). pH-mediated dissociation of E dimer and interaction of E monomers with target membrane at fusion loop peptide; (C). Initiation of E protein trimerization by relocation of DIII and conformational changes of the stem along DII; (D). Hemifusion of the outer leaflet membrane as DIII moves towards DII; (E). Final E protein trimer formation and opening of the fusion pore (Stiasny and Heinz 2006). Copyright obtained at RightsLink®.

ZIKV E protein domain III located at the C-terminal end of E protein, is an immunoglobulin-like domain, which has the same β -strand structure as the

other two domains. Domain III of E protein attracted a lot of research attentions in other flavivirus studies as well as in ZIKV studies because it is the host cell attachment domain of E protein. It can be expressed and purified using *E. coli* expression system and can fold independently. It has also been reported for DENV that antibodies neutralizing E protein domain III had high potency in inhibiting viral entry, but they are usually not cross-reactive (Crill and Roehrig 2001, Chávez, Silva et al. 2010, Li, Zhang et al. 2012).

The interface of E protein dimer is a very important epitope targeted by potent cross-neutralizing mAb (monoclonal antibodies). The fusion loop region of the dimer interface was known to be conserved across flaviviruses. A broadly neutralizing antibody 2A10G6 was reported to target this fusion loop region in 2016, it has also been reported to neutralize ZIKV infection in mice. The structure of immune complex formed between 2A10G6 and ZIKV E protein revealed the interaction between ZIKV E protein fusion loop and mAb (Dai, Song et al. 2016). One weak point of neutralizing antibody against structural protein of a virus is that viruses escape by mutations in the epitope region. Thus, it is crucial in antibody development to target mutationally-constrained regions of viral proteins. From past research done for other viruses, it was reported that an epitope can be mutationally-constrained if it is functionally constrained or structurally constrained (Peyerl, Barouch et al. 2003, Berkhoff, De Wit et al. 2005, Gorin, Du et al. 2017).

Antibody-dependent enhancement (ADE) phenomenon describes the enhancement of viral infectivity mediated by antibodies against that virus. ADE was firstly described in the 1960s by Hawkes (Hawkes 1964, Hawkes and Lafferty 1967), two mechanisms of enhancement for flavivirus were later

recorded: (1). mediated by cellular receptor for the Fc region of antibodies (FcR) and (2). mediated by receptors for components of the complement system (Halstead 1982, Cardoso, Porterfield et al. 1983, Westaway, Brinton et al. 1985). ADE was also connected with severity of subsequent infections for flavivirus thus the use of neutralizing antibodies for flavivirus including ZIKV must be carefully assessed to avoid ADE phenomenon.

Non-structural (NS) proteins

Besides three structural proteins that have been introduced, polypeptide of ZIKV is post-translationally cleaved into seven NS proteins as well, namely NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. In this section, the functions and structures of the NS proteins will be described, more attention will be given to NS3 and NS4B proteins, as the interaction between these two proteins is important for viral replication in DENV.

1.1.9.1 NS1 protein

NS1 protein has a molecular mass of 48 kDa, which is cleaved from E protein at N-terminus and from NS2A at C-terminus. Glycosylated NS1 interacts with lipids to form a lipoprotein. During infection, the NS1 hexamer lipoproteins are secreted to cytoplasm as an indication of severity of viral infection (Gutsche, Coulibaly et al. 2011, Brown, Akey et al. 2016). With such property, NS1 is generally regarded as a reliable antigen marker for detection of viral infection by ZIKV specifically (Kam, Leite et al. 2019).

1.1.9.2 NS2A and NS2B proteins

NS2A and NS2B are two small integral membrane proteins of ZIKV. They are part of the RNA replication complex. The proteins act as scaffold in

replication complex for non-structural enzymatic proteins to function in genomic replication. Besides, NS2B acts as a co-factor for NS3 protease region, which helps NS3 protease in post-translational cleavage of ZIKV polyprotein (Phoo, Li et al. 2016). Mutational studies in other flaviviruses have confirmed the essential role of NS2A and NS2B in genomic replication because viral replication can be disrupted when NS2A or NS2B protein is mutated (Mackenzie, Khromykh et al. 1998).

1.1.9.3 NS3 protein

NS3 protein is a multifunctional enzymatic protein in ZIKV. As a main component in the replication complex, NS3 protein conjugates with cofactor NS2B to function as viral serine protease, RNA triphosphatase as well as RNA helicase. The structure of NS2B-NS3 protease has been determined, indicating the importance of NS2B-NS3 complex for the NS3 protein to execute protease function (Xu, Sampath et al. 2005, Phoo, Li et al. 2016, Zhang, Li et al. 2016).

Helicase region of NS3 protein (residues 171-618) contains three functional subdomains where subdomain 1 and 2 mostly functions in ATP-binding and/or hydrolysis, while subdomain 3 is responsible for RNA binding. Combining the three subdomains, NS3 helicase functions in unwinding double-strand RNA. The unwinding of double-strand RNA prepares it for amplification by NS5 RdRp (RNA dependent RNA polymerase) protein. The structure of NS3 helicase has also been solved, which give us a good basic understanding in NS3 helicase structure and the orientation of its subdomains (Jain, Coloma et al. 2016). This work follows what was done originally for DENV NS3 helicase by the group of Lescar (Xu, Sampath et al. 2005, Luo, Xu et al. 2008). By overlapping ZIKV

helicase structure to Dengue 4 helicase structure, they can superimpose very well with r.m.s. deviations of 0.60 Å, 0.31 Å and 0.40 Å respectively for ZIKV NS3 helicase subdomains 1, 2 and 3 (Jain, Coloma et al. 2016). (**Figure 1-9**) In addition, the sequence identity of NS3 helicase for ZIKV and Dengue 4 is up to 72%. The similarity in structure indicates functional similarities.

Figure 1-9. Superimposition of ZIKV NS3 and DENV-4 NS3 helicase

Adapted from (Luo, Xu et al. 2008, Jain, Coloma et al. 2016). Copyright obtained at RightsLink®.

NS3 helicase is a potential drug target as the function of NS3 helicase is essential in viral replication (Lescar, Luo et al. 2008, Bollati, Alvarez et al. 2010). From previous studies on DENV, it has been discovered that among the viral replication complex, the interaction between NS3 helicase and NS4B is important for enhancing NS3 helicase selectivity towards double-stranded RNA (Umareddy, Chao et al. 2006). The interaction between NS3 helicase and NS4B occurs through the NS4B cytoplasmic loop region (between transmembrane domain 3 and 4) and NS3 helicase subdomains II and III. The interaction has

been proved to be essential for viral replication (Chatel-Chaix, Fischl et al. 2015, Zou, Wang et al. 2015). Inhibitors targeting the interaction have been studied as possible antiviral drug for DENV (Wang, Dong et al. 2015, Xie, Zou et al. 2015). To establish a possible similarity between ZIKV and DENV NS3 helicase protein, it will be very interesting to characterize the interaction between NS3 helicase and NS4B for ZIKV. Moreover, antiviral drugs against ZIKV that targets this interaction could have a great potential.

1.1.9.4 NS4A and NS4B proteins

As reported earlier by Liang et al., ZIKV NS4A and NS4B proteins are responsible for inhibiting neurogenesis and promoting autophagy. These two proteins are likely responsible for causing microcephaly in infants (Li, Saucedo-Cuevas et al. 2016, Liang, Luo et al. 2016). The connection between NS4A and NS4B proteins to neuronal disease like microcephaly highlighted their importance and the possibility to develop antiviral drugs against them. Through studies in DENV, NS4A and NS4B are both hydrophobic proteins with transmembrane domains, where NS4B is the largest viral hydrophobic transmembrane protein (Zmurko, Neyts et al. 2015).

Antiviral drug and neutralizing antibodies targeting NS4B protein of DENV have been reported in previous studies (Xie, Zou et al. 2014, Wang, Dong et al. 2015, Xie, Zou et al. 2015). Among them, one neutralizing antibody reported by our group previously, namely mAb 44-4-7, showed cross-reactivity across different DENV serotypes and WNV (Xie, Zou et al. 2014). Even more interestingly, the epitope of mAb 44-4-7 was reported to be also the cytoplasmic loop between NS4B transmembrane domains 3 and 4 as shown in schematic

structure of NS4B in **Figure 1-10**, which overlaps with the predicted interaction region between NS3 helicase and NS4B.

Figure 1-10. Topology of Flavivirus NS4B protein

As for now, it is unclear if the interaction between NS3 helicase and NS4B in ZIKV exists and if the interaction region remains the same as for DENV. Thus, we focused on this question for basic understandings and for helping antiviral drug interventions.

1.1.9.5 NS5 protein

Similar to other flaviviruses, NS5 protein is the largest NS protein encoded in ZIKV. The functions of NS5 protein include methyl transferase (MTase) and RdRp. MTase adds the N7 and 2' O methyl on the 5' cap of the viral mRNA. Viral mRNA needs to be stabilized by the 5' cap and it also helps in translation efficiency and evasion of host immune response (Dong, Fink et al. 2014). RdRp functions as the polymerase for amplification of viral RNA. Therefore, both functions of NS5 protein are critical for viral replication, which make NS5 a very attractive antiviral drug target (Li, Dong et al. 2013, Dong, Fink et al. 2014). Structure of full-length NS5 protein has been solved (Coloma,

Jain et al. 2016, Zhao, Yi et al. 2017). Currently, publications have revealed the connection between ZIKV NS5 and host interferon signaling pathway (Chaudhary, Yuen et al. 2017), which can be important for antiviral target identifications. Many researches are carrying out studies to look for antiviral drugs against NS5 protein for treatment of ZIKV infection.

1.1.9 Flavivirus life cycle

For flavivirus, the life cycle can be divided into a few stages including (1). Viral attachment and entry; (2). Translation and assembly of replication complex; (3). Genomic replication; (4). Translation of viral proteins; (5). Virion assembly and encapsidation; and (6). Virion maturation and release of viral particles (**Figure 1-11**).

Figure 1-11. Schematic representation of Flavivirus life cycle

(Suthar, Diamond et al. 2013). Copyright obtained at RightsLink®.

All the structural and non-structural proteins introduced previously help virus in completing its life cycle, thus more infectious viral particles can be produced. Upon transmission of infectious viral particles to host cells, viral particle enters host cell by receptor-mediated endocytosis (Lindenbach and Rice 2001). E glycoprotein is responsible for the attachment to host cell receptor. Once the viral particle has entered into endosome, the decrease of pH in environment will mediate fusion of viral particle with endosomal membrane through the help of viral E protein fusion loop, which leads to uncoating of viral particle and releasing of viral genome into cytoplasm (Fritz, Stiasny et al. 2008). (+) ss RNA is then translated into polyprotein and co- or post-translationally processed to yield the ten viral proteins with help of viral and host proteases. Nonstructural proteins will then form viral replication complex in charge of asymmetric and semi-conservative RNA synthesis. The production of (+) ss RNA is much more in amount than the production of (-) ss RNA (Suthar, Diamond et al. 2013). Upon genomic replication and protein production, the genomic RNA will be packed with capsid to form nucleocapsid and the virion will then be assembled in rough ER (Endoplasmic Reticulum) to form immature virion with prM protein in presence. Immature virions will undergo furin cleavage in Golgi apparatus to produce mature infectious virions (Suthar, Diamond et al. 2013). The mature and infectious virion will finally be transported to plasma membrane where it is released through exocytosis.

To prevent viral infection, there are a few steps in its life cycle and the responsible proteins can be targeted. Firstly, viral attachment and entry can be hindered by targeting E protein. Neutralizing antibodies are currently under developments for preventing viral infection by flaviviruses for DENV and JEV

(Chávez, Silva et al. 2010, Li, Zhang et al. 2012, Dai, Song et al. 2016). This strategy might be applied for preventing ZIKV infection as well. Secondly, we can interrupt translation and assembly of replication complex, for instance by inhibiting functions of NS5 protein (Malet, Massé et al. 2008, Lim, Noble et al. 2015, Wang, Thurmond et al. 2018). And thirdly, by disrupting the formation of viral replication complex thus preventing viruses from amplification. For this, NS4B as an essential intermediate protein was generally targetted for other flaviruses studies (Wang, Dong et al. 2015, Xie, Zou et al. 2015, Zmurko, Neyts et al. 2015). Among all drug discoveries targeting viral replication complex, drugs targeting the interaction between NS3 and NS4B protein seemed to have some potentials (Umareddy, Chao et al. 2006, Zou, Wang et al. 2015). Last but not least, we could target host factors that assist virus in completing its life cycle (Lescar, Soh et al. 2018).

In my thesis projects, I focused on two aspects of viral life cycle to study possible novel therapies against ZIKV infection. One part was to target viral attachment, entry and fusion by studying neutralizing antibody against E protein. The other part was to understand the interaction between NS3 helicase and NS4B protein thus to target viral replication complex for antiviral drug intervention.

1.2 Summary of structural information on ZIKV

3D structural information has always been essential for studies involving proteins, it helped a lot on our understandings of viral proteins, their interactions and especially on drug discovery. Ever since the beginning of 3D structure determination over 45 years ago, the use of structural information in drug discovery has been discussed (Congreve, Murray et al. 2005). For the past

decades, there have been a list of successfully marketed drugs discovered rely on structural information of its targeted protein including renin targeting aspartic protease, Angenerase and Viracept targeting HIV protease and so on (Blundell, Sibanda et al. 1983, Lapatto, Blundell et al. 1989, Miller, Schneider et al. 1989, Congreve, Murray et al. 2005). Besides, Cryo-EM as a counterpart to X-ray crystallography has enabled us to visualize virions as a whole and to have better understandings in viral life cycle for different types of viruses (Ranson and Stockley 2010).

For studies on ZIKV, structural information from X-ray crystallography and Cryo-EM has accelerated the research progress greatly. A simple search of ZIKV on EMBL-EBI (Europe's flagship laboratory for the life sciences-The European bioinformatics institute) website gives 155 macromolecular structures including 22 EM structures and 133 crystal structures (EMBL-EBI 2020). In this section, a brief summary of structures available for ZIKV proteins, protein complexes and virions will be discussed together with their impacts on ZIKV research.

1.2.1 X-ray crystallography structures for ZIKV proteins and protein complexes

As indicated in **Figure 1-12** below, X-ray crystallography has helped to reveal the structural information for most of ZIKV structural and non-structural proteins. The structure of ZIKV C protein provided insight information on its encapsulating function (Li, Zhao et al. 2018). The structure of E protein for ZIKV indicated its high similarity towards DENV E protein, thus enabled the studies of cross-reactive neutralizing antibodies such as Fab C10, C8 and A11 (Barba-

Spaeth, Dejnirattisai et al. 2016, Zhang, Kostyuchenko et al. 2016). Structures of NS3 protease and NS5 MTase or RdRp were extensively studied for structural guided drug discovery purposes. The structural insights for NS3 protease in complex with potential inhibitors such as dipeptide inhibitor, reverse peptide inhibitor and compound fragment have guided the antiviral drug researches (Lei, Hansen et al. 2016, Zhang, Li et al. 2016, Li, Zhang et al. 2018). With these valuable structural information, researchers are able to optimize the binding affinity of inhibitors towards targeted viral proteins and eventually succeed in producing antiviral therapies.

Figure 1-12. Summary of available structures (crystal and cryo-EM) on ZIKV

1.2.2 Cryo-EM structures of ZIKV virions and immune complexes

The structures solved by Cryo-EM are more on the whole virion level (**Figure 1-12**). A 3.8Å cryo-EM structure of ZIKV was firstly solved by Devika Sirohi et al. in 2016 (Sirohi, Chen et al. 2016). With the structural information, it was suggested that viral transmission pathway and diseases caused could be related to the changes of ~10 amino acids surrounding Asn 154 glycosylation site of E protein (Sirohi, Chen et al. 2016). Later, with the effort of Victor A. Kostyuchenko et al., thermal stability of ZIKV was proved with intact virion structure at temperature up to 40°C (**Figure 1-13**) (Kostyuchenko, Lim et al. 2016). This also explains the possibility of ZIKV to be transmitted sexually.

Figure 1-13. Thermally stable ZIKV particles under cryo-EM

Micrographs of ZIKV particles that were incubated in different temperatures (Kostyuchenko, Lim et al. 2016). Copyright obtained at RightsLink®.

Besides the ZIKV virion structures, cryo-EM has also been used to solve immune complex structure for ZIKV together with a few neutralizing antibodies including cross-reactive and ZIKV-specific antibodies (Zhang, Kostyuchenko et al. 2016, Tharakaraman, Watanabe et al. 2018). With these information, it improved our understandings on mechanism of action for neutralizing antibody candidates.

1.3 Vaccine developments and drug discovery for flavivirus prevention and treatment

As mentioned above, studies on drug discovery and vaccine developments against flaviviruses have been carried out throughout the decades. However, as compare to the treatment for chronic diseases caused by viral infections such as HCV and HIV, it tends to be harder to develop effective treatments for acute diseases caused by viral infections including DENV and ZIKV infections (De Clercq 2007, Lim, Wang et al. 2013). To combat viral infections, there are commonly two approaches we can take: (1). Development of vaccine that can be used as preventive measurement; (2). Discovery of anti-viral drugs that can treat infected patients.

1.3.1 Vaccine developments for flavivirus

Vaccine can stimulate human immune system to produce antibodies against less lethal forms of microbes and thus to protect human body from future infections by those microbes (Plotkin 2011, Han 2015). It is so far the most well-known precaution can be taken to combat viral infection. The developments of vaccine for viruses have started three centuries ago beginning with active immunization for smallpox, it was then developped to live attenuated vaccines, killed whole organisms, purified proteins of organisms, reassortants and eventually genetically engineered biological agents (Plotkin 2011).

For flaviviruses, vaccines for three of them have been developped and approved including: a series of live attenuated vaccines based on 17D lineage for YFV; 15 vaccines with different forms including live attenuated vaccines, inactivated Vero cell-derived vaccines and live chimeric vaccines were available

for JEV; for TBEV, there were 2 approved vaccines for western european and russian respectively (Oya 1988, Monath 2005, Publication 2011, Li, Zhang et al. 2012).

However, the vaccine development for DENV was much obstructed for more than 75 years by the presence of four serotypes and the existence of ADE phenomenon (Sabin and Schlesinger 1945). Therefore, an effective vaccine for DENV must be tetravalent to all four serotypes of DENV in order to avoid ADE phenomenon (Whitehead, Blaney et al. 2007). The only approved vaccine for Dengue was Dengvaxia® developed by Sanofi Pasteur (CYD-TDV), a live attenuated recombinant dengue vaccine based on the backbone of YFV 17D vaccine. CYD-TDV has been proved to be safe and efficacious for seropositive individuals, but it raises risk of developing severe dengue for seronegative individuals if they get infected by DENV after vaccination (Aguilar, Stollenwerk et al. 2016, Aguilar, Halstead et al. 2017, Halstead 2017, Thomas and Yoon 2019). Currently, Dengvaxia® is still approved by U.S. FDA (Food and Drug Administration) for prevention of DENV infection, though the usage is limited to individuals with laboratory confirmed previous DENV infection (Thomas and Yoon 2019). The issues with Dengvaxia® informed us that a better DENV vaccine was still much needed and more efforts should be taken to ensure the safety of vaccine. Fortunately, two other dengue vaccines in their phase 3 clinical trials namely TAK-003 (NCT02747927) and Butantan-DV (NCT2406729) could be the solutions to dengue vaccine developments.

The development of ZIKV vaccine was accelerated with the experiences from vaccine developments for DENV. By the year of 2017, two years after the genomic information has been confirmed for ZIKV, there were more than 45

vaccine candidates under development (Dredze, Broniatowski et al. 2016, Durbin and Wilder-Smith 2017, Barrett 2018, Shan, Xie et al. 2018). 16 of the vaccine candidates have entered clinical trials as listed below in **Table 1-1** (WHO 2019). The platforms for ZIKV vaccine development ranges from purified inactivated vaccine to mRNA vaccine platforms. However, no vaccine has been approved for ZIKV yet, the unpredictability of future outbreaks urges a successful development of vaccine against ZIKV (Durbin and Wilder-Smith 2017).

Table 1-1. Zika vaccines in clinical trials

Candidate vaccine	Developer	Platforms	Development Status	Clinical trial registration
GLS-5700	GeneOne Life Science, Inc.	DNA	Phase 1	NCT02809443
GLS-5700	GeneOne Life Science, Inc.	DNA	Phase 1	NCT02887482
AGS-v	NIH ₁	Peptide	Phase 1	NCT03055000
MV-Zika	Themis Bioscience	Recombinant viral vector	Phase 1	NCT02996890
mRNA-1325	Moderna Therapeutics	mRNA	Phase 2	NCT03014089
VRC-ZKADNA085-00-VP	NIAID ₂	DNA	Phase 1	NCT02840487
VRC-ZKADNA090-00-VP	NIAID	DNA	Phase 1	NCT02996461
VRC-ZKADNA090-00-VP	NIAID	DNA	Phase 2	NCT03110770
ZIKV PIV	NIAID	IWTO ₄	Phase 1	NCT02963909
ZIKV PIV	NIAID	IWTO	Phase 1	NCT02952833
ZIKV PIV	BIDMC ₃	IWTO	Phase 1	NCT02937233
ZIKV PIV	NIAID	IWTO	Phase 1	NCT03008122

PIZV or TAK-426	Takeda	IWTO	Phase 1	NCT03343626
VLA1601	Valneva Austria GmbH	IWTO	Phase 1	NCT03425149
rZIKV/D4Δ30-713	NIAID	Recombinant viral vector	Phase 1	NCT03611946
BBV121	Bharat Biotech	IWTO	Phase 1	CTRI/2017/05/008539

1 National Institutes of Health

2 National Institute of Allergy and Infectious Diseases

3 Beth Israel Deaconess Medical Center

4 Inactivated Whole Target Organism

1.3.2 Drug discovery for flavivirus

Besides vaccine developments, antiviral drugs are essential for treatment of symptomatic viral infections as well. To identify potential antivirals, scientists commonly use approaches including (1) drug design based on viral proteins's crystal structure or genomic RNA structures; (2) test and prove for FDA approved antiviral drugs against other viruses; (3) screen compounds library for novel antiviral targets; (4) chemical modification of known antiviral drugs and (5) neutralizing antibody therapies (Ray and Shi 2006). For antiviral drug discovery against ZIKV infection, drug repurposing approach has also been adopted beside novel drug discovery. FDA antivirals, FDA non-antiviral drugs that are active against viruses and all other FDA approved drugs are under repurposing studies (Ekins, Mietchen et al. 2016).

Challenges have been faced for antiviral drug discovery against DENV for the following reasons: (1). the drug candidate needs to be pan-serotypic to allow usage of drug without further differentiation in infected DENV serotype;

(2). it needs to be fast acting as the viremia in patient reduced by 100-fold within 48 hours upon infection (Libraty, Young et al. 2002, Lim, Wang et al. 2013, Nguyen, Tran et al. 2013); (3). it needs to be cost effective and (4). the form of delivery needs to be easy for admission. With a review on challenges faced and progresses made on antivirals discovery, it will help for future drug discoveries to combat flaviviruses like ZIKV and DENV.

Antivirals can be classed into two categories base on the nature of their targets: (1). Direct-acting antivirals (DAA) target viral proteins and (2) Host directed antivirals (HDA) target host factors that aided in viral infection.

The popular targets for DAA discovery are NS3 protein with protease and helicase functions and NS5 protein with MTase and RdRp functions. With combined efforts from academic institutes and industries like NITD (Novartis Institute of Tropical Diseases), a few antivirals against DENV have entered clinical trials including NITD-008 and Balapiravir as DAA targeting RdRp of DENV. RdRp as the target for antivirals has been validated for HIV and HCV treatments and is regarded as a suitable drug target (Rawlinson, Pryor et al. 2006, Lim, Noble et al. 2015). Drugs targeting RdRp can be divided into nucleoside/nucleotide inhibitor or non-nucleoside inhibitor (Lim, Wang et al. 2013). Both NITD-008 and Balapiravir are RdRp nucleoside analog inhibitors. NITD-008 was originally discovered inhibiting RdRp nucleoside, which did not pass phase I clinical trial due to side-effects observed in both rats and dogs (Lim, Wang et al. 2013). Balapiravir is a repurposed HCV drug candidate, which unfortunately ended its clinical trial against HCV due to hematological adverse effects in clinical participants (Nelson, Zeuzem et al. 2012). Nguyen et al. later repurposed it as a drug candidate for DENV as it is the prodrug of R1479 that

has anti-DENV activity, it thus entered a phase II trial directly for treating DENV (Nguyen, Tran et al. 2013). However, it also ended the clinical trial showing no improvement in clinical and virological parameters in patients. Non-nucleoside inhibitors are non-competitive compounds acting by blocking conformational changes that are needed for polymerase's initiation or elongation activities. Currently, there is no non-nucleoside inhibitors for DENV or ZIKV entered clinical trials.

For ZIKV, DAA discoveries were mostly focused on NS3 protein. Many groups are studying small molecule inhibitors and peptide inhibitors targeting ZIKV NS3 protease and NS2B-NS3 protease domain (Lei, Hansen et al. 2016, Zhang, Li et al. 2016, Domachowske, Khan et al. 2018). Though, no DAA against ZIKV NS3 protein has started clinical trial yet, all the valuable structural information obtained in complex with small molecule inhibitors and peptide inhibitors will guide further developments of DAA targeting NS3 protein for ZIKV.

During viral amplification, some host factors are recruited by viral proteins. These host factors are potential antiviral targets as well as they generally play vital roles in viral replication. There are various advantages for HDA including (1). Broad-spectrumity as same host factors may be recruited for different viruses; (2) lower chance of yielding escape mutation as host cells are not error prone as comparing to viral proteins; (3) there are some FDA approved HAD that can be repurposed for treating flavivirus infection (Krishnan and Garcia-Blanco 2014, Acosta and Bartenschlager 2016). Celgosivir is an imino sugar prodrug, which is a HAD discovered for HCV treatment. It inhibits α -glucosidase I, an enzyme plays vital role in processing of N-linked

oligosaccharides on prM, E and NS1 proteins. Celgosivir was then repurposed for treatment of DENV and entered clinical trial phase 1 (Sung, Wei et al. 2016). The outcome of clinical trial showed promising pharmacological effects, however it did not show statistically significant differences in secondary endpoints. To further investigate on it, scientists were to evaluate the effect of celgosivir on patients with a revised dosing regiment in another clinical trial (NCT02569827) (Sung, Wei et al. 2016). For HAD development, the control of cytotoxicity and side-effects need to be addressed as host factors generally are involved in multiple functions and some functions might be essential for cell survival and metabolism (Lim, Wang et al. 2013).

In summary, through the years of drug discoveries against flaviviruses especially DENV and ZIKV, lots of difficulties and challenges have been faced. Though there is still no antivirals successfully developed, the lessons learnt are valuable for further researches. For DAA and HAD developments, three aspects including drug safety, cost and admission pathway must be thoroughly considered in order to obtain a successful drug candidate. In the mean time, scientists are exploring other approaches for treatment of viral infection including the development of neutralizing antibodies.

1.4 Neutralizing antibodies and immune complex

One other antiviral approach becomes more and more popular now is neutralizing antibody (NAb). As the name suggests, NAb is a kind of antibody that functions differently from binding antibody (BAb). NAb can protect cell from infection by viruses through binding with viral proteins (antigens) and has been highly implemented for antiviral therapeutics (Heinz and Stiasny 2012).

The neutralizing effect of antibody is achieved through different mechanisms including (1) competing epitope that can bind to host cell receptor (2) inhibiting fusion of viral particle to endoplasmic membrane (3) Fc- γ -mediated clearance of viral particle (4) antibody-dependent cytotoxicity of infected cells (Pierson, Fremont et al. 2008). Although acquisition of different working mechanisms may affect antibodies' effectiveness, neutralizing antibodies can only function in protecting cell from viral infection if it can form a tight and specific immune complex with viral antigens.

Immune complex, also known as antibody-antigen complex, describes a complex formed by integral binding of antibody and antigen. The higher the binding affinity between antibody and antigen will lead to formation of a tighter immune complex. Thus in the process of getting effective neutralizing antibody as vaccine or immunotherapeutic agent, the structure of immune complex always needs to be studied for understanding the mechanism of neutralization. The structure can also reveal essential information like the structural origin of the binding affinity, the antibody-binding epitope and the possibilities for optimizing the interaction interface.

The best way to study immune complex is to get the X-ray crystal structure of the complex. To get the crystal containing both antibody and antigen, crystallography as a technique was often applied. For the case of immune complexes, Fab (antigen-binding fragment) of an antibody is commonly used to form complex with an antigen, where the complex as a whole is set for protein crystallization. Sometimes though, a smaller substitute of Fab the single-chain variable fragment (scFv) can also be used for the formation of immune complex.

Alternatively, cryogenic electron microscopy (cryo-EM), a more recently developed technique comparing to x-ray crystallography can also be adopted for studying immune complex structures. One advantage of cryo-EM over x-ray crystallography is that cryo-EM allows the observation of specimens without staining or fixing them in any way, thus it shows the structure of specimens in their native form (Kühlbrandt 2014). Therefore, cryo-EM is a more preferred technique nowadays for studies involving whole viral particles because structural information can be enhanced using symmetry redundancy. The tremendous advances in detector technology and software algorithms have also allowed Cryo-EM to solve structures with near to atomic resolution, which is comparable to the resolution we can gain from x-ray crystallography (Merk, Bartesaghi et al. 2016, Vonck and Mills 2017).

1.5 Open questions

There is neither neutralizing antibody nor antiviral drug available on the market to treat ZIKV infection. Meanwhile, therapies are needed because of the potentially severe neurological complications related to ZIKV infection. Hence, both fundamental and applied research are needed to find solutions for preventing or curing ZIKV infection. For the ZIKV E protein, a neutralizing antibody targeting the fusion loop or domain III could form an attractive option as there is still no antiviral drug that can inhibit viral entry. For the viral replication complex, the detailed interactions among NS proteins also remain unknown. Therefore, a deeper understanding of replication complex is needed as it can help in developing new therapeutic strategies

1.6 Aims and objectives

For my PhD studies, I will focus on two strategies that can be used to prevent or cure ZIKV infection with the following goals,

1. Study the immune complex formation between ZIKV E protein domain III with broad-spectrum DENV neutralizing antibody 513 or 9F12.
2. Solve the immune complex structure for ZIKV E protein with neutralizing antibody ZAb_FLEP.
3. Develop an interaction assay using a peptide ligase.
4. Study the interaction between ZIKV NS3 helicase and NS4B to understand more on viral replication complex formation.
5. Using the assay established in 4, screen for peptides that disrupt ZIKV NS3 helicase and NS4B interaction, thus, impeding formation of viral replication complex.

1.7 Organization of the work

In chapter II, I will summarize the materials and methods that were used. In chapter III, results and discussion on neutralizing antibody studies will be presented in detail. Chapter IV will list all results we have obtained on interaction between ZIKV NS3 helicase and NS4B as well as the application of peptide ligase in developing a new interaction assay. In the last chapter, I will draw conclusions on all the works done and discuss future directions.

Chapter II: Materials and Methods

2.1 Materials

2.1.1 Reagents and chemicals

Shown in **Table 2-1** below, the reagents and chemicals used for experiments as well as their suppliers are listed

Table 2-1. List of reagents and buffers

Reagents/Chemicals	Brand
10x PBS buffer	Vivantis
6x DNA loading dye	NEB
Agarose	1 st Base
Ampicillin	Gold Biotechnology
Bovine Serum Albumin (BSA)	Merk
Cellfectin™ II	Thermo Fisher Scientific
Chloramphenicol	Gold Biotechnology
Clarity™ ECL western blotting substrates	Bio-Rad
DEPC-treated water	Invitrogen
Difco™ LB Agar, Lennox	BD
Difco™ LB Broth	BD
Dithiothreitol (DTT)	Gold Biotechnology
DNA ladder – 1 kb / 100 bp	Thermo Fisher Scientific
ELISA 1x coating buffer	Thermo Fisher Scientific
Ethylenediaminetetraacetic acid (EDTA)	1 st Base
Fetal Bovine Serum (FBS)	Gibco
Gentamicin	Nacalai Tesque

Glycerol	Nacalai Tesque
Grace's insect medium	Gibco
GSH	Sigma-Aldrich
GSSG	Sigma-Aldrich
His Mag Sepharose™ Ni	GE Healthcare
Imidazole	Bio-Basic
Iodoacetamide	Sigma-Aldrich
Isopropyl β-D-1-thiogalactopyranoside (IPTG)	Bio-Basic
Kanamycin	Gold Biotechnology
L-Arginine	Sigma-Aldrich
L-Cysteine	Sigma-Aldrich
Na HEPES	Sigma-Aldrich
Ni-NTA beads	Thermo Fisher Scientific
Pierce™ BCA Protein Assay Kit	Thermo Fisher Scientific
Pierce™ Protein A agarose	Thermo Fisher Scientific
Protease Inhibitor	Nacalai Tesque
Protein marker	1 st Base and Genedirex
Sf-900™ II SFM medium	Thermo Fisher Scientific
Silver staining kit	Thermo Fisher Scientific
Sodium chloride (NaCl)	Merk
Sodium Dodecyl Sulfate (SDS)	Bio-Rad
Tetracycline	Sigma-Aldrich
Tris	1 st Base

Triton X-100	Sigma-Aldrich
Tween 20	Bio-Basic
Urea	Bio-Basic
Eco-Stain (DNA staining solution)	Bio-Basic
Glycine	1 st Base
Strep-Tactin® sepharose	IBA Lifetechnology

2.1.2 Enzymes and antibodies

Enzymes and antibodies that have been used in the studies with their suppliers are listed in **Table 2-2** below.

Table 2-2. List of enzymes and antibodies

Enzymes/Antibody	Usage	Brand
2x Phusion Flash Polymerase Master Mix	PCR	Thermo Fisher Scientific
KLD Enzyme Mix	Mutagenesis	NEB
Gibson Assembly® Master Mix	Gibson Assembly	NEB
Papain	Antibody Fragmentation	Sigma-Aldrich
Anti-His HRP antibody	Immunoblotting	Sigma-Aldrich

2.1.3 Composition of buffer solutions

In **Table 2-3** below, compositions of general buffers are summarized.

Table 2-3. List of buffers with their compositions

Buffer Name	Usage	Composition
10 x Tris-Glycine SDS-Running Buffer	Running of Tris-Glycine SDS-PAGES	<ul style="list-style-type: none"> • 250 mM Tris-HCl pH 8.3 • 1.92 M glycine • 1% (weight/vol) SDS
50 x Tris-Acetate- EDTA (TAE) Buffer	Running of DNA agarose gel	<ul style="list-style-type: none"> • 2M Tris-HCl • 50 mM EDTA • Acetic acid, adjust pH to 8.4
10x Wet Transfer	Immunoblotting	<ul style="list-style-type: none"> • 250 mM Tris-HCl pH 8.3 • 1.92 M glycine
10x TBS Buffer	Immunoblotting	<ul style="list-style-type: none"> • 0.5 M Tris-HCl pH 7.4 • 1.5 M NaCl
1x TBST Buffer	Immunoblotting	<ul style="list-style-type: none"> • 10x TBS Buffer • 1% Tween 20

2.2 Molecular cloning

Constructs used in the studies are listed in **Table 2-4** with properties of each construct. The methods used for preparation of above-mentioned constructs will be described in detail in the following subsections.

2.2.1 Preparation of competent cells

Frozen glycerol stock of bacterial cell was plated onto LB agar plate with antibiotics that the cell line is resistant to and incubated overnight at 37°C. A single colony was then picked and inoculated into 5 mL of LB broth with

respective antibiotics for shaking overnight in 37°C shaker as the starter culture. On the next day, start culture was diluted to LB at 1/100 volume dilution rate with respective antibiotics and cultured in 37°C shaker. Bacterial growth was monitored by measuring optical absorbance at 600 nm (OD₆₀₀). When OD₆₀₀ reached 0.4 to 0.6, culture was chilled on ice and then centrifuged for 15 mins at 5000 rpm at 4°C. The cell pellet was then resuspended gently with 1/5 of original culture volume of ice-cold TFB1 buffer (30 mM KOAc, 100 mM RbCl, 10 mM CaCl₂, 50 mM MnCl₂, and 15% Glycerol). After resuspension, it was incubated on ice for 1 hr prior centrifugation at 5000 rpm for 15 mins at 4°C. Cell pellet from centrifugation was then resuspended with 1/25 of original culture volume of ice-cold TFB2 buffer (10 mM MOPS, 10 mM RbCl, 75 mM CaCl₂ and 15% Glycerol) and incubated on ice for 30 mins. By then, the cells were competent chemically and were aliquoted and snap-frozen with liquid nitrogen. It was then stored at -80°C for further use.

2.2.2 Polymerase Chain Reaction (PCR) for molecular cloning

PCR was applied for all the insertion, deletion, site directed mutation for constructing the expression plasmids. Thermo Scientific Phusion Flash High-Fidelity PCR Master Mix was mainly used in the PCRs. A typical 50 µL of PCR mixture includes 2.5 µL of forward/reverse primer (10 µM), 1 µL of template (50 ng/µL), 25 µL of Phusion Flash Mater Mix (2x) and 19 µL of DEPC treated water. The thermo cycler program was determined based on the melting temperatures of primers and the size of expected PCR product. Typically, the annealing temperature used is 3°C higher than the lower melting temperature of primers according to manufacturer's manual. The extension time used was 15 sec for each

1 kb of PCR product. To maximize the amount of PCR product, thermo cycle number was set to 25 to 30.

To prepare the constructs listed in **Table 2-4**, primers were designed respectively. The list of primers with their respective products and the following molecular cloning method are shown below in **Table 2-5**.

2.2.3 Agarose gel electrophoresis

To confirm the identity of the PCR products, agarose gel electrophoresis was carried out. Agarose gel was prepared by dissolving desired amount of agarose in 1x TAE buffer through heating by microwave. Eco-stain was added into agarose gel solution for staining of the DNA (Deoxyribonucleic Acid) products. 1% (w/v) agarose gel is generally used for observation of PCR product with size ranging from 1 kb to 8 kb. Gel electrophoresis was run at constant voltage of 120 V for 20 mins to 30 mins. Observation of DNA was done using Bio-Rad ChemiDoc™ Imaging System with UV light.

2.2.4 PCR product purification and gel extraction

Before proceeding to cloning, PCR products were purified with Qiagen PCR purification kit where the product was diluted with 5 volumes of PB buffer. In some cases, unspecific PCR products might be obtained, so gel extraction was used to purify out the correct PCR product for cloning. For gel extraction, DNA band with correct size was cut from agarose gel after electrophoresis and measured with weighting balance. 300 µL of QG buffer was used for dissolving 0.1 g of gel by incubation at 50°C for about 10 mins. For both PCR purification and gel purification, the mixture was then loaded onto purification column and centrifuged at 14,000 rpm at room temperature for 1 min. Column was washed

with 750 μ L of PE buffer by centrifuging at 14,000 rpm for 1 min. Centrifugation of the column at 14,000 rpm for another 2 mins at room temperature was done to clean dry the column. 20 μ L of nuclease-free water was then added onto the column and incubated for 1 min before spinning down. Cleaned-up DNA products were collected for later use.

2.2.5 Q5® site directed mutagenesis

Q5® site directed mutagenesis is a way to carry out insertion, deletion or substitution without using overlapping primers. The usage of overlapping primers in other mutagenesis methods has low successful rate in obtaining correct PCR products as it gives high chance of obtaining primer dimers. Site directed mutagenesis in the other way has primers designed in end-to-end fashion (**Figure 2-1**), which largely improves the successful rate in obtaining correctly altered constructs. PCR products for site directed mutagenesis that had been purified were treated with KLD (Kinase, Ligase and DpnI) enzyme mix in a reaction mixture of 5 μ L containing 2.5 μ L of KLD reaction buffer, 0.5 to 2 μ L (about 50 ng) of PCR product depending on concentration, 0.5 μ L of KLD enzyme mix and nuclease free water if needed. Reaction was done at room temperature for 25 mins.

Figure 2-1. Schematic diagram of Q5® site directed mutagenesis

2.2.6 Gibson assembly

For constructions involving the introduction of a large DNA fragment into a backbone, for example the insertion of GST tag into plasmid containing ZIKV NS4B cytoplasmic loop gene, Gibson assembly was used instead of ligation with restriction enzyme sites. Gibson assembly makes use of a combination of three enzymes: exonuclease, DNA polymerase and DNA ligase, to combine up to 15 DNA fragments based on their sequence similarity without requiring paired restriction enzyme sites (Gibson, Young et al. 2009, Gibson 2011). In **Figure 2-2**, the process of Gibson assembly is described. Fragments for Gibson assembly can be prepared though PCR or restriction enzyme digestion.

Figure 2-2. Process of gibson assembly

2.2.7 Transformation

Upon finishing KLD reaction or Gibson assembly of DNA fragments, it was then transformed into competent cell (Top 10) for the formation of expression plasmid. A volume of 5 μ L of KLD mixture or Gibson assembly

mixture was added into a tube of chemically competent bacterial cell, mixed and incubated on ice for 30 mins. The competent cells were then heat shocked at 42°C for 45 sec, which opens pores on competent cell surface enabling entrance of DNA. It was cooled down on ice for 5 mins for closing of the pores before addition of 200 µL of LB broth for recovering of bacterial cells at 37°C shaker for 1 hr. Cells were spun down at 4500 rpm for 30 sec and supernatant was discarded with only 50 µL left for resuspension of cell pellet. Cells were finally plated onto LB agar plate containing respective antibiotics and incubated at 37°C incubator for bacterial colony growing.

2.2.8 Plasmid miniprep (Miniprep)

A single colony was picked from LB agar plate and inoculated into 5 mL of LB broth containing respective antibiotics for growing at 37°C shaker overnight. Bacterial cells harvested by centrifugation at 14,000 rpm for 1 min were used for extraction of plasmid with Qiagen QIAprep® Miniprep kit. Briefly, cell pellet was resuspended with 250 µL of P1 buffer in an Eppendorf tube (supplemented with 100 mg/mL RNase A). This was followed by 250 µL of P2 buffer and thorough but gentle mix by inverting the tubes 4 to 6 times until appearance of a viscous and clear solution. 350 µL of N3 buffer was then added and mixed immediately by inverting the tube 4 to 6 times, by this time, the denatured proteins, cDNA (chromosomal DNA) and cellular debris were precipitated together with SDS leaving plasmid DNA in solution. Centrifugation of the mixture at 14,000 rpm for 10 mins at room temperature to separate supernatants that contains plasmid DNA from precipitates was followed with loading of the supernatants onto QIAprep 2.0 spin column. After centrifuging the column for 1 min, flow through was discarded, and the column was washed with

750 μ L of buffer PE to wash the bound plasmid DNA. QIAprep column was centrifuged at high speed for another 2 mins to remove residual PE buffer prior loading nuclease free water for elution of plasmid DNA. Plasmid DNA was collected in Eppendorf tube and concentration measured with nanodrop. All constructs were confirmed by sequencing with 1st Base Sequencing service or BioBasic-Asia Sequencing service prior to using for protein expression.

Table 2-4. List of constructs used in the study

Construct Name	Description	Schematic Representation	Vector	Remarks
Cytoloop-His	For recombinant expression of ZIKV NS4B cytoplasmic loop with GST and His ₆		pNIC28-Bsa4	Cloned
GST-Cytoloop	For recombinant expression of ZIKV NS4B cytoplasmic loop with GST at N-terminus		pNIC28-Bsa4	Cloned
scFv 513	For recombinant expression of antibody scFv 513		pET26b	From JL lab
scFv 513-NAL	For recombinant expression of scFV513 with NAL at C-terminus		pET26b	Cloned
scFv 513-NGL	For recombinant expression of scFV513 with NGL at C-terminus		pET26b	Cloned
scFv 513-NHL	For recombinant expression of scFV513 with NHL at C-terminus		pET26b	Cloned
scFv 9F12	For recombinant expression of antibody scFv 9F12		pET 28	From JL lab

ZIKV E	For recombinant expression of ZIKV E protein		pFastBac / pET28b	Bio-Basic Asia
ZIKV EdIII	For recombinant expression of ZIKV E protein domain III		pET 22b	Cloned
ZIKV NS3	For recombinant expression of ZIKV NS3 full-length protein		pNIC28-Bsa4	Gifted from Dr. Luo Dahai
ZIKV NS3hel	For recombinant expression of ZIKV NS3 helicase protein		pNIC28-Bsa4	Cloned
ZIKV NS3hel sbd1 (vc003)	For recombinant expression of ZIKV NS3 helicase subdomain 1 protein		pNIC28-Bsa4	Provided by PPP
ZIKV NS3hel sbd2 (vc005)	For recombinant expression of ZIKV NS3 helicase subdomain 2 protein		pNIC28-Bsa4	Provided by PPP
ZIKV NS3hel sbd3 (vc008)	For recombinant expression of ZIKV NS3 helicase subdomain 3 protein		pNIC28-Bsa4	Provided by PPP
ZIKV NS3hel-NAL	For recombinant expression of ZIKV NS3 helicase protein with NAL at C-terminus		pNIC28-Bsa4	Cloned

ZIKV NS4B	For recombinant expression of ZIKV NS4B protein until transmembrane 4 region		pFastBac	From JL lab
ZIKV NS4B-Strep	For recombinant expression of ZIKV NS4B full-length protein with a twin-strep tag at C-terminus		pFastBac	Cloned

Table 2-5. List of primers used for PCR

Construct	Template	Forward Primer Sequence	Reverse Primer Sequence	Cloning Method
Cytoloop-His	GST-Cytoloop	5' - CAC CAC CAT TAA GGA TCC GAA TTC GAG C – 3'	5' – GTG GTG GTG CAG AGC GTT CAG CAG AAC -3'	Insertion
	Inserted GST-Cytoloop	5' – GAG AAC CTG TAC TTC CAA G -3'	5' - CAT ATG CCC CTG GAA CAG -3'	Deletion
GST-Cytoloop	GST Construct	5' - TTT AAG AAG GAG ATA TAC ATA TGA GCG ATA AAA TTA TTC ACT C - 3'	5' - CGA GCA GCA GCA GCC TGC AGA CCT TGG AAG TAC AGG TTC TC -3'	Gibson Assembly
	Cytoloop	5' – GGT CTG CAG GCT GCT G -3'	5' – ATG TAT ATC TCC TTC TTA AAG TTA AAC AAA ATT ATT TC -3'	
scFv 513 - NAL	scFv 513-NGL	5' – CCA CCA CAA TGC GCT GTG AGA TCC G -3'	5' – TGG TGG TGG TGC TCG AGC -3'	Mutation

scFv 513 - NGL	scFv 513	5' – GCT GTG AGA TCC GGC TGC TAA C -3'	5' – CCA TTG TGG TGG TGG TGG TGG TG -3'	Insertion
scFv 513 - NHL	scFv 513 - NGL	5' – CCA CCA CAA TCA CCT GTG AGA TCC GGC TG -3'	5' – TGG TGG TGG TGC TCG AGC -3'	Mutation
ZIKV NS3hel	ZIKV NS3	5'- GAG GAA GAA ACC CCG TT -3'	5' – CAT GGA TTG GAA GTA CAG G -3'	Deletion
ZIKV NS3hel-NAL	ZIKV NS3hel-NGL	5' – CGT GGT AAT GCG CTG TAA CAG TAA AGG TG -3'	5' – CTT GCC CGC CGC AAA CTC -3'	Mutation
ZIKV NS3hel-NGL	ZIKV NS3hel	5' – GCT GTA ACA GTA AAG GTG GAT ACG GAT CCG AAT TCG AGC -3'	5' – CCA TTA CCA CGC TTG CCC GCC GC -3'	Insertion
ZIKV NS4B-Strep	ZIKV NS4B	<p>1. NS4B Strep I F: 5'- AAG GGA GGT GGT TCC GGA GGA TAA CAG TAA AGG TGG ATA CGG ATC CGA ATT CGA GCT CC -3'</p> <p>2. NS4B Strep II F: 5'- CCT GGT CGC ATC CAC AGT TTG AAA AAT AAC AGT AAA GGT GGA TAC GGA TC -3'</p>	<p>1. NS4B Strep I R: 5'- TTC AAA TTG TGG ATG GGA CCA CGC GCC CGC TTC GCC CCA -3'</p> <p>2. NS4B Strep II R: 5'- CGC TGC CAC CGG ATC CTC CTC CGG AAC CAC CTC C -3'</p>	Insertion

2.3 Protein expression

To study the properties of viral proteins, a large quantity of proteins was needed. Recombinant protein expression allows to produce specific proteins with high yield and free of virus amplification. For protein expression, a few expression systems exist including bacterial expression, insect cell expression, mammalian cell expression and yeast expression. Bacterial expression is the most commonly used system in structural studies, antibody generations and so on. It is often chosen as it is easy to scale up protein production in bacterial expression system and it is economically efficient. Moreover, the timeline for protein expression with bacterial expression system is relative short as the doubling time for bacteria is generally only 15 mins to 30 mins. However, for some proteins with complex structures such as transmembrane proteins where integration with membrane is needed for proper protein folding, bacterial expression system might not be a good choice.

Another common protein expression system is using insect cells such as Sf9 cell. One obvious advantage of insect cell expression system is the similarity to mammalian protein processing including post-translational modifications such as glycosylation and membrane integration for transmembrane proteins. Bac-to-Bac® Baculovirus Expression System is an insect-cell based expression system that makes use of amplification of Baculovirus in insect cell (Hitchman, Possee et al. 2009). This expression system involves the generation of recombinant Baculovirus that contains the gene of interest (**Figure 2-3**). In **Figure 2-3** protein expression using Baculovirus expression system has been summarized (Hitchman, Possee et al. 2009, Chambers, Aksular et al. 2018). Many

investigations have been using this expression system for their production of recombinant proteins especially for the studies on viral proteins.

Figure 2-3. Diagram of the Bac-to-Bac® Baculovirus Expression System

Modified and reproduced from Bac-to-Bac® Baculovirus Expression System. Invitrogen Life Technologies, 2015.

Constructs with pFastBac as vector were expressed in Bac-to-Bac® Baculovirus Expression System with insect sf9 cells, while other constructs were expressed with *E. coli* expression system.

2.3.1 Protein expression in Baculovirus expression system

2.3.1.1 Cell culture

Cell line sf9 was maintained and passaged as suspension culture using Sf-900™ II SFM medium supplemented with 1% of FBS (Fetal Bovine Serum). Sf9 cells were growing in flasks with ventilation filter cap in non-humidified shaker at 28°C at 150 rpm. For sf9 cells, CO₂ exchange is not required unlike mammalian cells. The doubling time for sf9 cells is around 24 hours, they are passaged by diluting with pre-warmed growth medium (28°C) to cell density of 0.5 x 10⁶ cells/mL whenever the cell density reaches 6 x 10⁶ cells/mL. Cell line used for protein expression was maintained for not longer than 3 months for maintaining cell viability.

2.3.1.2 Transposition into Bacmid

To produce Bac-to-Bac® Baculovirus for desired constructs, the construct in pFastBac vector, for example ZIKV NS4B-Strep, must be transformed into bacmid formation competent cell for transposition of gene of interest into bacmid. DH10EMBacY (A gift from Prof. Daniela Rhodes's lab) was transformed with 1 ng of pFastBac plasmid by heat shocking at 42°C for 45 sec, it was recovered with 900 µL of LB broth by shaking at 37°C for 6 hours after transformation. The transformation would then be centrifuged at 5,000 rpm for 3 mins at room temperature to pellet down the cells and it was plated onto LB agar plate containing 50 µg/mL kanamycin, 7 µg/mL gentamicin, 10 µg/mL tetracycline,

40 µg/mL X-Gal and 40 µg/mL IPTG for blue white selection. Colonies were allowed to grow at 37°C incubator for 48 hours, allowing the expression of LacZ proteins for the blue white color display. White colonies were picked and streaked on to fresh LB agar plate to verify the phenotype by incubating at 37°C for 48 hours.

White colonies were checked with colony PCR using M13 forward (5' – CCC AGT CAC GAC GTT GTA AAA CG -3') and reverse (5' – AGC GGA TAA CAA TTT CAC ACA GG -3') primers to confirm transposed bacmid. As shown below in **Figure 2-4**, the size of colony PCR product depends on the size of gene of interest. For colonies with successful transposition, the size of PCR product is the size of gene insertion plus 2300 bp.

Figure 2-4. Schematic diagram of transposed Bacmid DNA

2.3.1.3 Isolation of recombinant Bacmid DNA

White colonies confirmed by colony PCR were inoculated into 5 mL of LB supplemented with 50 µg/mL kanamycin, 7 µg/mL gentamicin, 10 µg/mL tetracycline, grown at 37°C shaker for 18 hours. It was centrifuged at 14,000 rpm

for 1 min to collect the pellet. Similar to Miniprep described earlier, pellet was dissolved with P1, P2 and N3 buffers sequentially and centrifuged at 14,000 rpm for 10 mins to get clear supernatant containing bacmid DNA. Unlike Miniprep, bacmid DNA was precipitated by gently mixing 1 volume of isopropanol with supernatant and incubating on ice for 10 mins. The mixture was centrifuged at 14,000 rpm for 15 mins to collect bacmid DNA. Supernatant was discarded and 500 μ L of 70% ethanol was added to wash bacmid DNA precipitate. Final centrifugation at 14,000 rpm for 5 mins to remove ethanol and air dry at room temperature for 5 mins were done before dissolving bacmid DNA in 50 μ L of nuclease free water. Bacmid DNA was stored at -20°C.

2.3.1.4 Production of recombinant Baculovirus

Transfection of sf9 cell with bacmid DNA was done following manufacture's manual for Cellfectin™ II reagent. Briefly, 1 million cells per well were plated in 6-well cell culture plate and stored in 28°C incubator for 15 mins for cells to adhere to plate bottom. Cellfectin™ mixture was prepared by diluting 8 μ L of Cellfectin™ II with 100 μ L of Grace's medium (un-supplemented), this mixture can be kept at room temperature for up to 30 mins. In the meantime, bacmid DNAs (1 or 2 μ g) were diluted respectively with 100 μ L of Grace's medium gently. Diluted Cellfectin™ was mixed with diluted bacmid DNA and incubated at room temperature for 25 mins while cells were washed with 2 mL of Grace's medium (un-supplemented) for twice. Each well was then filled with 2 mL of Grace's medium. Transfection mixture (210 μ L) was added into each well dropwise with gentle twirling of the plate. Cells with transfection mixture were incubated in 28°C incubator for 6 hours before replacing the transfection mixture with 3 mL of Sf-900™ II SFM medium supplemented with 1% of FBS.

Cells were left to grow for 72 hours post-transfection for production of recombinant Baculovirus. Successful viral infection was confirmed by checking with fluorescence microscopy as YFP (yellow fluorescence protein) exists in bacmid DNA isolated from DH10EMBacY competent cell. In addition, cells would display other phenotypes indicating viral infection involving cessation of cell growth, granular appearance and detachment.

At this stage, P1 viral stock can be isolated from transfected well by collecting the medium containing virus and spinning it at 500 g for 5 mins to clarify cell debris. Supernatant containing P1 viral stock can be stored in dark at 4°C till further use. The theoretical viral titer for P1 virus was regarded as 5×10^6 pfu/mL. The viral titer was increased by amplifying viral stock to P3. To amplify P2 viral stock, suspension sf9 cells with density of 2×10^6 cells/mL with desired volume were infected with P1 viral stock at MOI (multiplicity of infection) of 0.1 for 72 hours. Harvested P2 viral stock was then used for amplifying P3 viral stock through the same procedure. Viral stock was limited at P3 as deleterious mutations might be acquired with each viral passage. P3 viral stock with a viral titer around 5×10^8 pfu/mL and was used to infect sf9 cells for protein expression.

2.3.1.5 Protein expression

Viral stock at P3 was used to infect sf9 cells at cell density of 2.5×10^6 cells/mL. MOI for infection was set at 1 as optimal for protein expression. Cell pellet containing expressed protein was harvested by centrifuging at 1000 g for 15 mins at 4°C. Cell pellets were stored at -20°C till protein purification.

2.3.2 Protein expression in bacterial expression system

Chemically competent BL21(DE3)-T1R cell was used as the bacterial strain for protein expression. Constructs were transformed into BL21(DE3)-T1R as described in section 2.2.7 and were plate onto LB agar plate with respective antibiotics for growing of single colonies, which were picked for inoculation in LB medium supplemented with antibiotics as starter culture. On the following day, starter culture was diluted 100 times into 1 L of LB medium for culturing at 37°C shaker till OD₆₀₀ reached 0.6 to 0.9. Bacterial culture was cooled down to 4°C prior to addition of 0.5 mM IPTG for induction of protein expression at 16°C shaker overnight. Cell pellets were harvested by centrifugation at 4,000 rpm for 15 mins at 4°C. Cell pellets were stored at -20°C until be used for protein purification.

2.4 Protein purification

In this section, three purification protocols will be described. First protocol is for purifying soluble proteins, second protocol is for purification of transmembrane protein ZIKV NS4B-Strep and third protocol is for purification of insoluble proteins.

2.4.1 Purification of soluble protein

To purify soluble proteins, a three-step purification protocol has been adopted including IMAC (Immobilized Metal Affinity Chromatography), reverse His-Trap and SEC (Size Exclusion Chromatography).

2.4.1.1 IMAC

Cell pellet was thawed and resuspended with lysis buffer (50 mM Na HEPES, pH 7.4, 500 mM NaCl, 1 mM DTT (Dithiothreitol) and 5% Glycerol) in addition of protease inhibitor to prevent degradation of proteins. For each 1 g of

cell pellet, 10 mL of lysis buffer was used for resuspension. Cells were lysed by sonication at 70% amp for 1 min/10mL with 3 sec on/off or by LM20 microfluidizer with 22,000 psi pressure for two rounds. Lysed samples were clarified by centrifugation at 20,000 rpm for 45 mins at 4°C. Supernatant containing all soluble proteins was collected and incubated with Ni-NTA beads for 3 hours with agitation at 4°C. Flow through was collected with purification column by gravity flow, followed by washing the beads with washing buffer (50 mM Na HEPES, pH 7.4, 500 mM NaCl, 1 mM DTT, 5% Glycerol and 10/20/50 mM Imidazole) sequentially. For each washing buffer, the volume used was 10 times the volume of Ni-NTA beads. Proteins with histidine-tag bound to Ni-NTA beads were eluted with elution buffer (50 mM HEPES, pH 7.4, 500 mM NaCl, 1 mM DTT, 5% Glycerol and 300 mM Imidazole) for 2 x beads volume each eluate for 6 eluates. Samples at each stage were checked by SDS-PAGE.

2.4.1.2 Protease cleavage and reverse His-Trap

Eluates containing target protein were pooled to incubate with TEV protease accompanied with dialysis against lysis buffer overnight at 4°C. By doing so, His₆ tag was cleaved off from target protein. The mixture of His₆ tag, TEV protease and target protein was then incubated with Ni-NTA beads with agitation at 4°C for 3 hours. Similar to IMAC, flow-through was collected as well as a wash with 10 mM Imidazole washing buffer. Since both TEV protease and His₆ tag were bound to Ni-NTA beads, they were eluted with elution buffer while target protein was in the flow-through. Sometime, trace of target protein was found in washing step due to nonspecific binding affinity towards Ni-NTA beads.

2.4.1.3 Size Exclusion Chromatography (SEC)

Upon this step, the target protein was already quite homogeneous except some minor contaminants. To get protein with purity above 95%, flow through collected from reverse his-trap was concentrated with protein concentrator of correct MWCO (Molecular Weight Cut Off) to a volume for injection for SEC, around 4 mL. MWCO was chosen according to the size of target protein, generally, MWCO should be less than half of the size of target protein. Through SEC, protein will be eluted at different volumes according to size in gel filtration buffer (20 mM Na HEPES pH 7.4, 150 mM NaCl, 1 mM DTT and 5% glycerol), the formation of oligomers can be differentiated as well. Purified protein was concentrated and snap frozen with liquid nitrogen. Protein were stored at -80°C till further use.

2.4.2 Purification of transmembrane protein

Transmembrane protein ZIKV NS4B in my study was purified according to the recent reported protocol that maintains membrane protein in their local lipid environment (Lee, Knowles et al. 2016). For this purification, styrene maleic acid (SMA) co-polymer (2:1) was used to form SMA lipid particles (SMALPs) with ZIKV NS4B protein. ZIKV NS4B cell pellet was thawed and resuspended in NS4B lysis buffer (50 mM Tris-HCl pH 8.0, 300 mM NaCl and 10% Glycerol) supplemented with protease inhibitor. It was lysed by sonication at 70% amp for 1 min per 10 mL with 5 s ON/OFF. Lysate was clarified by centrifuging at 8000 g for 15 mins at 4°C. Supernatant was collected and further centrifuged at 100,000 g for 45 mins at 4°C using ultra centrifuge machine. Membrane pellet brought down was collected and the weight was measured. Membrane pellet was broken with Sartorius™ Borosilicate Glass Homogenizer in NS4B lysis buffer (around 10 mL per 0.1 g of membrane pellet). Per 0.1 g of

SMA (2:1) was added for 10 mL of lysate and left to rotate at room temperature to form SMALPs with NS4B protein for 1 hr to 2 hr till the mixture became clear. Solution was then centrifuged at 100,000 g for 1 hr at 4°C to collect supernatant that contains the NS4B SMALPs.

Protein was purified with Strep Tactin beads where 6 eluates were collected. Unbound protein was then incubated with Ni-NTA beads for IMAC as the protein contains a His₆ tag as well. Both eluates from Strep Tactin purification and IMAC were combined and buffer exchanged to NS4B lysis buffer. It was concentrated with protein concentrator (10 kDa MWCO) and the concentration was checked with BCA assay kit. According to protocol published in Nature, purified protein in SMALPs can be kept at 4°C and is stable for months' time (Lee, Knowles et al. 2016).

2.4.3 Purification and refolding of insoluble proteins

ScFv is a portion of mAb that contains the entire antigen-binding region from heavy and light chains but without the constant regions. scFv has been proved to have similar binding affinity as compared to its parent antibody. In my studies for the immune complex structures, scFv of the antibody will mostly be used for the formation of immune complex. In Figure 1-11, the difference between different forms of antibody is shown. scFv can be produced in two forms (**Figure 2-5**), with the location of V_L and V_H reversed. In both forms of scFv, the V_L and V_H are connected with a short linker that is commonly high in glycine for flexibility, as well as high in serine and threonine for solubility (Schirrmann 2005). Bacterial expression system using *E. coli* often produces scFv as inclusion bodies where refolding is needed to get soluble scFvs. Whereas, *Drosophila* S2

cells can also be used for scFv expression, where the protein will be secreted out as a soluble protein (Gilmartin, Lamp et al. 2012).

Figure 2-5. Schematic information of mAb, Fab, Fc and scFv

A. A whole structure of mAb (IgG); **B.** Fab and Fc yielded when mAb is digested with papain; **C.** Two forms of scFv.

Insoluble protein including ZIKV EdIII and scFv proteins were purified by refolding from inclusion body with serial dialysis. The process is described as detailed below. Cell pellet was collected and resuspended in lysis buffer with protease inhibitor. It was then lysed by sonication and clarified by centrifugation at 20,000 rpm for 45 mins at 4°C. As the proteins were not soluble, pellet from centrifugation was collected to recover inclusion body.

To refold proteins, inclusion body was firstly prepared from insoluble pellet by washing it once with pellet washing buffer A (50 mM Tris-HCl pH 7.4/8.8, 1 mM EDTA, 1 M NaCl, 1% Triton X-100 and 2 M urea) and twice with pellet washing buffer B (50 mM Tris-HCl pH 7.4/8.8, 1 mM EDTA, 1 M NaCl and 2 M urea) to remove partial soluble protein contaminants. Inclusion body was then re-solubilized by incubating with solubilizing buffer (50 mM Tris-HCl pH 7.4/8.8, 1 mM EDTA, 150 mM NaCl, 50 mM glycine, 5 mM imidazole and 8 M urea) for overnight at 4°C with agitation. Re-solubilized proteins were then refolded following a modified step-wise dialysis protocol (ShengFeng, Ping et al.

2003, Chen, Huang et al. 2006, Arakawa and Ejima 2014, Yuasa, Koyama et al. 2014). Briefly, five rounds of dialysis were carried out against four dialysis buffer conditions to lower the urea concentration step-wisely to less than 0.5 mM for further experiments. For each dialysis step, the volume of dialysis buffer used was 10 times the volume of protein sample. The contents of dialysis buffers are dialysis buffer 1 (50 mM Tris-HCl pH 7.4/8.8, 0.5 M L-Arginine, 150 mM NaCl, 1 M urea, 0.25 mM GSSG and 2.5 mM GSH), dialysis buffer 2 (50 mM Tris-HCl pH 7.4/8.8, 0.5 M L-Arginine, 150 mM NaCl, 0.125 mM GSSG and 1.25 mM GSH), dialysis buffer 3 (20 mM Tris-HCl pH 7.4/8.8, 0.2 M L-Arginine, 150 mM NaCl, 0.125 mM GSSG and 1.25 mM GSH) and dialysis buffer 4 (20 mM Tris-HCl pH 7.4/8.8 and 150 mM NaCl). Protein sample was dialyzed twice against dialysis buffer 4 condition.

Upon refolding by dialysis, refolded proteins were concentrated and loaded for SEC. All fractions containing pure protein were pooled together accordingly and concentrated to a final concentration of 2 mg/mL with batch variations for storage and further usage. The concentration was determined by measuring OD280 with nanodrop and was calculated based on corresponding molecular weight and extinction coefficient.

2.5 SDS-PAGE electrophoresis

SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) is used for separation of charged molecules in a mixture by their molecular weight. For protein purification, samples from each purification were checked with SDS-PAGE to for sample purity and protein size. Tris-glycine SDS-PAGE gels were prepared according to previously established recipe (Harlow and Lane

1988, Mus-Veteau 2010). Proper percentage of SDS-PAGE gel was chosen according to molecular weight of sample loaded. Range of gel percentage goes from 10% to 15%. Electrophoresis was done in gel tank with 1x SDS-PAGE running buffer, it was run at constant voltage till the protein markers were separated as wanted. SDS-PAGE gel was then stained with staining solutions including coomassie blue staining solution, silver staining solution and so on. It may also be transferred for immunoblotting or be directly scanned for fluorescence images.

2.6 Immunoblotting

Immunoblotting is the approach to detect protein with antibodies. For immunoblotting in general, protein on SDS-PAGE gel was transferred to Immun-Blot® PVDF membrane (Bio-Rad), which was activated with 100% ethanol prior transfer. Transfer was done with Trans-Blot Turbo Transfer System (Bio-Rad) for 20 min at 25 v with 1x wet transfer buffer. Membrane was blocked with 5% bovine serum albumin (BSA) solution after transfer for 30 min at room temperature with gentle shaking to prevent unspecific binding of antibodies. Primary antibody was then incubated with membrane for 1 hour followed by three washes with 1x TBST buffer. Incubation with secondary antibody was done for 1 hour followed by three washes with 1x TBST buffer. Clarity™ ECL Western Blotting Substrates (Bio-Rad) was then added on to membrane with gentle pipetting over the membrane and brief incubation. Immunoblotting image was taken with ChemiDoc Imaging System (Bio-Rad). For immunoblotting with scFv 513 and scFv 9F12, both proteins were used as primary antibody and all the other steps remained the same.

2.7 Isothermal Titration Calorimetry

Isothermal titration calorimetry (ITC) assay is a biophysical assay for measuring protein-protein, protein-ligand or ligand-ligand interaction. The amount of energy needed for bringing temperature of experiment cell to the temperature of reference cell is recorded at each injection of ligand or protein to its binding partner. To carry out ITC, both samples were prepared in same buffer with no presence of DTT and percentage of glycerol less than 5%. Sample for injection syringe was prepared at concentration of 10 times the concentration of sample in cell. Sample for cell was loaded into experiment cell at 200 μL , the volume of sample loaded into syringe was at 70 μL .

MicroCal PEAQ-ITC of Malvern was used to performing ITC assay for checking the binding affinity between ZIKV EdIII and scFv 9F12 or scFv 513. All the samples were dialyzed to same buffer condition in ITC assay buffer (20 mM Tris-HCl pH 7.4 and 150 mM NaCl) before loading into MicroCal PEAQ-ITC. As ZIKV EdIII with lower molecular weight is easier to be concentrated to high molar concentration, it was prepared to concentration of 100 μM as the titrate to be loaded in syringe for the injection. While for scFv 9F12 and scFv 513, the proteins were concentrated to 10 μM as the receptor and were loaded in cell. The titrate in syringe was injected into cell 20 times with 2 μL of injection volume and was mixed with receptor with 1000 rpm. For reference, ITC assay buffer was loaded and tested with ZIKV EdIII. ITC was also performed for checking binding affinity between ZIKV NS3 helicase and ZIKV NS4B cytoplasmic loop peptide and peptide P16. Amount of proteins used will be mentioned in result section.

2.8 Co-crystallization

For co-crystallization, two proteins (Fab ZAb_FLEP and ZIKV E protein) were mixed together for formation of complex and then concentrated to 8 mg/mL. Crystallization screening was set up with sitting drops and hanging drop with conditions listed in **Table 2-6** below.

Table 2-6. Buffer conditions used for Co-crystallization screening

3.5 M Na Formate 0.1 M Tris pH8.5	0.1 M Tris pH 8 15% PEG 8000 0.2 M Li ₂ SO ₄	0.1 M Tris pH 8 18% PEG 8000 0.2 M Li ₂ SO ₄	0.1 M Tris pH 8 20% PEG 8000 0.2 M Li ₂ SO ₄
	0.1 M Tris pH 8.5 15% PEG 8000 0.2 M Li ₂ SO ₄	0.1 M Tris pH 8.5 18% PEG 8000 0.2 M Li ₂ SO ₄	0.1 M Tris pH 8.5 20% PEG 8000 0.2 M Li ₂ SO ₄
	0.1 M Tris pH 8.6 15% PEG 8000 0.2 M Li ₂ SO ₄	0.1 M Tris pH 8.6 18% PEG 8000 0.2 M Li ₂ SO ₄	0.1 M Tris pH 8.6 20% PEG 8000 0.2 M Li ₂ SO ₄
	0.1 M Tris pH 8.8 15% PEG 8000 0.2 M Li ₂ SO ₄	0.1 M Tris pH 8.8 18% PEG 8000 0.2 M Li ₂ SO ₄	0.1 M Tris pH 8.8 20% PEG 8000 0.2 M Li ₂ SO ₄

Protein sample was added into a drop of reservoir buffer with three ratios 2:1, 1:1 and 1:2 respectively with a final volume of 3 μ L. For hanging drop approach, cover slips with crystallization mixture were then sealed onto wells containing 750 μ L of same reservoir buffer with high vacuum grease. The use of grease was to protect reservoir buffer and crystallization drops from drying up. For sitting drops, crystallization mixture was prepared in the same fashion but in wells on plate not on cover slips. Plastic seals was used to seal plates with reservoir buffer and corresponding crystallization mixtures. Crystallization plates were incubated at 20°C for crystal growing. The plates were checked regularly with stereo microscope for crystal hits.

Figure 2-6. Schematic representation of Fab preparation by papain digestion and fragmentation

2.9 Cryo-EM

To carry out Cryo-EM for immune complex formed between ZIKV and Fab ZAb_FLEP. All the samples have to be prepared in good quality as well as the Cryo-EM grids. In this section, I will describe the materials and methods used for a Cryo-EM experiment.

2.9.1 Enzymatic digestion of IgG and purification of Fab fragment

To obtain Fab from IgG, papain was used for the digestion, papain digest whole IgG above its Fc region to separate Fab fragments from Fc region of heavy

chain (**Figure 2-6**). For papain digestion, papain slurry was firstly activated with 1M L-Cysteine, for 2.1 μ L of papain slurry, 2.2 μ L of fresh prepared L-cysteine was used with 302 μ L of Tris buffer (0.1 M Tris-HCl pH 8, 2 mM EDTA) for the activation at 37°C for 30 mins. The final concentration of L-Cysteine for papain activation is 10 mM Upon activation, papain solution became clear from the original white slurry, it was then used to incubate with IgG sample for digestion reaction. IgG sample was supplemented with L-Cysteine to a final concentration of 10 mM as well prior mix with activated papain. Typically, around 10 mg of mAb ZAb_FLEP was diluted with Tris buffer to final volume of 500 μ L, which was then supplemented with 5 μ L of 1M L-Cysteine. The presence of L-Cysteine as a reducing agent is to maintain a thiol group in the active site of papain. Next, all mAb sample was mixed with 1/50 volume of activated papain, for 500 μ L of mAb ZAb_FLEP, 10 μ L of activated papain was added. The digestion was performed by incubation at 37°C for 6-7 hours. Digestion was checked with SDS-PAGE before it was stopped by addition of iodoacetamide with final concentration of 20 mM and left incubate at 37°C for 30 mins. Fab fragments were then separated from Fc region with Pierce Protein A Agarose (Thermo Fisher Scientific). 1 mL column volume of protein A agarose was prepared and equilibrated before loading of digested sample into it. Digestion sample was diluted 1:1 with Tris buffer and loaded onto protein A agarose in a column. Flow-through was collected containing Fab fragments as Fc was bound onto protein A agarose. 3 washes was done with 2 times column volume of Tris buffer and collected respectively. Fc domain was eluted with 5 mL of 1x elution buffer (Thermo Fisher Scientific) and collected 1 mL fractions for 5 fractions. For each 1 mL of elution, 100 μ L of Tris-HCl pH 9 was added to neutralize pH. All

samples were checked on SDS-PAGE to decide what the washes were to be collected. Collected Fab samples were concentrated and loaded for SEC with Superdex 75 10/300 GL (GE Healthcare) to change storage buffer to 1x PBS. Final product of Fab ZAb_FLEP was concentrated to 3.5 mg/mL and stored at -80°C.

2.9.2 Viral particle preparation

The biosafety measures were followed according to our collaborators (Prof. Ooi Eng Eong) at Duke-NUS SOP for the preparation of viral particles. Cell line *Aedes albopictus* C6/36 was used for amplification of ZIKA virus (ZIKV). The precipitation and purification was done following previously published virion preparation protocol (Tan and Lok 2014). Briefly, virus was harvested from infected C6/36 cells 4 days post-infection by centrifuging virus-containing media at 9,000 g for 45 min. Virus was then precipitated with addition of 8% (w/v) polyethylene glycol (PEG) 8,000 in NTE buffer (10 mM Tris-HCl pH 8.0, 120 mM NaCl and 1 mM EDTA). Virus precipitate was pelleted by centrifugation at 14,000 g for 1 hr after overnight incubation. Pellet containing virus was then purified by 24% (w/v) sucrose cushion and 10-30% w/v potassium tartrate gradient. The virus band was visualized with a torch light in the linear potassium tartrate gradient and extracted with syringe. It was then concentrated, and buffer exchanged to NTE buffer with 100 kDa molecular weight cut-off filter. The yield and purity of viral E protein were checked with SDS-PAGE using BSA standard. Special thanks to Dr. Hwee Cheng Tan (Duke-NUS) for her help in preparing pure ZIKV viral particles.

2.9.3 Preparation of ZIKV-Fab ZAb_FLEP Complex

A molar ratio of 1:2 between ZIKV E protein and Fab ZAb_FLEP was used for the preparation of ZIKV-Fab ZAb_FLEP. The concentration of E protein was measured to be 0.25 mg/mL from pure ZIKV viral particle while the concentration of Fab ZAb_FLEP was 3.5 mg/mL. A volume of 10 μ L of ZIKV was mixed with 1.4 μ L of Fab ZAb_FLEP for the formation of complex by incubating at 37°C for 30 mins. The mixture was then kept on ice for 1 hour before it was applied to grids for negative staining and Cryo-EM.

2.9.4 Negative staining, Cryo-EM grids preparation and data collection

For negative staining grids, 1 μ L of complex sample was diluted with 3 μ L of 1x PBS buffer, then 3.5 μ L of diluted sample was applied to carbon-coated grid that was prepared with Carbon Coater. The sample was allowed to incubate on grid for 1 min at room temperature followed by 10 seconds blotting with filter paper. 2% UA (Uranyl Acetate) was used to stain the grid for twice with 10 seconds blotting each time. Micrographs for negative staining were collected with T12 Spirit electron microscope (FEI). Negative staining is an easy and rapid technique for checking the quality of TEM (Transmission Electron Microscopy) sample and for determining the concentration of TEM sample (Bozzola and Russell 1999, Laue 2010).

After obtaining the micrographs from negative staining, we decided to use 2.5 μ L of complex sample for the preparation of Cryo-EM grids. Four grids were prepared with amount of sample and blotting time listed below in **Table 2-4**. For Cryo-EM grids preparation, glow-discharged 2 nm carbon coated holey grid (R1/2, Quantifoil GmbH, Germany) was used. Sample on grids was allowed to incubate for 30 seconds before the grid was blotted using Vitrobot (FEI). The

grids were then immediately frozen in liquid N₂-cooled liquid ethane. 4 grids were loaded to a GEI Tecnai Arctica cryo-transmission electron microscope for screening of grids, where only a few micrographs were taken to evaluate the quality of grids prepared. Out of 4 grids as listed in **Table 2-7**, grids No.1 and No.4 were chosen for Cryo-EM data collection.

The chosen Cryo-EM grids were stored in liquid N₂ till it was used for data collection. Cryo-EM data was collected with Arctica cryo-transmission electron microscope that was operated at 200 kV and equipped with a back-thinned Falcon III direct electron detection device. Special thanks to Prof. Shashi Bhushan (NTU-SBS-NISB) for his help in Cryo-EM data collection.

Table 2-7. Conditions for Cryo-EM grids preparation

Grid Number	Sample Amount (μL)	Blotting Time (Sec)	Quality
No.1	2.5	2	Good
No.2	2.5	2.5	Bad
No.3	2.5	2.5	Bad
No.4	1.5	2	Good

2.9.5 Image processing and structure determination of the ZIKV-Fab ZAb_FLEP complex

Particles were picked from 287 selected micrographs with EMAN2 (Bell, Chen et al. 2016), followed by data processing with RELION 1.4 (Scheres 2012). 4,256 particles in total were subjected to reference-free 2D classification to discard bad particles. After 2D classifications, 1,482 selected particles were used for 3D classifications using a 60 Å filtered ZIKV EM map (EMDB: EMD-8116)

(Sirohi, Chen et al. 2016). After 3D refinement with icosahedral symmetry, 925 particles were selected and an EM density map at 9.7 Å resolution was solved.

Chimera was used to fit the 3.8 Å resolution Cryo-EM structure of ZIKV (EMDB: EMD-8116) into our EM density map. The asymmetric unit structure of ZIKV (PDB: 5IRE) on the three E protein molecules was used to form ½ of the raft that comprises 3 adjacent E monomers. The remaining 59 copies in the symmetry-related orientations were generated using the 5-3-2 symmetry after aligning the three chains in EM density map. Fv (variable fragment) of ZAb_FLEP was then docked onto each of 120 sites after the viral assembly structure has been generated. 120 sites were determined due to the lack of Fab density at 5-fold axes as observed in cryo-EM density map. The orientation of Fv in relative to Fab density was assessed in Chimera.

2.10 Inter-molecular and intra-molecular ligation with *OaAEP*

Protein post-translational modification is an interesting approach for synthesis of highly complex peptides, protein and protein conjugates (Schmidt, Toplak et al. 2017). Throughout the past decades, much research has been done to have efficient, selective and broadly applicable techniques that can be used for ligation of peptides and proteins. Major breakthroughs include the discovery of sortases, trypsin related enzymes, subtilisin-derived variants and the discovery of a group of asparaginyl endopeptidases (AEPs) including *OaAEP* and Butelase-1 (Harris, Durek et al. 2015, Nguyen, Qiu et al. 2016, Schmidt, Toplak et al. 2017). The advantages of AEPs are that they can be recombinantly expressed. AEPs are also more efficient in inter- and intra-molecular ligation as compared to other enzyme-mediated ligation techniques like sortases. Reported in 2017, with the

help of structural information, peptide ligase can be engineered also to have improved activity (Yang, Wong et al. 2017).

Many applications of peptide ligase have been introduced recently including engineering of live bacterial cell surface (Bi, Yin et al. 2017). There is still potential for applications of peptide ligase in simplifying various research techniques.

For inter-molecular ligation, such as ligation of fluorescein peptide to ZIKV NS3hel-NAL, 1/25 weight of *OaAEP* was incubated with ZIKV NS3hel-NAL and fluorescein peptide (1.5 times molar amount of fluorescein peptide as compared to ZIKV NS3hel-NAL). The ligation reaction was left at 4°C for over 72 hours with gentle agitation and protection from light. It was then purified with IMAC to have pure ligated product.

For intra-molecular ligation such as the cyclization of ZIKV NS4B cytoplasmic loop peptide, 1/25 weight of *OaAEP* was added into ZIKV NS4B cytoplasmic loop peptide sample and left at 4°C for overnight for cyclization.

The product of inter-ligation was checked with SDS-PAGE. For ligation of fluorescein peptide, the product was also checked with Typhoon scan for the presence of fluorescence signal on ligated product. For cyclization, the cyclized product was checked and confirmed with MALDI-TOF (Matrix-assisted Laser Desorption/Ionization–Time of Flight) and SDS-PAGE.

2.11 MALDI-TOF mass spectrometry (MALDI-TOF MS)

MALDI-TOF MS was carried out following the method generally used for monitoring peptide cyclization in peptide ligase studies (Nguyen, Qiu et al. 2016). In brief, 0.5 µL of sample was mixed with 0.5 µL of MALDI matrix (10

mg/mL solution of α -cyano-4-hydroxycinnamic acid in 60% ACD, 0.05% TFA (vol/vol)). 05 μ L of the mixture was then spotted onto a target plate. ABI 4800 MALDI-TOF/TOF system (Applied Biosystems) was used to perform MS. The Raw Spectrum Filtering/Peak Detection was set according to the molecular weight of sample to be analysed. Special thanks to my colleague Hu Side for his help in teaching me on performing MALDI-TOF MS.

2.12 Direct ELISA

ELISA is an analytical biochemistry assay that was firstly introduced by Engvall and Perlmann in 1972 (Engvall and Perlmann 1972). It was originally designed to study binding affinity between antibody and antigen. But with modifications made to the assay, it can also be used as a biochemical method to study the interaction between two macromolecular binding partners. The absorbance measured with serial increment of one binding partner can be recorded and used to get dissociation constant K_d as an indication of binding affinity. For previously reported ELISA-based binding assay, antibodies were needed as well as substrates for detection of absorbance at certain wavelength (Syedbasha, Linnik et al. 2016). Thus it requires target-specific antibodies available in order to study a protein-protein interaction with ELISA.

However, in many cases, antibodies targeting a protein of interest might not be available. To solve this issue, we developed a modified direct ELISA technique using a peptide ligase and this direct ELISA was then used to study the interaction between ZIKV NS3 helicase and NS4B proteins. Moreover, a modified competitive miniaturized ELISA based on the direct ELISA was also

introduced to characterize the interaction sites and to screen for competitors as possible antiviral drug targets.

As so, ELISA is a way to measuring interaction between proteins in vitro with low amount to proteins and where proteins are in their native folding conformation. For our newly developed direct ELISA, ZIKV NS4B protein or cytoplasmic loop peptide were diluted in 1x coating buffer to 1.5 ng/ μ L and 100 μ L was added into 96-well Nunc MaxiSorp ELISA plate for coating overnight at 4°C. Samples were discarded on the next day and wells were blocked with 5% milk in 1x PBS for overnight at 4°C. Wells were then washed with washing buffer containing 0.1% Triton X-100 in 1x PBS for 3 times at 15 mins each time. Interacting protein for instance ZIKV NS3hel was added to wells with serial dilution for incubation at 4°C for 6 hours. As ZIKV NS3hel was labelled with fluorescein, the plate was covered to keep it in the dark during the incubation. Upon incubation, labelled ZIKV NS3hel was discarded and wells were washed with washing buffer 3 times for 15 mins each time. For negative control, wells were coated with 5% milk while signaling protein was added same as experimental wells. Plate was read with Tecan plate reader with excitation wavelength of 473 nm and emission wavelength of 520 nm. To analyze the interaction strength, fluorescence readings from experiments were subtracted by readings of negative control. GraphPad Prism was then used for analyzing data with 'One Site-Specific Binding' method. Dissociation constant K_d was calculated as a measurement of the binding strength. (**Figure 2-7**).

2.13 Competitive ELISA

For competitive ELISA, plates were coated with ZIKV NS4B same as direct ELISA. Competition was detected as the decrease in fluorescence reading when competitor was added with same amount of ZIKV NS3hel. Upon coating, wells were blocked for avoiding non-specific binding with 5% milk for overnight at 4°C. Mixture of ZIKV NS3hel at same amount with various competitors involving ZIKV NS3 subdomain proteins, peptides, fragments and the peptide of ZIKV NS4B cytoplasmic loop was prepared and kept at 4°C for 1 hour. During the incubation time, wells were washed with ELISA washing buffer for 3 times at 15 mins each time. The mixtures of ZIKV NS3hel with competitor were then added into wells accordingly to be incubated for 6 hours. Tecan plate reader was used to read the fluorescence intensity after washing wells with washing buffer.

The data collected are fluorescence readings from Tecan plate reader. The following equation was used to calculate the percentage of bounded NS3hel* to NS4B when competitors are presenting in the measuring:

$$(\text{NS3hel* bound to NS4B}) \% = \frac{\text{Experimental Reading} - \text{Empty Reading}}{\text{NS3hel Only Reading} - \text{Empty Reading}}$$

The percentages of NS3hel* bound to NS4B for serial dilution of competitors were then analyzed with GraphPad Prism Non-linear regression: [Inhibitor] vs normalized response equation.

Figure 2-7. Flow chart of direct ELISA

2.14 Pull-Down assay

Pull-down assay was used to check the direct interaction between proteins. To carry out pull-down assay, His Mag Sepharose™ Ni was used. Beads were firstly washed and equilibrium with gel filtration buffer. One protein contains affinity tag like His₆ tag in my case and the other protein does not contain the affinity tag. Mixture of two proteins or the control protein (without His₆ tag) were incubated with same amount of Ni beads at room temperature for

2 hours. The sample was then removed as flow through with the magnetic stand attracting magnetic Ni beads. Buffer with 10 mM imidazole was then added to Ni beads for incubation for 10 minutes with agitation for washing the beads followed by washing with 50 mM imidazole buffer. Each wash sample was collected with help of the magnetic stand. Protein was finally eluted with 300 mM imidazole buffer. All samples were then checked with SDS-PAGE by Coomassie blue staining and silver staining.

Chapter III: Neutralizing Monoclonal Antibodies

3.1 Background and introduction

Similar to other viruses from *Flaviviridae* family, E protein of ZIKV is responsible for host cell interaction. Thus, it is the primary target for neutralizing antibodies to stop viral infection at the entry stage (Crill and Roehrig 2001, Chávez, Silva et al. 2010, Schieffelin, Costin et al. 2010). From previous studies, some neutralizing antibodies have been identified against DENV infection and two neutralizing antibodies namely Ab513 and Ab9F12 were reported by our group with good broad-spectrum neutralizing activity towards all four serotypes of DENV and even towards WNV (Rajamanonmani, Nkenfou et al. 2009, Robinson, Tharakaraman et al. 2015, Wong, Kumar et al. 2018). With regard to around 55% of amino acid sequence identity between ZIKV and DENV E protein, it would be interesting to find out if these antibodies can bind to ZIKV E protein as well (Priyamvada, Quicke et al. 2016). Meanwhile, neutralizing antibody is an active area for vaccine development against viral infection including human immunodeficiency virus (HIV) (Li, O'Dell et al. 2011, Haynes and Bradley 2015). A neutralizing antibody that can specifically target ZIKV infection will be a valuable candidate for vaccine development to prevent future ZIKV epidemics. One shortcoming of neutralizing antibody however is that virus can develop escape mutants against the antibody thus to survive from it (Hessell, Hangartner et al. 2007, Wang, Bardelli et al. 2017). To prevent virus from developing escape mutants, bi-specific antibody can be used where two antibodies targeting different epitopes are joined together, making it harder for virus to develop escape mutants (Wang, Bardelli et al. 2017). A new way reported by several groups including Prof. Ram Sasisekharan's group at MIT was epitope-driven engineering of antibodies thus the antibodies could be designed to target

mutationally constrained epitope (Robinson, Tharakaraman et al. 2015, Tharakaraman, Subramanian et al. 2015, Nimrod, Fischman et al. 2018, Tharakaraman, Watanabe et al. 2018).

To understand the mechanism of neutralizing antibodies, studies on its interaction with targeting protein are essential. With structural information, neutralizing antibodies can be optimized to have higher neutralizing effect against viruses. Therefore, structural studies on neutralizing antibody are needed. The studies on neutralizing antibodies for ZIKV have not started until the epidemic outbreak in 2015. Nonetheless, with the fundamental studies done for DENV throughout the years, a few neutralizing antibodies either cross-reactive to ZIKV or specific to ZIKV have been reported targeting E protein (Barba-Spaeth, Dejnirattisai et al. 2016, Sapparapu, Fernandez et al. 2016, Zhao, Fernandez et al. 2016, Wang, Bardelli et al. 2017). However, for Ab513 and Ab9F12, it was not known if they could be good neutralizing antibodies against ZIKV as well. So, the first objective for my study on neutralizing antibody against ZIKV was to study the interaction between Ab513/Ab9F12 and ZIKV E protein domain III.

Besides, through a multiway collaboration with Duke-NUS, SMART and Tychan (a start-up set up by Prof. Eng Eong Ooi and Prof. Ram Sasisekharan), we were given a neutralizing antibody that targets a mutationally constrained surface (namely FLEP region) formed between two ZIKV E protein monomers. This antibody ZAb_FLEP was chosen by identifying E protein epitopes that are highly constrained for mutation (Tharakaraman, Watanabe et al. 2018). ZAb_FLEP was checked for *in vitro* neutralization against three different strains

of ZIKV with comparison to two other neutralizing antibodies PGT124 and 4G2 (Figure 3-1), showing neutralizing effects towards all three strains of ZIKV.

Figure 3-1. *In vitro* neutralization of ZIKV strains with ZAb_FLEP, 4G2 and PGT124

A. *In vitro* neutralization assay for antibodies against **A.** ZIKV (H/PF/2013), **B.** ZIKV (PF13) and **C.** ZIKV (MR766). ZAb_FLEP can neutralize all three strains with good PRNT₅₀ ranges 0.022 – 5.092 µg/mL. (Tharakaraman, Watanabe et al. 2018)

ZAb_FLEP has also been tested to efficiently prevent vertical transmission of virus infection and to protect fetuses in pregnant mice. This property of ZAb_FLEP made it a very good candidate for further applications in treating ZIKV infection as it can prevent severe complications such as microcephaly. Moreover, there was no escape mutant generated after serial

passaging of ZIKV with supplement of ZAb_FLEP. Taken into account of all properties of ZAb_FLEP mentioned above, this neutralizing antibody has high potential for curing ZIKV infection as well as for lasting neutralization against ZIKV. However, the mechanism of neutralization for ZAb_FLEP was elusive without structural information detailing its interaction with the complete viral particle. Therefore, the second objective of my project was to understand the mechanism of neutralization for ZAb_FLEP using structural approaches.

3.2 Study on interaction between ZIKV E protein Domain III and neutralizing antibodies Ab513 and Ab9F12

Ab513 and Ab9F12 are the two neutralizing antibodies reported by our group (Rajamanonmani, Nkenfou et al. 2009, Robinson, Tharakaraman et al. 2015). To produce recombinant antibodies with *E. coli* expression system, scFv constructs of both antibodies have been constructed. In the section below, results that I have obtained for the interaction between ZIKV E protein domain III and scFv 513/ scFv 9F12 will be described in detail.

3.2.1 Construction, expression and purification of proteins

Expression plasmids of scFv 513 and scFv 9F12 were obtained from our group's plasmid depository (courtesy to Yee Hwa Wong). For ZIKV E protein domain III (ZIKV EdIII), the DNA fragment was purchased from IDT® with NcoI and XhoI restriction enzyme sites. Molecular cloning was done to insert ZIKV EdIII into pET 22b vector. The construct was confirmed with 1st Base sequencing services. All three proteins were expressed and purified by refolding

as described in chapter II. As shown in **Figure 3-2** below, all proteins were purified with more than 99% of purity as judged from SDS-PAGE.

Figure 3-2. SDS-PAGE and SEC chromatogram of ZIKV EdIII, scFv 513 and scFv 9F12

A. ZIKV EdIII protein; **B.** scFv 513 protein and **C.** scFv 9F12 protein.

3.2.2 Immunoblotting showing interaction between ZIKV EdIII and scFv 513 or scFv 9F12

Immunoblotting was done to investigate the interaction between ZIKV EdIII and scFv proteins. Using scFv 513 or scFv 9F12 as primary antibody to probe ZIKV EdIII without His₆ tag, then anti-His HRP antibody as secondary antibody, immunoblotting signal can only be observed if scFv protein can bind to ZIKV EdIII. As shown in **Figure 3-3** below, both scFv 513 and scFv 9F12 were able to bind ZIKV EdIII specifically in immunoblotting, where ZIKV NS4B proteins with/without His₆ tag were used as negative controls. This shows that scFv 513 and scFv 9F12 can interact with ZIKV EdIII as well, however, it did not reveal the binding affinity between ZIKV EdIII and scFv 513 or scFv 9F12. To further characterize the interactions, ITC and analytical FPLC were carried out.

Figure 3-3. Immunoblotting of ZIKV EdIII with scFv 513 and scFv 9F12

Appearance of ZIKV EdIII without His₆ tag protein band for immunoblotting with scFv 513 or scFv 9F12 as primary antibody. Red arrows indicate the band of ZIKV EdIII.

3.2.3 ITC for scFv proteins and ZIKV EdIII

ITC assays were carried out for ZIKV EdIII and scFv 513 or scFv 9F12 respectively, however precipitations were observed for both ITC assays due to thermal instability of refolded proteins and we were unable to determine the interaction affinity (**Figure 3-4 B/C**). Analytical FPLC was done as well for scFv 9F12 and scFv 513 with ZIKV EdIII, however, immune complex could not be separated from the mixture (**Figure 3-4 A**). All in all, despite the good neutralizing effect and binding affinity towards E protein domain III of DENV, both antibodies scFv 513 and scFv 9F12 did not seem to interact in good affinity with ZIKV E protein domain III. This made us think what the best way is for developing neutralizing antibodies against ZIKV. Though ZIKV belongs to flavivirus group and it has high sequence identity towards DENV, it behaves very differently from DENV in many ways such as its ability in passing BBB (blood-

brain barrier) (Li, Xu et al. 2016, Organization 2016). Thus, a virus-specific neutralizing antibody targets ZIKV itself might be a better choice for treating ZIKV infection even for development of vaccine for ZIKV.

Figure 3-4. Interaction assays of ZIKV EdIII with scFv 513 and scFv 9F12
(A). Analytic FPLC for complex formed between scFv 9F12 and ZIKV EdIII, no obvious peak shift was observed for the mixture of sc9F12 and ZIKV EdIII as comparing to single protein FPLC peaks; **(B).** ITC graph of scFv 513 injected into ZIKV EdIII; **(C).** ITC graph of sc Fv9F12 injected into ZIKV EdIII, both ITC assay gave no binding affinity.

3.3 Study on Interaction between ZIKV E protein and neutralizing antibody ZAb_FLEP

ZAb_FLEP has great neutralizing effect towards strains of ZIKV and prevents ZIKV in generating escape mutants (Tharakaraman, Watanabe et al. 2018). In this section, I will describe in detail what we have done to study the interaction between ZIKV E protein and ZAb_FLEP structurally thus aiding in understanding its mechanism of neutralization.

3.3.1 Expression and purification of ZIKV E protein

Gene coding ZIKV E protein was ordered from BioBasic-Asia for expression in *E. coli* expression system. Expression and purification were carried out with standard protocol, however as shown in **Figure 3-5**, the overexpression level of ZIKV E protein in *E. coli* was very poor with only trace of overexpressed E protein. In order to improve protein expression level, E protein gene was also ordered in pFastBac vector from BioBasic-Asia for expression in Baculovirus expression system. However, despite the observable expression in infected sf9 cells, most of protein expressed was insoluble and could not be purified for crystallization works. Purified ZIKV E protein from Suriname strain was then purchased from The Native Antigen Company, which was pure and in good quality as judged from SDS-PAGE in **Figure 3-7**. The protein was produced in 293 human cells and was stored in buffer containing 20 mM NaH₂PO₄ pH 7.8, 210 mM NaCl. This purchased E protein was then used to form immune complex with antigen binding region of ZAb_FLEP (Fab ZAb_FLEP).

Figure 3-5. Expression and purification of ZIKV E protein

A. SDS-PAGE showing purification of ZIKV E from *E. coli* expressed pellet, as highlighted with red arrow, ZIKV E protein is in minor population from purification; **B.** Western blot showing expression of ZIKV E protein in sf9 insect cells, red arrow indicates the expressed his-tagged E protein.

3.3.2 Enzymatic digestion and Purification of Fab ZAb_FLEP from mAb

mAb form of ZAb_FLEP was provided by Prof. Ram Sasisekharan's group (MIT, Tychan). To obtain the Fab fragment of ZAb_FLEP, papain digestion was carried out as described followed by protein A affinity and SEC purification. Upon papain digestion, Fab was cleaved from Fc with size around 50 kDa when native loading dye was used for sample loading. When reducing loading dye was used, the inter-chain disulfide bond in Fab fragment was disrupted, the size corresponded to the separate light and heavy chain around 25 kDa. During protein A affinity purification, Fc bound to the protein A beads

leading to pure Fab collected from flow-through and washes. Purified Fab ZAb_FLEP was concentrated to 3.5 mg/mL and stored in -80°C.

Figure 3-6. Papain digestion of mAb and purification of Fab ZAb_FLEP

Blue arrow indicates product of Fc, red arrow indicates product of Fab and green arrow indicates papain enzyme.

3.3.3 Co-crystallization of Fab ZAb_FLEP with ZIKV E protein

Upon successful preparation of Fab fragment of ZAb_FLEP, it was mixed with ZIKV E protein with 1:1 molar ratio. The mixture was incubated at 4°C for 3 hours with agitation before it was injected for SEC to check if there was formation of immune complex. According to result shown below in **Figure 3-7**, we observed a peak at A8 to A10, which contains both proteins when run on SDS-PAGE. This indicates there might be formation of immune complex between ZIKV E protein and Fab ZAb_FLEP. More Fab ZAb_FLEP and ZIKV

E protein were then mixed and incubated to form immune complex for crystallization. However, no crystal was obtained from the screening conditions.

Figure 3-7. Formation of immune complex between Fab ZAb_FLEP and ZIKV E protein

A. SDS-PAGE showing purchased ZIKV E protein; **B.** SDS-PAGE showing mixture of ZIKV E and Fab ZAb_FLEP after SEC of the mixture; **C.** SEC chromatography of ZIKV E and Fab ZAb_FLEP mixture.

Though the immune complex was formed between Fab ZAb_FLEP and ZIKV E protein, we were unable to obtain crystals with the crystallization conditions reported for neutralizing antibody C8 with recombinant ZIKV E protein (Barba-Spaeth, Dejnirattisai et al. 2016). A review of publications of all the reported neutralizing antibodies against ZIKV or cross-reactive to ZIKV (Barba-Spaeth, Dejnirattisai et al. 2016, Dai, Song et al. 2016, Zhao, Fernandez et al. 2016, Wang, Bardelli et al. 2017), revealed that at the time of the thesis, there were only a few of the structures available for the immune complex formed between neutralizing antibody and the whole viral particle of ZIKV. Because a structure of native immune complex formed between the Fab of neutralizing antibody and viral particle is most informative to understand the structural basis for the mechanism of neutralization, we therefore proceeded to obtain the

structure of the complete ZIKV viral particle bound to Fab of ZAb_FLEP using cryo-EM.

3.3.4 Production of ZIKV viral particle

The viral particle of ZIKV was produced with the help of Dr. Hwee Cheng Tan (Duke-NUS). Viral particle was purified by the method described earlier for cryo-EM viral particle preparation (Tan and Lok 2014). Viral particle concentration (E protein concentration) was quantified by SDS-PAGE with BSA standard (**Figure 3-8**). The concentration of E protein for purified ZIKV viral particle was calculated to be 0.25 mg/mL. The measured E protein concentration was used in calculating the amount of antibody to form the immune complex.

Figure 3-8. SDS-PAGE for pure ZIKV viral particles with BSA standards

Figure credits to Dr. Hwee Cheng Tan (Duke-NUS).

3.3.5 Cryo-EM structure of the immune complex formed between ZIKV and Fab ZAb_FLEP

Knowing the concentration of E protein from viral particle sample, we were able to calculate the molar concentration of it. Molar ratio of 1:2 between ZIKV E protein and Fab ZAb_FLEP was applied for formation of immune complex. This ratio of 1:2 was used to have an excess amount of Fab with respect to E proteins on viral particle in order to saturate the viral particles with Fab. The complex was allowed to form for 30 min at 37°C before it was kept on ice and applied onto Cryo-EM grids. Negative staining results of immune complex as shown in **Figure 3-9** were obtained. For negative staining, 1 μ L of complex was diluted to 4 μ L, within which 3.5 μ L was applied to carbon-coated grid. The size of viral particle observed from negative staining results was revealed to be corresponding to the size of ZIKV virion as shown in **Figure 3-9**. The level of density observed from negative staining also indicated the concentration of immune complex was suitable for preparation of cryo-EM grids. Images collected from negative staining were processed with EMAN2 to have an initial 2D classification as well as reconstructed model as shown in **Figure 3-9**. From the scale bar, we can judge the size of particles to be around 50 nm, which corresponds to the size of ZIKV virion. The density of particle was also suitable for picking particles.

The density of particles observed on cryo-EM grids is generally lower than that on negative staining grids due to the lack of carbon-coating for cryo-EM grids. Therefore, with the information from negative staining, we decided to use 2.5 μ L of complex sample for preparation of cryo-EM grids. Four grids were prepared with different blotting times as described in chapter II and were

Figure 3-9. Negative staining micrographs, 2D classification and 2D resconstruct model of ZIKV-Fab ZAb_FLEP complex

A. 4 micrograph from negative staining of immune complex; B. 2D classification sorted out by processing micrographs with EMAN2; C. 2D resconstructed model obtained from micrographs for ZIKV-Fab ZAb_FLEP complex.

screened to check the quality of grids. As shown in **Figure 3-10**, two grids were selected for Cryo-EM data collection after screening, which gave good quality micrographs. The micrographs collected from the two well-prepared Cryo-EM grids had particles with nice density and without ice rings. Meanwhile, by comparing micrographs from Cryo-EM screening with previously published micrographs of ZIKV alone and ZIKV bound to Fab C10, it can be observed that the particles from cryo-EM micrographs were more similar to the particles of ZIKV with Fab C10 (Zhang, Kostyuchenko et al. 2016). The particles observed

had small spikes that might correspond to Fab density (instead of very smooth sphere for ZIKV viral particles alone).

Figure 3-10. Cryo-EM micrographs from grids screening and reference Cryo-EM micrographs

A. Two micrographs obtained from ARTICA cryo-EM screening of ZIKV-Fab ZAb_FLEP complex from the two well-prepared grids; **B.** Micrographs of ZIKV alone and ZIKV + Fab C10 as reference (Zhang, Kostyuchenko et al. 2016), red arrows identify spikes on particles.

The two Cryo-EM grids were then used for data collection with the help of Prof. Shashi Bhushan. A total of 304 micrographs were recorded and 287 micrographs were selected for data processing. A final Cryo-EM 3D map of ZIKV-Fab ZAb_FLEP complex was computed at resolution of 9.7 Å according

to the gold-standard Fourier Shell Correlation (FSC) of 0.143 criteria (EMDB: EMD-7613) (**Figure 3-11 E**). Interestingly in the present cryo-EM structure, only 120 units of Fab ZAb_FLEP were observed around the icosahedral 2-fold and 3-fold axes (**Figure 3-11 A**) but not around the 5-fold axis which accounts for the 60 copies of Fab ZAb_FLEP missing in the virus-Fab complex. The same occupancy of Fab ZAb_FLEP was observed when we did a reconstruction with a Fab-free model, where only ZIKV virion and cryo-EM density map were used for the reconstruction. This suggests that ZAb_FLEP can effectively neutralize ZIKV with RFNT50 value of 0.14 nM without fully covering the viral particle. For Fab C10 that was previously reported to be a good ZIKV neutralizing antibody with reported FRNT50 value of 0.063 nM, 180 units of Fab C10 were bound per ZIKV viral particle (Zhang, Kostyuchenko et al. 2016). According to literature, the measured FRNT50 value is generally 2-fold lower than the PRNT50 value for same neutralizing target (Vaidya, Brown et al. 2010). Thus ZAb_FLEP is a more potent neutralizing antibody as lower occupancy of ZAb_FLEP is needed as compared to Fab C10 for reaching similar neutralizing effect.

To understand the origin for the absence of binding around the 5-fold icosahedral axes of the viral particle, we also modeled a complex with 180 units of Fab ZAb_FLEP bound to viral particle. This modeling revealed that: (1) ZAb_FLEP makes inter-dimer contacts and (2) the binding of Fab ZAb_FLEP near the 5-fold axis is hindered sterically by the adjacent E protein at 5 fold assembly (**Figure 3-11 C and D**), which also explains why only 120 units of Fab ZAb_FLEP were observed in cryo-EM density map.

In summary, the different arrangement of E protein near the 2, 3 and 5-fold icosahedral vertices (**Figure 3-11 D**) and the topological nature of ZAb_FLEP epitope account for the occupancy of 120 units of ZAb_FLEP to ZIKV. The spacing between bound Fab fragments then suggests the possibility of a bivalent binding pattern of ZAb_FLEP towards ZIKV, wherein nearby 2-fold and 3-fold icosahedral axes were bound by two arms (Fab) of a single IgG (**Figure 3-11 B**). Together the epitope of ZAb_FLEP maps to the fusion loop peptide and its ability to lock the intra-dimer and inter-dimer interfaces suggests a mechanism of neutralization by preventing essential conformational changes required during viral fusion to endosomal membrane of host cells.

Figure 3-11. Cryo-EM structure of ZIKV-Fab ZAb_FLEP complex

A. Electron density map of ZIKV-Fab ZAb_FLEP complex is shown with ZIKV E protein fitted in the density, the view is along the 5-fold axis. No occupancy of Fab ZAb_FLEP is observed around the 5-fold axes; **B.** ZAb_FLEP epitope on E protein raft. Fab ZAb_FLEP is displayed as surface. Two arms of Fab bind bivalently to E protein, where one arm engages the 2-fold epitope and the other engages the 3-fold epitope. **C.** Electron density of ZIKV-Fab ZAb_FLEP with fitting of 180 copies of E protein. Notice the different orientation of E protein around 5-fold axis; **D.** Model of a complex with 180 units of Fab ZAb_FLEP, steric hinderance can be observed around the 5-fold axes. E monomers that form the pentamer are displayed as surface (magenta) and the variable dimer (VH-VL) that occupy the sites around 5-fold axes are in orange and yellow color. Overlap of VH-VL with E protein monomers at 5-fold axis can be observed; **E.** FSC chart of the final EM density map.

To further investigate the difference in interaction between ZAb_FLEP and Fab C10 with ZIKV E protein, we have looked insight into the structural comparison for Fab C10 and ZAb_FLEP in complex with E protein. As shown in **Figure 3-12 A** below, ZAb_FLEP adopts different orientation with respect to ZIKV E protein asymmetric unit. Taking view angle down the 2-fold symmetry axis, Fv of ZAb_FLEP orients 30° counterclockwise relative to Fv of C10, as such the vestigial loops of ZAb_FLEP VH is brought nearer to the adjacent dimer of ZIKV E protein. In addition, with highlighting the non-overlapping residues of ZAb_FLEP and Fab C10 (**Figure 3-12 B**), ZAb_FLEP is noticed to make more contacts with the adjacent dimer region as compared to C10. On the other hand, C10 has more contacts with E protein monomer. This difference in orientation further explains the lower occupancy of ZAb_FLEP on ZIKV virion as clash could happen between units of ZAb_FLEP.

Figure 3-12. Structural comparison for ZAb_FLEP and C10 Fv in complex with ZIKV E protein

A. Comparison between the orientations of ZAb_FLEP and C10 Fv in respective to E protein surface. Heavy and light chains of the two antibodies are labelled and represented in surface format. **B.** Comparison of the contacting epitope residues by ZAb_FLEP and C10. Overlap region is indicated as grey in color, residues unique to ZAb_FLEP are shaded in blue while residues unique to C10 are shaded in red.

3.4 Conclusions and discussions

A potent neutralizing antibody candidate could provide a solution for treating ZIKV infection. In this project, we characterized a good neutralizing antibody against ZIKV namely ZAb_FLEP. With the structural information we obtained, we are able to propose a mechanism of neutralization for ZAb_FLEP. And thus, future studies can be done to promote the development of ZAb_FLEP into a potential antibody therapy against ZIKV infection. In fact, a patented antibody based on ZAb_FLEP namely Tyzivumab is currently undergoing phase 1 of clinical trials according to ClinicalTrials.gov. Furthermore, structural information can guide the further optimization of ZAb_FLEP to improve its neutralizing ability.

Though ZAb_FLEP showed high neutralizing activity against ZIKV and it is currently undergoing clinical trials, we should not be too optimistic and neglect the intrinsic challenges one could face in implementing neutralizing antibody therapies for treating acute diseases caused by viral infections. The limitations of antibody therapies according to previous studies and clinical trials performed on Influenza include (1). The development cost for antibody is considerably high while dose needed for treatment is high as well, thus antibody therapies are unlikely to be considered as cost-effective; (2). Generally, antibodies are thermally instable with short half-life; (3). The form of delivery for most antibody candidates in clinical trials are intravenous injection over a few hours, as such hospitalization is needed, and it raises burden to healthcare system especially in developing countries (Sparrow, Friede et al. 2016). For ZAb_FLEP, it has addressed the first limitation in cost effectiveness as the development lead time for ZAb_FLEP as an engineered antibody is much shorter comparing to the development of conventional mAbs thus the cost is much lower. As pandemic outbreaks of ZIKV tend to happen in developing countries, we will face the limitations in healthcare system thus it will be challenging if the form of delivery remains as intravenous injection. Moreover, the stability and half-life need to be improved.

Currently, MEDI8897, a mAb for the treatment of RSV (respiratory syncytial virus) is the only antibody therapy for acute disease caused by viral infections, which is in the final phase of clinical trial (AstraZeneca 2019). The outstanding property of MEDI8897 is its extended half-life from typical 21 – 28 days to more than 150 days (Griffin, Khan et al. 2017). This was achieved with engineering at its Fc region, where 3 amino acid changes have been introduced

(Domachowske, Khan et al. 2018). This could be a guideline for our further optimization of ZAb_FLEP to increase its preference as antiviral drug for ZIKV treatment. Besides, it needs to be optimized in binding affinity with ZIKV E protein thus to lower down the effective dose. Last but not least, form of delivery needs to be improved from intravenous injection to intramuscular inject to release the burden to healthcare system.

Even though there is still a long journey for ZAb_FLEP and other antibody therapies to go through, they will certainly become widely used after addressing these limitations. In addition, studies are carrying out on peptides and small molecule antiviral drug developments as good alternatives in treating viral infections.

**Chapter IV: Interaction between ZIKV NS3 Helicase
and NS4B**

4.1 Background and introduction

RNA replication is essential for virus amplification, and it is a well-regulated and organized process. RNA replication is mediated by a replication complex formed with viral non-structural proteins and host proteins. Besides the functions of individual protein, multiple protein-protein interactions among these non-structural proteins are also crucial for the formation of replication complex (Klema, Padmanabhan et al. 2015). The interaction between NS3 helicase and NS4B was studied for DENV, and proved to be essential for virus amplification (Zou, Wang et al. 2015). However, ZIKV is different from DENV in many aspects including the pathology and the thermostability of viral particles (Kostyuchenko, Lim et al. 2016). This may extend to other differences as well like differences in interactions between non-structural proteins.

How the non-structural proteins interact with each other in ZIKV replication complex is unknown at the moment. Moreover, NS4B of ZIKV was reported to inhibit neurogenesis and induce autophagy thus leading to microcephaly in infants (Liang, Luo et al. 2016). Given the important role played by ZIKV NS4B, it is interesting to understand the protein interactions involving NS4B. In this project, I will test the interaction between ZIKV NS4B and NS3 helicase and map this interaction. Additionally, I wish to screen for competitors targeting this interaction as antiviral drug candidates.

4.2 Results and discussions

4.2.1 Construction, expression and purification of proteins

4.2.1.1. ZIKV NS3hel-NAL

The plasmid with full length gene of ZIKV NS3 was a kind gift from Dr. Luo Dahai (LKC SCMed). In order to express recombinant NS3 helicase protein with peptide ligase recognition motif, protease region was removed, followed by insertion of recognition motif at C-terminus. A short recognition motif is needed for peptide ligase to function consisting of one asparagine (Asn) and two other amino acids as the leaving group (Harris, Durek et al. 2015, Yang, Wong et al. 2017). The dipeptide ‘GL’ was the leaving group reported from previous studies on *OaAEP* for intra-molecular ligations (Yang, Wong et al. 2017). ‘NAL’ was chosen as the recognition motif for *OaAEP* because we observed the best ligation efficiency for ‘NAL’ as compared to ‘NGL’ and ‘NHL’. Sequence of final construct was confirmed with 1st Base sequencing service. Protein was purified and characterized to be monomer with SEC (**Figure 4-1**).

Figure 4-1. Purification of ZIKV NS3hel-NAL

A. SDS-PAGE of ZIKV NS3hel-NAL protein; **B.** SEC Chromatogram of ZIKV NS3hel-NAL protein.

4.2.1.2. Subdomains of ZIKV NS3 helicase

Constructs of ZIKV NS3 helicase subdomains were obtained from Protein Production Platform (PPP), NTU. These subdomains were purified in order to map the interaction regions on NS3 helicase. All subdomains were cloned with N-terminal His₆ tag for purification. Judging from SDS-PAGE (**Figure 4-2**), all subdomains were prepared with high purity above 95%.

Figure 4-2. Purification of ZIKV NS3hel subdomains

A. Schematic representation of ZIKV NS3hel subdomains with relation to full-length NS3hel (FL); **C.** SDS-PAGE of final products of ZIKV NS3hel subdomain 1/2/3.

4.2.1.3. ZIKV NS4B

The gene of ZIKV NS4B was cloned into pFastBac vector with a His₆ tag at N-terminus and a Dual-Strep tag at C-terminus (see **Table 2-4** and **Table 2-5**). The correct construct was confirmed with 1st Base sequencing service. After transposition of ZIKV NS4B-Strep/pFastBac into DH10EMBacY competent cell, white colonies were selected and screened with colony PCR. Transfection of bacmid into sf9 cells was done successfully and confirmed by fluorescence microscopy. Fluorescence can be observed in cells with recombinant Baculovirus as DH10EMBacY bacmid has yellow fluorescence protein (YFP) gene that is expressed during the replication of Baculovirus (**Figure 4-3**).

A.

B.

Figure 4-3. Schematic drawing of DH10EMBacY bacmid and result showing successful transfection

A. Bacmid organization for DH10EMBacY; **B.** Sf9 cells under bright field and fluorescence microscope 3 days post-transfection. Fluorescence was observed suggesting successful formation of recombinant Baculovirus.

As the protein expressed and purified was to be used for interaction assays, detergent was avoided to keep the protein in its native fold. Instead, SMA polymer (2:1) was used for the purification of ZIKV NS4B as described in chapter II. As a result, pure NS4B protein was obtained, and its concentration was confirmed with Bicinchoninic assay (BCA) instead of nanodrop since SMA polymer has absorbance at 280 nm as well (Walker 2009). **(Figure 4-4)** The purified protein was stored at 4°C and used within four weeks.

Figure 4-4. Purification of ZIKV NS4B protein

A. Complex unit formed with transmembrane protein and SMA polymer; **B.** Sequence alignment of NS4B protein among ZIKV and four serotypes of DENV; **C.** SDS-PAGE of purified NS4B protein with observed mass around 40 kDa.

4.2.1.4. Cytoplasmic loop peptide of ZIKV NS4B

The interaction region on ZIKV NS4B with other viral and cell proteins is also unknown. According to the topology of NS4B protein (**Figure 1-9**), most regions of NS4B are buried inside membrane. Cytoplasmic loop from G₁₂₆ to L₁₇₅ is the only exposed region and might therefore be responsible for interacting with other non-structural proteins. Cytoplasmic loop region in DENV was also reported to be responsible for interacting with NS3 helicase (Zou, Wang et al. 2015). The gene of ZIKV NS4B cytoplasmic loop region was prepared from ZIKV NS4B-Strep/pFastBac construct. It was then cloned into PNIC28-Bsa4 vector for expression in bacterial expression system. The expressed cytoplasmic loop peptide however was insoluble (data not shown). In order to increase the solubility of ZIKV NS4B cytoplasmic loop peptide, a new construct was then designed with a GST tag at its N-terminus as shown in **Figure 4-5**. The construct was confirmed by BioBasic-Asia sequencing service. The presence of GST tag made the cytoplasmic loop peptide soluble and purifiable with GST affinity purification. GST tag and His₆ tag were then removed by proteolysis. As shown in **Figure 4-5**, pure cytoplasmic loop peptide was obtained. However, since there is not any amino acid containing tryptophan within this peptide, no absorbance peak at 280nm was observed in SEC chromatogram. Absorbance at 215nm that represents the absorbance of peptide bonds was then used as the indication for the presence of peptide. The final concentration of peptide was measured with BCA assay.

The cytoplasmic loop region in native NS4B was cyclized with both sides buried in membrane (**Figure 1-9**). However, the peptide we purified was not cyclized but linear. Another construct was then designed for cyclization of the

peptide with *OaAEP*. To enable the separation between linear and cyclized cytoplasmic loop peptide, His₆ tag was moved from N-terminus to C-terminus as shown in **Figure 4-5 A**. The new construct namely ‘Cytoloop-His’ was prepared and its sequence was confirmed by BioBasic-Asia sequencing service. It was expressed and purified similar to GST-Cytoloop construct and was stored at -80°C (**Figure 4-5**).

Figure 4-5. Purification of ZIKV NS4B cytoplasmic loop peptides

A. Schematic representations of GST-Cytoloop and Cytoloop-His constructs; **B.** SDS-PAGE of purified peptides from two different constructs; **C.** The SEC chromatogram of peptides, absorbance peak can only be observed at 215nm.

4.2.1.5.scFv 513-NAL/NGL/NHL

scFv 513 with different C-terminal recognition sites for peptide ligase were constructed from original scFv 513 by insertion and mutation. Proteins were expressed and prepared by refolding as described in section 2.4.3. Shown in **Figure 4-9**, all three proteins were purified and stored at -80°C with high purity judged from SDS-PAGE.

4.2.2 Direct and competitive ELISA for identifying interaction region on ZIKV NS3 helicase

To test the interaction between ZIKV NS3 helicase and NS4B, a proper interaction assay is needed. However, NS4B as a transmembrane protein limits the choices of assays. ITC is not suitable as large amount of proteins are required for the assay, while the yield for transmembrane proteins is generally low (Parker and Newstead 2014). Even with the considerable high yield from insect cell expression system, the yield of ZIKV NS4B protein was at 0.65 mg per 1 L of insect cell culture. ELISA on the other hand requires minimum amount of proteins, and it is a high-throughput assay with usage of 96-well or 384-well plates. Nonetheless, for commercial ELISA, target-specific antibodies are often unavailable when the target proteins are novel. Moreover, the chance of random errors tends to be high as a series of antibodies and substrates need to be added into the assay (**Figure 4-6 A**). Therefore, we introduced a modified direct ELISA as illustrated in **Figure 4-6 B**. In brief, protein B labelled with fluorescein peptide serves as the interacting partner as well as reporter molecule. ELISA plate was coated with protein A followed by incubation with protein B. Signal of fluorescein peptide (protein B) is detected with Tecan plate reader, which is an

Figure 4-6. Schematic representation of A. Commercial ELISA; B. Direct ELISA and C. Competitive ELISA.

Rod A and B represent interaction partners A and B respectively. Rod labelled with C/D/E represents competitors. For commercial ELISA, interaction partner B is not labelled. For direct and competitive ELISA, binding partner B is labelled with fluorescein, thus it is the interaction partner as well as the reporter molecule.

indication of protein B bound to coated protein A. Only the two interacting macromolecules were used during the assay, which makes the measurement of interaction to be more accurate and reduces the chance of random errors.

As mentioned, protein B needs to be labelled with fluorescein peptide so to serve as the reporter molecule. Protein labelling was done with help of peptide ligase (*OaAEP*). The working principle of *OaAEP* for inter-molecular ligation is illustrated in **Figure 4-7**. Briefly, *OaAEP* recognizes the C-terminal 'NAL' motif

where it cleaves the peptide bond between Asn and Ala, a new peptide bond was then formed between protein and acceptor peptide (Nguyen, Qiu et al. 2016). *OaAEP* was prepared with thanks to Ms. Yee Hwa Wong. Fluorescein peptide was synthesized by Dr. Kalyan Pasunooti (Prof. Liu Chuan Fa Laboratory, SBS), which was designed with sequence of ‘GLGGK*HHHHHH’ so that the labelled proteins can be separated from non-labelled ones by metal affinity purification (**Figure 4-7 A**). Fluorescein was coupled at lysine residue (**Figure 4-7 B**).

Figure 4-7. Working principle of inter-molecular ligation with *OaAEP* (A) and chemical structure of fluorescein peptide (B).

4.2.2.1 Protein labelling with *OaAEP* and purification

For inter-molecular ligation with *OaAEP*, the best residues for leaving group have not been identified yet. In order to have improved ligation efficiency, we checked three recognition motifs with different leaving groups including ‘NAL’, ‘NGL’ and ‘NHL’. ‘NGL’ was used as the benchmark (Yang, Wong et al. 2017). Purified scFv 513 NAL/NGL/NHL were ligated with fluorescein peptide by *OaAEP* respectively. Reaction time and conditions were kept the same for all three ligations. According to the result showing in **Figure 4-8**, the best ligation efficiency (above 60%) was observed for recognition motif of ‘NAL’ while the lowest ligation efficiency was observed for ‘NGL’. Therefore, recognition motif ‘NAL’ was used for all the constructs that were to be ligated with *OaAEP*.

Purified ZIKV NS3hel-NAL was incubated with fluorescein peptide and *OaAEP* for 72 hours at 4°C for labelling. A mixture of ZIKV NS3hel-NAL and ZIKV NS3hel* (ZIKV NS3hel with fluorescein peptide) was separated with Ni-NTA affinity purification and SEC. Shown in **Figure 4-8**, more than 99% of the final product was ZIKV NS3hel*. The fluorescein molecule used can be excited at 473nm and it emits at 520nm.

Figure 4-8. Protein labelling with *OaAEP*

A. SDS-PAGE was stained with comassie blue and imaged with Typhoon imager showing labelled and unlabelled scFv513-NAL/NGL/NHL; **B.** Percentage of labelled protein over total protein; **C.** SDS-PAGE showing labelling and purification of ZIKV NS3hel*.

4.2.2.2 Interaction between ZIKV NS3hel and NS4B

The interaction between ZIKV NS3 helicase and NS4B was tested and measured by the modified direct ELISA. Briefly, the ELISA plate was coated with full-length NS4B and 5% milk respectively. Blocking of wells was done with 5% milk in 1x PBS. The captured ZIKV NS4B was then incubated with 2-fold serial dilutions of ZIKV NS3hel*. As shown in **Figure 4-9**, ZIKV NS3

helicase (ZIKV NS3hel*) bound to ZIKV NS4B in a dose-dependent manner. The dissociation constant (Kd) was analyzed to be $200.7 \text{ nM} \pm 61.11 \text{ nM}$ with GraphPad Prism ‘one-site specific binding’ equation, indicating a moderate affinity interaction. This result confirmed direct interaction between ZIKV NS3 helicase and NS4B as only these two proteins were used for the assay. This modified direct ELISA was also confirmed to be applicable in measuring interaction affinity between ZIKV NS3 helicase (ZIKV NS3hel*) and ZIKV NS5 RdRp (by Dr. Aicha Gharbi Ayachi, personal communication).

4.2.2.3 NS3 helicase subdomain 3 mediates interaction between ZIKV NS3 helicase and NS4B

Upon validating the interaction between NS3 helicase and NS4B in ZIKV, we further mapped the interaction region on ZIKV NS3 helicase with competitive ELISA. The principle of competitive ELISA is shown in **Figure 4-6**. In brief, amount of bound ZIKV NS3hel* decreases with presence of competitors. Since the competitors were unlabeled proteins, signal detected also dropped. With excess amount of subdomain proteins, the competition reaches a saturation, thus allowing us to calculate IC₅₀ where 50% of NS3hel* remains bound with NS4B with GraphPad Prism ‘Non-linear regression: [Inhibitor] vs normalized response’ equation. The result of competitive ELISA shown in **Figure 4-10** suggested ZIKV NS3 helicase subdomain 3 was responsible for interacting with ZIKV NS4B with subdomain 2 assisting in the interaction, while subdomain 1 only minimally disrupts the interaction. The IC₅₀ value of ZIKV NS3 helicase subdomain 3 was $346.4 \text{ nM} \pm 92.3 \text{ nM}$. For DENV, the interaction region on NS3 helicase was only characterized to be domain 2+3 (Zou, Wang et al. 2015).

My results show that ZIKV NS3 helicase interacts with NS4B and the interaction region is mainly on subdomain 3. Peptides were then designed to bind with ZIKV NS3 helicase or ZIKV NS3 helicase subdomain 3 as potential competitors against this interaction. Next, we also tried to identify the interaction region on ZIKV NS4B.

	ZIKV NS4B	Cytoplasmic Loop
One site -- Specific binding		
Best-fit values		
Bmax	199.7	13.00
Kd	200.7	-5.795
Std. Error		
Bmax	17.50	5.061
Kd	61.11	10.97
95% Confidence Intervals		
Bmax	162.6 to 236.8	2.271 to 23.73
Kd	71.16 to 330.3	-29.04 to 17.45
Goodness of Fit		
Degrees of Freedom	16	16
R square	0.8413	-0.09478
Absolute Sum of Squares	10662	4778
Sy.x	25.81	17.28
Number of points		
Analyzed	18	18

Figure 4-9. Interaction between ZIKV NS3 helicase and NS4B investigated by modified direct ELISA

Wells coated with 5% milk were used as negative control, where the readings were subtracted. Cytoplasmic loop peptide was also coated with no interaction observed. Analysis data is listed in the table.

	1	2	3	Without Competitor
[Inhibitor] vs. normalized response				Perfect fit
Best-fit values				
IC ₅₀	8.400	1.761	0.3464	2.172e+016
logIC ₅₀	0.9243	0.2457	-0.4604	16.34
95% CI (profile likelihood)				
IC ₅₀	5.051 to 16.59	1.418 to 2.198	0.2541 to 0.4726	
logIC ₅₀	0.7034 to 1.220	0.1516 to 0.3420	-0.5950 to -0.3255	
Goodness of Fit				
Degrees of Freedom	19	13	19	19
R squared	0.5761	0.9407	0.9278	1.000
Sum of Squares	2949	567.8	2564	0.000
Sy.x	12.46	6.609	11.62	
Constraints				
IC ₅₀	IC ₅₀ > 0	IC ₅₀ > 0	IC ₅₀ > 0	IC ₅₀ > 0
Number of points				
# of X values	20	20	20	20
# Y values analyzed	20	14	20	20

Figure 4-10. Identification of the NS3 helicase domain responsible for interacting with NS4B by competitive ELISA

Percentage of ZIKV NS3hel* bound to NS4B was almost fully competed by addition of ZIKV NS3 helicase subdomain 3. The IC₅₀ calculated for ZIKV NS3 helicase subdomain 3 is 346.4 nM $\pm$ 92.3 nM. Analysis data is listed in the table.

4.2.3 Identification of interaction region on ZIKV NS4B

Based on the topology information of flavivirus NS4B and the previously reported interaction between DENV NS3 helicase and NS4B, the hypothesized interaction region on NS4B protein was the cytoplasmic loop region (Zou, Wang et al. 2015, Li, Wong et al. 2016). Therefore, we have prepared the cytoplasmic loop peptide as described. To check the interaction between ZIKV NS4B cytoplasmic loop and ZIKV NS3 helicase, a few assays were done including direct ELISA, ITC and pull-down assay.

4.2.3.1 NS4B cytoplasmic loop peptide does not interact directly with NS3 helicase

As shown in **Figure 4-9**, ZIKV NS3hel* did not bind to coated ZIKV NS4B cytoplasmic loop peptide in a dose-dependent manner. This suggested ZIKV NS3 helicase did not interact with NS4B cytoplasmic loop peptide directly unlike what have been observed for DENV in previously reported study (Zou, Wang et al. 2015).

To confirm this finding, ITC was also carried out between ZIKV NS4B cytoplasmic loop peptide and ZIKV NS3 helicase. 230 μ M of ZIKV NS3 helicase was injected into cell containing 25 μ M of ZIKV NS4B cytoplasmic loop peptide for 20 times. According to the ITC result showing in **Figure 4-11**, the interaction could not be measured as well despite good quality of protein and peptide used (**Figure 4-11 A**). The contrary finding was also confirmed with pull-down assay between ZIKV NS3 helicase and ZIKV NS4B cytoplasmic loop peptide (**Figure 4-12**). There was no band of cytoplasmic loop peptide observed when ZIKV NS3 helicase was used to pull down the peptide. All in all, we can conclude that the

cytoplasmic loop peptide is not responsible for the direct interaction with ZIKV NS3 helicase or that the expressed cytoplasmic loop is not folded properly.

Figure 4-11. ITC for ZIKV NS4B cytoplasmic loop peptide and ZIKV NS3 helicase

A. SDS-PAGE showing proteins used for ITC; **B.** ITC graph of buffer injected into cytoplasmic loop peptide; **C.** ITC graph of ZIKV NS3 helicase injected into cytoplasmic loop peptide.

A possible explanation for this finding is that the interaction between ZIKV NS3 helicase and NS4B is more conformation-dependent compared to DENV. The cyclized conformation of cytoplasmic loop in full-length ZIKV NS4B is hypothesized to be responsible for interacting with ZIKV NS3 helicase. However, the purified peptide is in linear conformation. To confirm the hypothesis of conformation-dependent interaction between ZIKV NS3 helicase and ZIKV NS4B cytoplasmic loop, *OaAEP* was used to produce cyclized ZIKV NS4B cytoplasmic loop peptide.

Figure 4-12. Pull-down assay for ZIKV NS4B cytoplasmic loop peptide and ZIKV NS3 helicase

A. SDS-PAGE of pull-down assay stained with coomassie blue; **B.** SDS-PAGE of pull-down assay stained with silver stain. Red arrows indicate the expected band of cytoplasmic loop peptide.

4.2.3.2 Cyclization of ZIKV NS4B cytoplasmic loop peptide

Cytoloop-His construct was expressed and purified successfully as shown in **Figure 4-5**. *OaAEP* was used for intra-molecular ligation (cyclization), the principle of intra-molecular ligation is illustrated in **Figure 4-13 A**. For Cytoloop-His peptide, the cyclization is slightly different as indicated in **Figure 4-13 B**. Recognition motif 'NAL' locates in front of a His₆ tag instead of at C-terminus. Cyclization of peptide was done with this arrangement of recognition motif enabled us to separate cyclized peptide from linear peptide by affinity towards Ni-NTA beads. Purification of cyclized Cytoloop-His peptide was confirmed with SDS-PAGE and MALDI-TOF MS (**Figure 4-13 C, D**). The concentration of cyclized Cytoloop-His was measured with BCA assay as well.

Figure 4-13. Intra-molecular ligation to cyclize NS4B Cytoloop-his peptide

A. Schematic representation of intra-molecular ligation with peptide ligase in general; **B.** Cyclization of ZIKV NS4B cytoplasmic loop peptide with *OaAEP*; **C.** SDS-PAGE showing the bands of linear and cyclized ZIKV NS4B cytoplasmic loop peptide; **D.** MALDI-TOF profiles of linear and cyclized ZIKV NS4B cytoplasmic loop peptide. Mass of 6568 Da represents the mass of original cytoplasmic peptide. Small peptide ‘ALHHHHHH’ was removed from cyclized peptide, therefore the mass became 5550 Da.

Both linear and cyclized Cytoloop-His peptides are being used for direct ELISA assays with ZIKV NS3hel protein. Preliminary data has shown interaction affinity between cyclized Cytoloop-His peptide and NS3 helicase to be much higher than the interaction affinity between linear Cytoloop-His peptide and NS3 helicase (data not shown). However, to draw a conclusion on this hypothesis, biological repeats and a series of other experiments with linear and cyclized cytoplasmic loop peptides need to be carried out.

4.2.4 Screening of competitor peptides against interaction between ZIKV NS3 helicase and NS4B

The interactions between non-structural proteins were known to be essential for the formation of replication complex (Umareddy, Chao et al. 2006, Lescar, Luo et al. 2008, Chatel-Chaix, Fischl et al. 2015, Zou, Wang et al. 2015). Therefore, peptides that can disrupt the interactions by competitive interaction could be good antiviral drug candidates. Moreover, antiviral drug targeting the interactions between non-structural proteins instead of functional viral proteins could reduce the generation of escape mutants. Functional viral proteins generally obtain higher mutation rate for RNA viruses (Dai, Du et al. 2016, Sanjuán and Domingo-Calap 2016).

We collaborated with Dr. Su Seong Lee's group (IBN-A*STAR) aiming to develop peptide therapies targeting the interaction between ZIKV NS3 helicase and NS4B. Dr. Joo-Eun Jee (IBN-A*STAR) initiated peptide library screening targeting ZIKV NS3 helicase or ZIKV NS3 helicase subdomain 3. The peptide library screening was based on combinatorial one-bead-one-compound (OBOC) peptide libraries (Lam, Salmon et al. 1991, Jee, Li Ang et al. 2014). In

order to obtain positive beads from the screening, fluorescent dye-labeled proteins were used in the screening with improved fluorescent dyes (Jee, Lim et al. 2014). Pure proteins provided by us were used for screening and validation of peptides. A total of 15 peptides targeting ZIKV NS3 helicase (P1-P13 and P15-P16) and 3 peptides targeting ZIKV NS3 helicase subdomain 3 (PP1 – PP3) were validated and synthesized. All 18 peptides were then used as competitors with a final concentration of 100 μ M for competitive ELISA against ZIKV NS3hel*. A threshold of 50% competition was set for the selection of competitor peptides. As shown in **Table 4-1**, with addition of P16, percentage of NS3hel bound to NS4B dropped to 39%, which indicated P16 competed more than 60% towards the interaction between ZIKV NS4B and NS3 helicase. This level of competition is comparable to what NS3hel subdomain 3 has. The high level of competition by P16 together with the small size of it (less than 2 kDa) make P16 a potential antiviral drug candidate. Small size of P16 can help it in penetrating cell membranes thus to reach viral replication complex. Meanwhile, the percentage of NS3hel bound to NS4B dropped to 62% with addition of P15, which made P15 a potential candidate as well. Since the sequences for P15 and P16 are very different from each other, it provides us the option to conjugate both peptides together to improve its competition activity.

Table 4-1. Screening of peptides in competitive ELISA

Peptide Name	Peptide Sequence	Molecular Weight (Da)	Percentage of ZIKV NS3hel bound to NS4B
NS3hel subdomain 3			18%
P16	GYLQRWHRG	1927	39%
P15	GYHLKNEGG	1729	62%
P10	GNGRKLSTG	1644	75%
P9	GDVKRWHVG	1808	78%
P11	GNLDDKLSG	1673	78%
P13	GTLLGLFKG	1660	87%
P12	GRLFLRNDG	1802	92%
P8	GYSEVEEDG	1740	101%
PP3	GKFSFNIYG	1787	128%
P7	GKFKDATRG	1735	142%
PP1	GDVYIKTHG	1744	146%
P6	GNYDFYQIG	1832	154%
P5	GYLHLSWKG	1816	164%
P1	GEQYDDRLG	1808	176%
P3	GDEERIFKG	1806	182%
P2	GRELEEGIG	1715	197%
P4	GGYILTHFG	1720	219%
PP2	GFVVVKIFG	1734	279%

Direct ELISA between P16 and ZIKV NS3hel confirmed the interaction between these two binding partners. K_d was measured to be 672.2 ± 493.5 nM (Figure 4-14). P16 as we designed indeed interact with ZIKV NS3hel directly.

Figure 4-14. Interaction between P16 and ZIKV NS3hel investigated by modified direct ELISA

K_d for P16 is 672.2 ± 493.5 nM. R squared is 0.59 indicates improvement in data set is needed.

As indicated by the K_d between P16 and ZIKV NS3hel, the interaction is not strong, which might be the reason for the limited competition ability towards ZIKV NS4B and NS3 helicase interaction. In order to improve the competition ability of P16 and to have more competitor peptides, Dr. Joo-Eun Jee (IBN-A*STAR) is currently optimizing peptides screening and validation base on the sequence of P16. Upon optimization, more peptides will be screened for their competition towards the interaction between ZIKV NS3 helicase and NS4B. Chosen peptides will also be checked *in vitro* for their effects on virus replication. Moreover, as ZIKV NS3hel directly interacts with P16, a complex

crystal structure of peptide with ZIKV NS3hel will help to localize the interacting residues.

4.3 Conclusions and discussions

In this study, we mapped the interaction between ZIKV NS3 helicase and NS4B protein. Moreover, we also aimed to screen for competitors against this interaction as antiviral drug candidates.

Firstly, we managed to establish, optimize and validate the modified direct ELISA and competitive ELISA, which simplifies studies on macromolecular interaction. Secondly, the interaction between ZIKV NS3 helicase and NS4B was confirmed. The interaction region on NS3 helicase has also been narrowed down to subdomain 3 region. Thirdly, we tried to characterize the interaction region on ZIKV NS4B protein, and interestingly the previously reported cytoplasmic loop peptide region was examined to be not interacting with NS3 helicase directly. Instead, the interaction was hypothesized to be conformation dependent. With the application of *OaAEP* and addition of His₆ tag after recognition motif, cyclized peptides can be successfully purified, which enables studies on conformation-dependent interaction between ZIKV NS3 helicase and ZIKV NS4B. Last but not least, peptide P16 screened and synthesized to target ZIKV NS3hel was checked to be a potent competitor against NS3 helicase and NS4B interaction out from a pool of 18 peptides. The discovery of P16 as a good competitor opens up our discovery in antiviral therapies against ZIKV infection by targeting non-structural protein interactions.

Ever since Bruce Merrifield invented Solid Phase Peptide Synthesis (SPPS) in 1963, the process for peptide synthesis has been eased up a lot and the

usage of peptides in drug discovery has been promoted (Loffet 2002). Peptides were considered as the drugs of the future due to the advantages they have in better safety and lower development cost as comparing to small-molecule and antibody-based antiviral drugs (Loffet 2002, Yu, Deng et al. 2017). Enfuvirtide is such a successful example of peptide inhibitor, which is in the market for treatment of HIV-1 infection. The lead peptide in Enfuvirtide was originally discovered by Shibo Jiang et al. in 1993, corresponding to fusion domain binding peptide of the HIV-1 glycoprotein gp41 (Jiang, Lin et al. 1993). It gave 50% effective doses (ED_{50}) as low as 101 nM and low cytotoxicity as well (Kilby, Hopkins et al. 1998). Similar approaches for peptide inhibitors discovery have been adopted for ZIKV antiviral drug discovery including anti-ZIKV peptide Z2, P5 and boronate inhibitor cn-716. Both Z2 and P5 are from stem region of ZIKV E protein, which inactivate ZIKV virions by blocking E protein (Chen, Liu et al. 2017, Yu, Deng et al. 2017). Cn-716 on the other hand is an inhibitor for ZIKV NS2B-NS3 protease (Lei, Hansen et al. 2016). Here, we introduced another approach for peptide inhibitor by targeting protein-protein interaction thus to inhibit viral amplification. Peptide P16 showed potentials in competing binding with ZIKV NS3 helicase but with high IC_{50} in micromolar range. In order to have successful peptide inhibitor developed from P16, a few rounds of optimizations will be needed in the aspects to improving its safety, development cost as well as the form of delivery.

Chapter V: Conclusions and Future Directions

5.1 Neutralizing monoclonal antibodies against ZIKV

5.1.1 Conclusions

Three neutralizing antibodies, namely 513, 9F12 and ZAb_FLEP were studied for their interaction with ZIKV E protein or E protein domain III. These neutralizing antibodies were chosen for their outstanding neutralizing effects towards DENV or ZIKV. With the interaction assays and structural studies, we tried to understand the mechanism of neutralization for these antibodies.

From the results, we knew that scFv 513 and scFv 9F12, despite their significant neutralizing effects against DENV, the interaction with ZIKV E protein domain III seemed weak. This, on the other hand, suggested there were fundamental differences between ZIKV and DENV. For ZAb_FLEP, it is a highly potent neutralizing antibody targeting mutation-constrained epitope of ZIKV. Structure of the immune complex formed between ZAb_FLEP and ZIKV viral particle has been solved with cryo-EM, which aided in understanding its mechanism of neutralization as constraining the conformational changes needed during viral fusion with endosome membrane. It was also revealed that only 120 units of antibody were needed for neutralizing one viral particle. However, we were only able to reconstruct the electron density map to a resolution of 9.8 Å as the number of particles collected from micrographs were not enough.

5.1.2 Future directions

With the structural information obtained for ZAb_FLEP, future directions can be approached to aid in developing ZAb_FLEP into antibody therapy against ZIKV infection,

- Firstly, we could improve the resolution of Cryo-EM structure for ZAb_FLEP in complex with ZIKV by collecting more data from more Cryo-EM grids. With this, it will reveal more details on the contacting region between ZAb_FLEP Fv and ZIKV E protein, which can then be used for further engineering of ZAb_FLEP to increase its binding affinity towards ZIKV E protein.
- Secondly, ZAb_FLEP can be engineered on its Fv binding region to establish better binding affinity to ZIKV E protein. I would suggest focusing on the residues established interaction with intra-dimer interface. With the optimization in binding affinity, effective dose of ZAb_FLEP can be lowered down.
- Thirdly, ZAb_FLEP can be engineered on its Fc region. As inspired by MEDI8897, we could mutate similar residues to increase half-life of ZAb_FLEP.
- Last but not least, explore more neutralizing antibody candidates against ZIKV with computational engineering methods.

5.2 Interaction between ZIKV NS3 helicase and NS4B

5.2.1 Conclusions

To explore alternative approach for developing antiviral drugs in treating ZIKV infection, we studied the interaction between ZIKV NS3 helicase and NS4B. This interaction was known to be essential for replication complex formation in DENV. With the application of peptide ligase (*OaAEP*) in inter-molecular and intra-molecular ligation, we managed to develop a modified direct ELISA for measuring the interaction between these two proteins. The method

extended to competitive ELISA for characterizing the interaction regions on ZIKV NS3 helicase. Subdomain 3 of ZIKV NS3 helicase was determined to be responsible for interacting with NS4B. Peptides were then screened and synthesized in order to compete with this interaction.

5.2.2 Future directions

In order to clearly map down the interaction regions (residues) on ZIKV NS4B and to identify more promising competitor peptides other than P16. A series of future directions can be perused to make this study more comprehensive,

- Firstly, map interaction regions on ZIKV NS4B cytoplasmic loop with direct ELISA and ITC. In order to do so, more constructs of ZIKV NS4B cytoplasmic peptides will need to be produced.
- Secondly, to confirm the hypothesis of conformation-dependent interaction between ZIKV NS3 helicase and ZIKV NS4B by direct ELISA with cyclized and non-cyclized cytoplasmic loop peptides.
- Thirdly, co-crystallization of ZIKV NS3 helicase and inhibiting peptide candidates needs to be carried out. The interaction details can only be revealed with structural information available. In addition, structural information can help on further optimization of inhibition peptide.
- Fourthly, to optimize the interaction affinity and specificity of peptide P16 to target ZIKV NS3 helicase and NS4B interaction. We could screen peptides that can interaction with ZIKV NS3 helicase subdomain III with high binding affinity as we already knew subdomain III was in charge of interacting with ZIKV NS4B.

- Last but not least, cell-based *in vitro* studies need to be carried out to obtain ED₅₀ value as well as cytotoxicity profile in order to proceed in development of peptide inhibitor.

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Appendixes

Appendix 1. Amino Acid Sequences for ZIKV EdIII and ZIKV E

ZIKV EdIII:

MGHHHHHHENLYFQSVSYSLCTAAFTFTKIPAETLHGTVTVEVQYAGTDGPCK
VPAQMAVDMQTLTPVGRLITANPVITESTENSKMMLELDPPFGDSYIVIGVGE
KKITHHWHRSG-

ZIKV E:

MIRCIGVSNRDFVEGMSGGTWVDVVLEHGGCVTVMAQDKPTVDIELVTTTVSN
MAEVRSYCYEASISDMASDSRCPTQGEAYLDKQSDTQYVCKRTLVDRGWGNGC
GLFGKGS�VTCAKFACSKMTGKSIQPENLEYRIMLSVHGSQHSGMIVNDTGH
ETDENRAKVEITPNSPRAEATLGGFGSLGLDCEPRTGLDFSDLYYLTMNNKHW
LVHKEWFHDIPLPWHAGADTGTPHWNKEALVEFKDAHAKRQTVVVLGSQEGA
VHTALAGALEAEMDGAKGRLSSGHLKCRLKMDKLRLKGVSYSLCTAAFTFTKI
PAETLHGTVTVEVQYAGTDGPCKVPAQMAVDMQTLTPVGRLITANPVITESTE
NSKMMLELDPPFGDSYIVIGVGEKKITHHWHRSGSTIGK-

Appendix 2. Amino Acid Sequences for ZIKV NS3^{hel} and ZIKV NS4B-Strep

ZIKV NS3^{hel}:

MHHHHHHSSGVDLG TENLYFQSMEEETPVECFEPSMLKKKQLTVLDLHPGAGK
TRRVLP EIVREAIKTRLRTVILAPTRVVAEMEEALRGLPVRYMTTAVNVTHS
GTEIVDLMCHATFTSRLLQPIRVPNYNLYIMDEAHFTDPSSIAARGYISTRVE
MGEAAAI FMTATPPGTRDAFPDSNSPIMDTEVEVPERAWSSGFDWVTDHSGKT
VWFVPSVRNGNEIAACLTKAGKRVIQLSRKTFETEFQKTKHQEWD FVVTDIS
EMGANFKADRVIDSRCLKPVILDGERVILAGPMPVTHASAAQRRGRIGRNPN
KPGDEYLYGGGCAETDEDHAHWLEARMLLDNIY LQDGLIASLYRPEADKVA AI
EGEFKLRTEQRKTFVELMKRGDLPVWLAYQVASAGITYTDRRWCFDGTNNTI
MEDSVPAEVWTRHGEKRV LKPRWMDARVCSDHAALKSFKEFAAGKRG-

ZIKV NS4B-Strep:

MGHHHHHHSSGVDLG TENLYFQSMASADLEDN WETLNDNLKVIEKADNAAQVK
DALTKMRAAALDAQKATPPKLEDKSPDSPEMKDFRHGFDILVGQIDDALKLAN
EGKVKEAQAAAEQLKTTRNAYIQKYL VPRGSQ RSPQDNQMAIIIMVAVGLLGL
ITANELGWLERTKSDL SHLMGRREEGATIGFSMDIDL RPASAWAIYAALTTFI
TPAVQH AVTTSYNNYSLMAMATQAGVLFGMGKMPFYAWDFGVPLLMIGCYSQ
LTPLTLIVAIILLVAHYMYLIPGLQAAAARAAQKRTAAGIMKNPVVDGIVVTD
IDTMTIDPQVEKKMGQVLLIAVAVSSAILSR TAWGWGEAGAWSH PQFEKGGGS
GGSGGSAW SH PQFEK-

Appendix 3. Amino Acid Sequences for ZIKV NS3hel Subdomains

ZIKV NS3hel sbd 1 (VC003):

MHHHHHHSSGVDLG TENLYFQSMEEETPVECFEPSMLKKKQLTVLDLHPGAG
KTRRVLPEIVREAIKTRLRTVILAPTRVVAEMEEALRGLPVRYMTTAVNVTH
SGTEIVDLMCHATFTSRLLQPIRVPNYNLYIMDEAHFTDPSSIAARGYISTRV
EMGEAAAI FMTATPPGTRDAF –

ZIKV NS3hel sbd 2 (VC005):

MHHHHHHSSGVDLG TENLYFQSMAFPDSNSPIMDTEVEVPERAWSSGFDWVTD
HSGKTVWFVPSVRNGNEIAACLTKAGKRVIQLSRKTFETEFQKTKHQEWDFVV
TTDISEMGANFKADRVIDSRCLKPVILDGERVILAGPMPVTHASAAQRRGRI
GRNPKNKPGDEYLYGGGCAETDEDHAH –

ZIKV NS3hel sbd 3 (VC008):

MHHHHHHSSGVDLG TENLYFQSMHWLEARMLLDNIYLLQDGLIASLYRPEADKV
AAIEGEFKL RTEQRKTFVELMKRGDLPVWLAYQVASAGITYTDRRWCFDGTN
NTIMEDSVPAEVWTRHGEKRV LKPRWMDARVCS DHAALKSFKEFAAGKRG NGL
–

Appendix 4. Amino Acid Sequences for Cytoloop-His and GST-Cytoloop

Cytoloop-His:

MSDKIIHSPILGYWKIKGLVQPTRLLEYLEEKYEEHLYERDEGDKWRNKKFE
LGLEFPNLPYYIDGDVKLTQSMAIIRYIADKHNMLGGCPKERAETISMLEGAVL
DIRYGVSR IAYS KDFETLKVDFLSKLP EMLKMFEDRLCHKTYLNGDHVTHPDF
MLYDALDVVLYMDPMCLDAFPKLVCFKKRIEAI PQIDKYLKSSKYIAWPLQGW
QATFGGGDHPPKLEVL FQGHMENLYFQGI PGLQAAAAARAAQKR TAAGIMKNPV
VDGIVVTDIDTMTIDPQVEKKMGQVLLNALHHHHHHH-

GST-Cytoloop:

MSDKIIHSPILGYWKIKGLVQPTRLLEYLEEKYEEHLYERDEGDKWRNKKFE
LGLEFPNLPYYIDGDVKLTQSMAIIRYIADKHNMLGGCPKERAETISMLEGAVL
DIRYGVSR IAYS KDFETLKVDFLSKLP EMLKMFEDRLCHKTYLNGDHVTHPDF
MLYDALDVVLYMDPMCLDAFPKLVCFKKRIEAI PQIDKYLKSSKYIAWPLQGW
QATFGGGDHPPKLEVL FQGHMHHHHHHHENLYFQGLQAAAAARAAQKR TAAGIMK
NPVVDGIVVTDIDTMTIDPQVEKKMGQVLLNAL-

Appendix 5. Full text for publication related to Chapter III

Article

Cell Host & Microbe

Rational Engineering and Characterization of an mAb that Neutralizes Zika Virus by Targeting a Mutationally Constrained Quaternary Epitope

Graphical Abstract

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In Brief

Tharakaraman et al. describe the engineering and validation of a neutralizing anti-Zika antibody (ZAb_FLEP) that targets a mutationally constrained surface epitope formed by the envelope protein. ZAb_FLEP neutralizes ZIKV strains *in vitro* and protects mice and unborn pups from Zika infection *in vivo*, indicating its potential as a therapeutic candidate.

Highlights

- Engineered anti-ZIKV mAb ZAb_FLEP targets a mutationally constrained quaternary epitope
- ZAb_FLEP neutralizes multiple ZIKV strains belonging to different lineages
- ZAb_FLEP therapy prevents vertical transmission and protects fetuses in pregnant mice
- Cryo-EM structure of ZIKV: ZAb_FLEP Fab complex explains mechanism of neutralization

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Rational Engineering and Characterization of an mAb that Neutralizes Zika Virus by Targeting a Mutationally Constrained Quaternary Epitope

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SUMMARY

Following the recent emergence of Zika virus (ZIKV), many murine and human neutralizing anti-ZIKV antibodies have been reported. Given the risk of virus escape mutants, engineering antibodies that target mutationally constrained epitopes with therapeutically relevant potencies can be valuable for combating future outbreaks. Here, we applied computational methods to engineer an antibody, ZAb_FLEP, that targets a highly networked and therefore mutationally constrained surface formed by the envelope protein dimer. ZAb_FLEP neutralized a breadth of ZIKV strains and protected mice in distinct *in vivo* models, including resolving vertical transmission and fetal mortality in infected pregnant mice. Serial passaging of ZIKV in the presence of ZAb_FLEP failed to generate viral escape mutants, suggesting that its epitope is indeed mutationally constrained. A single-particle cryo-EM reconstruction of the Fab-ZIKV complex validated the structural model and revealed insights into ZAb_FLEP's neutralization mechanism. ZAb_FLEP has potential as a therapeutic in future outbreaks.

INTRODUCTION

Zika virus (ZIKV) has recently emerged as a global arboviral threat that has caused an estimated 30,000 human infections worldwide in the last 1 year alone (Lessler et al., 2016; Musso, 2015; Zhang et al., 2017). The rapid spread of the virus, aided in part by its cellular tropism, diverse transmission modes, and ability to bypass the human type I interferon response, calls for the rapid development of effective countermeasures (Rodriguez-Morales et al., 2016). While an effective vaccine against

ZIKV is still many years away (Barouch et al., 2017), monoclonal antibodies have shown promise in controlling infection in animal models (Sapparapu et al., 2016; Wang et al., 2016; Zhao et al., 2016), indicating that they could serve as a potential immunotherapy option for infected or at-risk individuals.

Before the time of ZIKV emergence in humans, an increasing number of studies were reporting highly potent broad-spectrum neutralizing monoclonal antibodies against Dengue virus (DENV, a related flavivirus) that were either engineered or isolated from infected patients (Beltramello et al., 2010; de Alwis et al., 2011; Lai et al., 2013). The progress in the DENV field has led to a rapid acceleration in the identification of neutralizing antibodies for ZIKV. Like DENV, the surface envelope (E) protein of ZIKV is responsible for viral entry and fusion, which makes it the main target of neutralizing antibodies (Heinz et al., 1994). Examples of anti-ZIKV antibodies include DENV antibodies that cross-react with ZIKV and ZIKV-specific antibodies (generated in murine models or isolated from infected human patients) that target different regions of the E-protein with neutralization potencies (PRNT₅₀) ranging from 0.014 to 6.560 μ g/mL (Barba-Spaeth et al., 2016; Sapparapu et al., 2016; Wang et al., 2016; Zhao et al., 2016). Despite these developments, no prior approaches have described antibody engineering from the standpoint of targeting specific epitope surfaces on the whole ZIKV spherical assembly. Such an approach may be highly desired, for instance, in order to target highly conserved regions of the E-protein assembly and therefore minimize the chance that the virus will escape the neutralizing antibody response. Indeed, a previous study isolated escape viruses to anti-ZIKV monoclonal antibody therapy by using an *in vivo* animal model and by means of serially passaging the virus in cell culture (Wang et al., 2017). Additionally, since these outbreaks are sporadic and short-lived, obtaining affinity matured antibodies from the peripheral blood mononuclear cells of ZIKV-infected individuals can be challenging. Consequently, a rational approach for engineering antibodies against mutationally constrained epitopes with potencies similar to previously reported monoclonal antibodies will likely be valuable for combating future outbreaks.

Figure 1. Computational Identification of Highly Constrained E-Protein Epitopes

(A) Average (per residue) network score and SASA of Zika antibody epitopes in the 2-fold, 3-fold, and 5-fold axes of the ZIKV cryo-EM assembly. Values in each column are colored into lighter shades (lower value) and darker shades (higher value).

(B) Putative FLEP epitope depicted on an asymmetric unit in a raft structure. The raft contains three E-protein dimers forming two asymmetric units. The three E-chains that are oriented parallel to each other in the raft are labeled A, B, and C, and those oriented in the opposite direction, A', B', and C', respectively. The rhombus that encloses the six chains is represented by a black line. The domains are color coded as follows: orange, domain I; yellow, domain II; blue, domain III. The 532 axes of symmetry are highlighted by blue polygons. Residues predicted to form the epitope are represented by spheres in one of the asymmetric units (chains labeled B-C-C'). The FLEP epitope proximal to the 3-fold axis of symmetry is highlighted by an oval. Note the proximity of the epitope residues to the adjacent dimer, which greatly influences the epitope's surface geometry.

(C) Flexibility and conformational changes of E-protein. PDB structures corresponding to the different E-protein conformations were selected (PDB: 1K4R, mature; PDB: 1UZG, IM(1); PDB: 1TG8, IM(2); PDB: 1OK8, PF; PDB: 5U4W, immature) and aligned, where the alignment is based on the DI-DII domain. The N- and C-terminal residues are represented by spheres and colored in brown and yellow, respectively. While DI-DII domains (e.g., FLEP epitope) appear to be largely invariant between the structures, significant conformational differences were observed in the DI-DIII region (vis-à-vis ZV67 epitope), particularly in the hinge region connecting the two domains. IM, intermediate; PF, postfusion.

(D) Illustration of rational engineering of antibodies targeting FLEP-like surface. Overview of antibody scaffold selection and paratope engineering (left); representative scaffolds targeting FLEP-like surface (right).

(E and F) Computationally docked model of Fv interacting with the E-protein asymmetric unit. Views perpendicular (E) and along (F) the 2-fold axes are shown. Select residues that make contact with Fv are shown as spheres.

Herein, we first defined potential epitope surface residues of the E-protein based on residue mutability constraints in the context of the entire spherical assembly of ZIKV. Toward this goal, we built a 3D homology model of E-protein ZIKV assembly to correlate the structural and network properties of the epitope surfaces with *in vitro* neutralization data obtained using a panel of ZIKV-specific and cross-reactive antibodies. Based on this, we demonstrated that antibodies that target highly networked, solvent accessible, inter-chain epitope surfaces are associated with more potent neutralization than antibodies that target intra-domain epitopes. Importantly, we identified a quaternary epitope surface proximal to the fusion loop (FLEP) that is the most structurally constrained among the various epitope surfaces. Driven by this careful epitope selection procedure, we then applied a computational approach to engineer an antibody (ZAb_FLEP) that specifically targets the FLEP quaternary epitope surface in ZIKV. Based on the computational analyses, we postulated that this antibody would show potent neutralization and that the evolution of

ZIKV escape mutants that could evade neutralization would be unlikely.

We first validated our hypothesis by performing biochemical binding studies and PRNT-based *in vitro* neutralization assays. Notably, ZAb_FLEP demonstrated neutralization of ZIKV strains from different lineages and circulation periods. Second, passive transfer of ZAb_FLEP in an *in vivo* lethal mouse model of ZIKV infection conferred 100% survival. Third, administration of ZAb_FLEP to pregnant mice in a post-exposure challenge resolved maternal-fetal placental transmission and fetal demise, highlighting ZAb_FLEP's potential for treating or preventing ZIKV fetal disease in humans. Fourth, serial passaging of ZIKV in the presence of ZAb_FLEP failed to generate viral escape mutants, which is consistent with our prediction that FLEP epitope is mutationally constrained. Finally, determination of the cryoelectron microscopy (cryo-EM) structure of ZIKV in complex with the antibody-binding fragment (Fab) of ZAb_FLEP validated the theoretical structural model and helped explain the neutralization mechanism of the engineered antibody.

Table 1. Binding and Neutralization Characteristics of Antibody Scaffolds

	Antibody	DENV-1 (EDEN2402)	DENV-2 (ST)	DENV-3 (EDEN863)	DENV-4 (EDEN2270)	ZIKV (H/PF/2013)	ZIKV (PF13)	ZIKV (MR766)
Affinity (Kd - μ g/mL)	4G2	0.192	0.149	2.482	0.814	10.290	6.405	0.042
	PGT124	no/weak binding	no/weak binding	no/weak binding	no/weak binding	no/weak binding	388.200	no/weak binding
	ZAb_FLEP	0.604	2.665	36.740	0.925	22.990	19.320	0.029
Neutralization PRNT50 (μ g/mL)	4G2	no neutralization	no neutralization	no neutralization	no neutralization	>500	no neutralization	no neutralization
	PGT124	no neutralization	no neutralization	no neutralization	no neutralization	>500	no neutralization	no neutralization
	ZAb_FLEP	<3.900	<3.900	15.600	15.600	3.579	5.092	0.022

ZAb_FLEP binds and neutralizes ZIKV from all three lineages tested. Reflecting its binding affinities, ZAb_FLEP most strongly neutralizes African strain (MR766), followed by the French Polynesian (H/PF/2013) and Brazilian (Paraiba) strains. In contrast, the widely used 4G2 antibody showed no neutralization of ZIKV, despite binding. We also examined the neutralization potency of ZAb_FLEP against clinical isolates representative of DENV serotypes 1–4. ZAb_FLEP was able to potentially neutralize DENV-1 and -2 and to a lesser extent DENV-3 and -4. DENV, Dengue virus; EDEN, Early Dengue Infection and Outcome Study; ZIKV, Zika virus.

RESULTS

Structure-Guided Analysis of Potential E-Protein Quaternary Epitopes

Optimal epitope selection is a critical first step in the engineering of a neutralizing antibody. Examples of important factors to consider during epitope selection include sequence conservation of the epitope across strains, accessibility of the epitope (in the context of the viral assembly), and the epitope's conformational flexibility. To gain a systematic understanding of how these epitope characteristics affect the neutralization ability of antibodies that target them, we examined the accessibility, conservation, mode of binding (e.g., occupancy), and binding affinity of a panel of different DENV antibodies and correlated these factors with the reported PRNT50 values (STAR Methods). The analysis revealed that the inter-chain locking potential (i.e., the ability of the antigen binding fragment to engage two or more chains), solvent accessibility, and network score are dominant factors that correlate with PRNT50 (Figure S1). In other words, antibodies that target quaternary epitopes spanning more than one chain are generally the most potent. Among the quaternary epitope targeting antibodies, those that target intra-dimer epitopes also exhibit a relatively high degree of epitope sequence conservation and limited conformational flexibility, highlighting their potential as promising therapeutic candidates. However, given the limited viral structural information for ZIKV (during the start of this work), we built the 3D model of E-protein ZIKV assembly starting from the homology model of soluble E-protein in order to understand the various neutralizing antibody epitope surfaces in the context of an assembled virus (STAR Methods). Simultaneously, we performed a PRNT-based neutralization of ZIKV for a panel of chosen antibodies (Figure S2). This analysis showed that the inter-chain locking potential is the most dominant factor that correlates with PRNT50, similar to what was observed with DENV (Table S1). Further, the analysis permitted us to define thresholds for network score and accessibility based on matching the corresponding values of the different antibodies (STAR Methods).

Taking advantage of this information, we employed a computational method that uses a residue interaction network based on graph theory in the context of the modeled E-protein ZIKV assembly to identify epitope surfaces that exhibit inter-chain locking potential, a high degree of residue interconnectivity, and epitope accessibility (STAR Methods). Accordingly, each residue in the assembly is assigned three variables: (1) a network score that reflects the constraints imposed on it by its environment; (2) its location relative to the 5-fold, 3-fold, and 2-fold symmetry axes; and (3) solvent and antibody accessibility in the viral assembly. The geometrical parameters are especially important when identifying potential quaternary epitopes that span or fall near E-protein dimer interfaces, which are extensively influenced by the membrane curvature and inter-chain interface orientations about symmetry axes of the assembly (STAR Methods). A potential epitope surface is defined as a set of spatially clustered, surface-exposed residues that are highly inter-connected by non-covalent interactions (STAR Methods).

Our analyses of the modeled E-protein ZIKV assembly revealed that the region involving the fusion loop (E-DII) of one monomer and a small portion of E-DI and E-DIII of the adjacent monomer is highly networked and accessible compared with other neutralizing antibody epitopes (Figures 1A and 1B). Consistent with the observed extensive inter-residue interactions in the fusion loop epitope-proximal region, only seven amino acid mutations were observed in the FLEP epitope in the 254 ZIKV strains found in GenBank (Figure S3). Notably, the fusion loop epitope-proximal epitope (FLEP) we define is enclosed within a dimer region, which indicates that the entire assembly presents 180 sites available for binding (at full occupancy) (Figure 1B). Furthermore, FLEP appears to be structurally invariant in the different pH-sensitive viral forms, indicating that the antibody is unlikely to dissociate from the antigen even in the low pH compartments of the endosome (Figure 1C). The quaternary nature of the FLEP epitope led us to postulate that antibodies that target this region would be capable of neutralizing intracellularly by blocking the fusion process and therefore would lower any risk of antibody-dependent enhancement or ADE (Chan et al., 2011).

Figure 2. In Vitro Neutralization of Zika Strains by Illustrative Antibodies

While 4G2 and PGT124 did not neutralize Zika virus efficiently (A), ZAb_FLEP neutralized all three strains with dose response (PRNT₅₀ = 0.022–5.092 μ g/mL) (A–C). Data points represent averages of $n = 3$ experiments with error bars representing SD. See also Figure S5.

different strains, MR766 (1947/Uganda virus), H/PF/2013, and PF13 (both French Polynesia, 2013), via ELISA (STAR Methods). In parallel, we also evaluated the engineered antibodies for their ability to inhibit ZIKV infection using a PRNT-based neutralization assay (STAR Methods). A significant number of the engineered antibodies failed to show binding, but the analyses yielded a few ZIKV binding antibodies with varying affinities against the three strains. After multiple iterations of testing (STAR Methods), ZAb_FLEP was obtained and showed neutralization of ZIKVs at PRNT₅₀ values of 0.022–5.098 μ g/mL (Table 1).

Based on the theoretical structural model of antibody binding at the 2-fold and 3-fold vertices, the variable region displayed potential to make contacts with the adjacent E-dimer, thereby effectively locking two adjacent dimers (Figures 1E and 1F). On the other hand, the theoretical model predicted that binding near the 5-fold vertices would be prevented due to steric hindrance from the adjacent dimer (STAR Methods). The *in vitro* neutralization potential of ZAb_FLEP approaches the potency of select Zika antibodies (e.g., PRNT₅₀ of DIII binding antibody ZV67 against H/PF/2013 measured in-house is 1.966 μ g/mL; data not shown). Interestingly, ZAb_FLEP showed better binding to ZIKV (MR766) relative to the more recent ZIKV (H/PF/2013) and ZIKV (PF13) strains (Table 1). This could be due to the absence of N-glycosylation at Asn154 of MR766 (Musso and Gubler, 2016), which may promote better accessibility to the fusion loop epitope. Despite the lower binding activity, ZAb_FLEP showed useful ZIKV neutralization activity against all strains tested (Figure 2). Additionally, ZAb_FLEP cross-reacted and neutralized the four serotypes of DENV (Low et al., 2006), albeit weakly (Table 1). We opted to focus on ZAb_FLEP for further studies given its ability to neutralize a wide variety of genetically diverse strains (Table 1; Figure 2).

In Vivo Efficacy of ZAb_FLEP in A129 Mice

To assess the prophylactic and therapeutic efficacy of ZAb_FLEP, A129 mice (8–11 weeks old, $N = 4$), which lack the receptor for type I interferon (IFN α/β), were given 50 μ g (~ 1.7 mg/kg) antibody or control immunoglobulin (IgG) and infected intraperitoneally on -1 (day post-infection [d.p.i.]) and $+1$ (d.p.i.) with H/PF/2013 strain at 10^3 plaque-forming units (PFU) (STAR Methods). Analysis of this ZIKV infection model shows that virus infection

While some antibody epitopes possess higher network score or solvent accessible surface area (SASA) than the FLEP region, none of the known epitopes possess inter-chain locking potential as well as higher network score and SASA values (Figure 1A). For instance, Z3L1 (Wang et al., 2016) targets a highly accessible region but its epitope residues are poorly networked and, hence, susceptible to viral escape. On the other hand, ZV-2 (Zhao et al., 2016) targets a highly networked epitope, but the region is partially hidden on the viral assembly, thereby increasing the risk of ADE (Figure 1A). Based on FLEP's promising characteristics taken together with the repertoire of antibodies that engage this region, we chose this epitope as the target for our antibody engineering effort as discussed below.

Rational Engineering of Neutralizing Antibodies against the FLEP Epitope

Herein, we took a two-pronged approach for antibody engineering, which included (1) scaffold selection and (2) paratope engineering. First, scaffolds targeting fusion-loop-like structures were used as anchoring points to develop the initial ZIKV antigen:antibody structural models (STAR Methods). In paratope engineering, we investigated complementarity-determining region (CDR) substitutions that provided optimal contacts with the epitope surface, using the approach described in the DENV studies (STAR Methods). The engineering approach took into consideration important differences in the accessibility and network scores of epitope residues, as these parameters vary across different icosahedral vertices on the spherical assembly. The engineered antibodies were tested for binding to three

Figure 3. In Vivo Efficacy of ZAb_FLEP

ZAb_FLEP (50 μ g) reduces viremia (A and B) and protects A129 mice against weight loss (C and D) and death (E and F) when administered -1 or $+1$ d.p.i. $n = 5$ for each condition, experiment performed twice, error bars indicate SD of the data, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. See also Table S2.

ZAb_FLEP Therapy Prevents Vertical Transmission and Protects Fetuses in Pregnant Mice

Next, we evaluated the potential of ZAb_FLEP to prevent vertical infection and fetal mortality in pregnant A129 mice. Others and we have observed placental and fetal infection and fetal mortality in pregnant A129 mice that are infected with ZIKV (Sapparapu et al., 2016). A total of $N = 18$ pregnant A129 mice that were mated to male mice for 4 days (starting on day 0 evening and separated on day 4 morning) were injected with 10^3 PFU ZIKV (H/PF/2013 strain of Asian lineage) intravenously on day 10 (corresponding to embryonic day 7–10; i.e., E7–E10). These mice were treated with 50 μ g ZAb_FLEP ($N = 9$) or an isotype control IgG ($N = 9$) 24 hr (E8–E11) after infection. The mice were sacrificed on day 17 (E14–E17) and viral RNA levels were analyzed on day 12 (blood) and day 17 (blood, placenta, and fetal compartments)

(Figure 5A) (STAR Methods). When compared with isotype control IgG-treated mice, the ZAb_FLEP-treated mice had substantially lower levels of viral RNA in blood on day 12 ($p < 0.0001$; two-sided t test) and day 17 ($p = 0.0018$; two-sided t test) (Figures 5B and 5C). Significantly, ZAb_FLEP treatment reduced placental ($p < 0.0001$; two-sided t test) and fetal ($p < 0.0001$; two-sided t test) infection and provided protection against fetal mortality. Notably, fetuses harvested from dams in the control group had 100% lethality (Figures 5D–5F). Similarly, treatment with the isotype control led to significant levels of placental and fetal infection, with concomitant fetal mortality by day 17 (Figure 5D). Fetuses harvested from dams that were treated with ZAb_FLEP showed signs of normal embryo development without any developmental impairment; on the other hand, fetuses from isotype control IgG treated dams were dead before harvesting, revealing a stark morphological difference compared with normal fetuses (Figures 5G and 5H).

Analysis of the Mechanism of Neutralization of ZAb_FLEP

To investigate the mechanism of neutralization and to validate the structural model of the ZAb_FLEP:E-protein interaction, we determined the 9.7 Å structure of Fab ZAb_FLEP in complex with ZIKV using cryo-EM (STAR Methods; Figure S4). Interestingly, we observed a total of 120 Fab fragments bound to the viral

causes 100% mortality by day 10 post-infection, accompanied with signs of neurological deficits, as well as high viral loads in brain, spinal cord, serum, spleen, and testes (data not shown). Efficacy of the antibody was monitored by assessing mortality, weight loss, and viremia reduction. While mice administered control IgG died by day 11, 100% of ZAb_FLEP-administered mice survived until day 30 (Figure 3). It must be noted that in the antibody protection studies published thus far, survival is monitored only up to 15–20 days (Sapparapu et al., 2016; Wang et al., 2016; Zhao et al., 2016). Furthermore, ZAb_FLEP prevented weight loss and reduced viremia by ~ 2 –3 logs when compared with the control antibody, explaining the reason behind the survival rate (Figures 3A–3D). In light of these data, we monitored weight loss and viral load reduction in A129 mice with 10 mg/kg (~ 300 μ g), 2 mg/kg (~ 60 μ g), and 0.2 mg/kg (~ 6 μ g) doses of ZAb_FLEP under a therapeutic challenge (+1 d.p.i.) with H/PF/2013 strain at 10^3 PFU (Figures 4A–4C). Remarkably, higher doses of ZAb_FLEP protected mice without resulting in cytotoxicity (Figures 4A–4C). Importantly, we did not see differences in viremia or accelerated death even when significantly lower doses (0.2 mg/kg ~ 6 μ g) of ZAb_FLEP were administered. This contrasts with the findings of dengue antibody studies in A129/AG129 mice that showed that lower doses can result in enhanced disease progression resulting in increased or accelerated death due to ADE (Balsitis et al., 2010).

Figure 4. Examining the Effects of ZAb_FLEP Dosing on In Vivo Efficacy

Evaluating the effect of ZAb_FLEP dosing on weight loss (A), viremia (B), and survival (C) in A129 mice. A total of 2 mg/kg (~60 μ g) ZAb_FLEP reduces viremia and provides complete protection against weight loss and survival. Remarkably, there were no observable differences in viremia or accelerated death even when significantly lower doses (0.2 mg/kg ~ 6 μ g) of ZAb_FLEP were administered. N = 5 for each condition, experiment performed twice, error bars indicate SD of the data, *p < 0.05, ***p < 0.001.

(Figure 6D). Additionally, ZAb_FLEP makes more contacts with the adjacent dimer residues than C10 (Figure 6E), although the contributions of these residues toward the binding energetics may still be marginal relative to the intra-dimer epitope residues. In summary, the topological nature of ZAb_FLEP epitope combined with the differences in the arrangement of the E-protein near the different icosahedral vertices potentially restricts the occupancy sites to 120. The spacing between the bound Fab fragments suggests the possibility of a bivalent binding pattern of ZAb_FLEP antibody, wherein the two arms (Fab) of a single IgG contact the epitopes near the 2-fold and 3-fold icosahedral axes, respectively (Figure 6B). The binding mode of the Fabs and the distances between the heavy-chain C terminus of adjacent Fab fragments have been used by others to evaluate such a bivalent mode of engagement (Ye et al., 2016). The epitope specificity of ZAb_FLEP mapping to the fusion loop peptide combined with its ability to lock the intra-dimer and inter-dimer interfaces suggests that the monoclonal antibody (mAb) neutralizes by preventing the necessary conformational changes required for the fusion event.

To verify the mechanism of action of ZAb_FLEP, we evaluated the neutralization potential of ZAb_FLEP in Fc γ R-expressing THP-1.2S monocyte cells and used 4G2 antibody as a reference, as 4G2 has been well established as a fusion-inhibiting antibody (Watterson et al., 2016). Studies carried out in our laboratory have shown that antibodies that are able to inhibit virus fusion with phagosomal membranes are most likely to prevent infection of Fc γ R-expressing cells (Chan et al., 2011). Different concentrations of antibody were incubated with ZIKV (strain H/PF/2013) for 1 hr prior to infecting THP1.2S monocytes. Three days later, the virus replication in culture supernatants was measured by plaque assay on BHK21 cells. We observed complete neutralization following treatment with ZAb_FLEP and near-complete neutralization with 4G2, but not with a weak-affinity ZAb_FLEP variant (K_d > 50 μ g/mL) (Figure S5A). These results provide mechanistic support for the anti-fusion activity of ZAb_FLEP. We also noted that a weak-affinity variant of ZAb_FLEP failed to neutralize the virus even at the highest concentration, resulting in 3-fold higher peak titers compared with ZAb_FLEP

particle around the icosahedral 2-fold and 3-fold axes (Figure 6A). Remarkably, there were no Fab fragments bound around the twelve 5-fold axes, which accounts for the absence of 60 Fab molecules (Figure 6A). The reconstruction process performed with a Fab-free model converged to the same solution with 120 Fab molecules, confirming very low to zero occupancy of Fab around the 5-fold axis. Thus, ZAb_FLEP is able to efficiently neutralize ZIKV infection even without completely covering the viral particle (Figure 6B). We fit 180 copies of E-monomer atomic structure onto the density map and docked the ZAb_FLEP binding fragment (Fv) against the E-protein using the predicted docked model as the reference (STAR Methods). Although the exact orientation of heavy and light chains could not be discerned from the density map, the Fv fitted into the electron density with good correlation, thus validating the structural modeling procedure. Modeling of a complex with 180 Fv bound gave rise to some exciting observations: (1) ZAb_FLEP makes inter-dimer contacts, as predicted by the computational modeling, and (2) while the Fab is nicely accommodated at the 2-fold and 3-fold vertices, binding of the Fab near the 5-fold assembly (Figure 6C). The recently described envelope dimer epitope human monoclonal antibodies (C8, C10, A11, and B7) target overlapping regions (Dejnirattisai et al., 2016; Barba-Spaeth et al., 2016), suggesting that the region is in fact immunogenic in humans. However, comparison between the cryo-EM complex structures of C10, which binds to all 180 sites on the viral assembly, and ZAb_FLEP shows differences in the orientation of ZAb_FLEP with respect to the E asymmetric unit

Figure 5. In Vivo Efficacy of ZAb_FLEP in a Maternal Transfer Mouse Model

(A) The overall study design.
(B) Viral load reduction in the blood collected from pregnant A129 mice on day 12 and day 17. In each case, significance was established by comparing viral RNA copies from the ZAb_FLEP treated ($n = 9$) and isotype control IgG treated ($n = 9$) mice.
(C) Fetal survival observed by day 17.

(Figure S5B). The potent neutralizing activity of ZAb_FLEP in Fc γ R-expressing cells combined with its epitope specificity (which, as observed by cryo-EM, maps to a region spanning the fusion loop peptide) provides a basis and a rationale for the fusion blocking mechanism of ZAb_FLEP.

Since ZAb_FLEP targets a highly networked region, we hypothesized that there should be a limited opportunity for the emergence of viral escape mutants. To test this hypothesis, we attempted to select for neutralization escape mutants after serial passaging of viruses in cell culture (STAR Methods). Briefly, we propagated H/PPF/2013 French Polynesian ZIKV strain in Vero cells at 37°C with 5% CO₂ for 48–72 hr in the presence of neutralizing concentration of antibody (90% effective concentration [EC₉₀]). EC₉₀ was chosen to ensure that there was sufficient selection pressure for the generation of escape mutants. The choice of EC₉₀ is consistent with previously published reports (Beltramello et al., 2010; de Alwis et al., 2011; Lai et al., 2013). The combination of selection and propagation was repeated for a total of seven passages. At each passage, the viral RNA was extracted and the E gene was amplified and sequenced. Importantly, we did not identify any escape mutants against ZAb_FLEP. Although this does not guarantee that escape mutation will not arise, it does imply the existence of underlying structural and/or evolutionary constraints that confer resistance against the antibody selection pressure.

DISCUSSION

There are a few important features of our epitope-driven engineering approach that were critical to the overall effectiveness of ZAb_FLEP. First, a key challenge in developing effective antiviral therapies is the development of resistance due to viral evolution (Low et al., 2017; Morens and Fauci, 2013; Ter Meulen, 2011), so we chose to enforce low epitope mutability and an inter-chain locking mechanism as strict constraints during the initial epitope selection. Maintaining the desired epitope engagement is likely what conferred ZAb_FLEP with its neutralizing ability that approaches that of those isolated from convalescent Zika patients (Barba-Spaeth et al., 2016; Robbiani et al., 2017; Sappapapu et al., 2016; Wang et al., 2016; Zhao et al., 2016). It must be noted that conventional epitope scanning methods using sequence-based approaches or analysis of a single domain or E-protein dimer did not lead to the selection of the FLEP epitope. As mentioned earlier, there was limited structural information available at the start of this work, and as a consequence we had to develop a homology model of the E-protein ZIKV assembly, which is validated by several recent structural studies (Kostyuchenko et al., 2016; Sirohi et al., 2016; Zhang et al., 2016). This is significant because it was important to capture geometrical parameters when identifying potential quaternary epitopes that span or fall

(D and E) Viral RNA found in fetal (D) and placental (E) compartments by day 17 in ZAb-treated ($n = 29$ samples) and isotype control IgG-treated ($n = 17$ samples) mice.

(F) Difference in placental viremia observed between ZAb_FLEP-treated and control mice.

(G and H) Fetuses harvested on day 17 from ZAb_FLEP-treated (H) and isotype control IgG (G) mice.

Error bars indicate SD of the data, ** $p < 0.01$, *** $p < 0.001$.

Figure 6. Cryo-EM Structure of 9.7 Å ZIKV: ZAb_FLEP Fab Complex

(A) Electron density of ZIKV:Fab complex is shown where the view is along a 5-fold axis. The density map highlights the lack of occupancy around the 5-fold axis.

(B) ZAb_FLEP epitope on E-protein raft. The ZAb_FLEP Fv is shown in surface format. The spacing between the C-termini of the Fvs suggests bivalent binding pattern where one arm engages the 2-fold epitope and the other engages the 3-fold epitope.

(C) Modeling of a complex with 180 Fv bound indicates steric hindrance as a reason for the lack of binding around the 5-fold axes. Shown in the figure is a view of the complex along the 5-fold where the E-monomers that form the pentamer are in surface format (magenta), and the Fv molecules that occupy the sites around the 5-fold axes are in orange and yellow. Notice that the footprint of the 5-fold Fv overlaps with the E-protein monomers in the adjacent raft.

(D) Comparison between the orientations of C10 and ZAb_FLEP Fv with respect to the E surface. The heavy and light chains of the two Fvs are represented in surface format and labeled. When viewed down the 2-fold symmetry axis, ZAb_FLEP Fv is oriented 30° counterclockwise relative to C10 Fv, which brings the vestigial loops of its VH nearer to the adjacent dimer.

(E) Comparison between the epitopes recognized by ZAb_FLEP and C10. Overlapping residues are shaded in gray, whereas residues unique to C10 and ZAb_FLEP epitopes are shaded in red and blue, respectively. View is looking down the 2-fold axis of the E-dimer.

See also Figure S4.

near E-protein dimer interfaces, as these are enormously influenced by the membrane curvature and inter-chain interface orientations about symmetry axes of the assembly.

The recently published quaternary epitope targeting anti-Zika antibody ZIKV-117 showed *in vivo* efficacy in a similar maternal transfer model, albeit in a prophylaxis setting (Sappapapu et al., 2016). While both ZIKV-117 and ZAb_FLEP recognize different epitopes, they potentially neutralize the virus by locking the E-monomers in the raft (Hasan et al., 2017). Similar to ZAb_FLEP, binding of ZIKV-117 Fab to the 5-fold vertex is restricted due to steric hindrance between the Fab and the E-monomer from the adjacent raft. It seems that the potency of ZIKV-117 and ZAb_FLEP is partly due to their ability to lock multiple antigens on the surface. Dosing studies indicate that ZAb_FLEP has the potential to provide partial protection even at low concentrations without increasing viremia associated with disease progression. Aside from the quaternary epitope, we believe that the potency of ZAb_FLEP is due to a combination of factors including binding affinity, epitope accessibility, and stoichiometry. Indeed, the fact that ZAb_FLEP conferred 100% survival

of mice for a significantly longer period of time than reported by previous reports strongly indicates its therapeutic potential (Table S2).

Finally, the emergence of infectious disease outbreaks such as ZIKV and DENV calls for the rapid development and clinical testing of vaccines or therapies. In addition to ZIKV and DENV, the ongoing threat of a yellow fever outbreak in Brazil (Goldani, 2017) and the re-emergence of Ebola in West Africa (Dudas et al., 2017) could lead to substantial worldwide morbidity and mortality. Because our epitope selection method does not rely on any characteristics intrinsic to ZIKV or flaviviruses but instead leverages well-defined structural metrics, we believe that it can be applied to any virus assembly, which can then guide appropriate antibody engineering efforts regardless of the platforms that might be used to actually generate the antibody.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and two tables and can be found with this article online at <https://doi.org/10.1016/j.chom.2018.04.004>.

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AUTHOR CONTRIBUTIONS

K.T., S.W., K.R.C., S.G.V., E.E.O., and R.S. conceived and designed the study experiments. K.T., D.Q., A.R., and R.S. conducted computational analyses, including epitope prediction and antibody engineering. K.R.C., V.S., Y.H.C., M.M., E.Z.O., E.S.G., H.C.T., and E.E.O. performed the biochemical, *in vitro*, and regular A129 *in vivo* experiments. S.W., K.R.C., E.Z.O., E.S.G., H.C.T., and S.G.V. designed and conducted the pregnant A129 mice experiments. J.H., A.T., S.B., and J.L. solved the cryo-EM structure of virus:Fab complex and completed structural analysis. K.T., A.R., S.G.V., J.L., E.E.O., and R.S. wrote the manuscript.

DECLARATION OF INTERESTS

MIT and Duke-NUS own intellectual property related to anti-Zika monoclonal antibodies that are described in part in this manuscript. E.E.O. and R.S. are board members of Tychan Pte. Ltd., which is conducting clinical studies related to anti-Zika therapeutics.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
ZAb_FLEP	This paper	RRID: AB_2725794
Goat anti-Hu IgG HRP Conjugated secondary antibody	Abcam	ab98624
Bacterial and Virus Strains		
Zika virus H/PF/2013 strain (French Polynesia)	European Virus Archives	NCBI (KJ776791)
Zika virus MR766 (1947/Uganda virus)	Duke-NUS infectious clones	NCBI (LC002520)
Zika virus PF13 (both French Polynesia, 2013)	Duke-NUS infectious clones	NCBI (KX369647)
Dengue virus-1 (EDEN2402)	EDEN study (Low et al., 2006)	DENVDB (158851730)
Dengue virus-2 (ST)	Singapore General Hospital	NCBI (JF327392)
Dengue virus-3 (EDEN863)	EDEN study (Low et al., 2006)	DENVDB (158851650)
Dengue virus-4 (EDEN2270)	EDEN study (Low et al., 2006)	NCBI (GQ398256)
Chemicals, Peptides, and Recombinant Proteins		
Mouse recombinant 4G2	PDB	RRID: AB_10903311; PDB: 1UYW
Human recombinant PGT124	PDB	RRID: AB_2725801; PDB: 4R26
Human recombinant C8	PDB	RRID: AB_2725799; PDB: 5LBS
Mouse recombinant ZV67	PDB	RRID: AB_2725795; PDB: 5KVG
Mouse recombinant ZV48	PDB	RRID: AB_2725796; PDB: 5KVE
Human recombinant Z23	PDB	RRID: AB_2725797; PDB: 5GZR
Human recombinant Z3L1	PDB	RRID: AB_2725798; PDB: 5GZN
Human recombinant C10	PDB	RRID: AB_2725800; PDB: 5H30
Deposited Data		
Cryo electron microscopy	Electron microscopy database (EMDB)	EMDB: EMD-7613
Experimental Models: Cell Lines		
BHK-21 (baby hamster kidney)	ATCC	CCL-10
C6/36 cells	ATCC	CRL-1660
Vero cells	ATCC	CCL-81
Freestyle 293 cells	N/A	N/A
Experimental Models: Organisms/Strains		
Mouse: Sv/129 mice deficient in type I IFN receptor (A129)	B&K Universal	N/A
Oligonucleotides		
ZIKV amplification and sequencing for escape mutant studies (Forward primer)	AIT Biotech	5' GCTTGGCTTTTG GGAAGCTC 3'
ZIKV amplification and sequencing for escape mutant studies (Reverse primer)	AIT Biotech	5' GGGGGAGTC AGGATGGTACT 3'
Additional ZIKV sequencing primer for escape mutant studies	AIT Biotech	5' AACATGGCS GAGGTAAGATC 3'
Software and Algorithms		
Discovery Studio	Accelrys	Molecular modeling software (http://accelrys.com/products/collaborative-science/biovia-discovery-studio/)
PyMOL v2.0	PyMOL	Molecular visualization software (https://pymol.org/2/)

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Multivariate Logistic Regression (MLR), Amino acid Interface Fitness (AIF)	Laboratory of Sasisekharan R (Tharakaraman et al., 2013)	http://www.pnas.org/content/110/17/E1555.full
EMAN v2	Baylor college	http://ncmi.bcm.edu/ncmi/software/software_details?selected_software=counter_222
Prism v7	GraphPad	Statistical analysis software (https://www.graphpad.com/scientific-software/prism/)
Chimera	UCSF	Visualization software (https://www.cgl.ucsf.edu/chimera/)
Other		
NUNC Maxisorp flat-bottom	Thermo Fisher	44-2404-21
TMB Substrate	Thermo Fisher	34021
QIAamp Viral RNA Mini kit	Qiagen	52906
One-Step Superscript III RT-PCR System with Platinum Taq	Invitrogen	12574018
MinElute Gel Extraction Kit	Qiagen	28606
Tissue Lyser	Qiagen	85300

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Ram Sasisekharan (rams@mit.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Ethics Statement

Mouse studies were conducted at the DUKE-NUS Medical School in Singapore following approval from the Institutional Animal Care and Use Committee (IACUC).

Vertebrate Animals

Sv/129 mice deficient in type I IFN receptor (A129), purchased from B&K Universal (UK), were housed in the BSL-2 animal facility at Duke-NUS, Singapore and all animal experiments (protocol 2016/SHS/1197) were approved by the Institutional Animal Care And Use Committee at Singapore Health Services. Eight to eleven week-old male A129 mice were used to assess prophylactic or therapeutic efficacy of antibodies. For this, Mice were injected intraperitoneally (ip) with antibodies one day before (prophylactic) or after (therapeutic) infection with 5×10^4 pfu of H/PF/2013 ZIKV strain (ip). Mouse blood was collected from facial vein on days 1-21 pi to measure serum viremia level by real-time PCR. Mouse weight and survival were monitored until 30 days post-infection (pi). To assess the protective efficacy of the antibody against fetal infection in pregnancy, 9-10 weeks old female A129 mice were housed with 9-10 weeks old male A129 mice in same cages (1 female with 1 male) for 4 days. Female mice were infected intravenously (iv) with 1×10^3 pfu of H/PF/2013 strain on day 10 (corresponding to embryo day 7-10). The mice were injected iv with 50 μ g of antibodies one day pi. Blood was collected from facial vein on day 2 pi to measure viremia. Mice were euthanized by CO₂ inhalation on day 7 pi and blood was collected from the post caval vein. Placenta and whole fetuses were harvested and snap-frozen in liquid nitrogen. Frozen samples were then homogenized by TissueLyser (Qiagen) in the presence of 500-1000 μ l of PBS, and the supernatants after centrifugation were used to measure tissue viral load. For the viral load measurement, total RNA was extracted from the homogenates using the TRIzol extraction method. 80ng of RNA was used to measure the viral load by real-time PCR.

METHOD DETAILS

Structural Analysis of DENV Antibody Complexes

Our goal was to define residues that constitute an epitope surface on the entire viral assembly by taking into account the distinct local environments of the residues. In the assembly of E-proteins in a closed viral particle surface (or in complexes of viral surface with antibodies), there are distinct interfaces. These include i) the interface of 2-fold, 3-fold, 5-fold axis along planar and curved regions of the viral surface, ii) location on outer or inner surface of the viral particle, and iii) location at a known paratope-epitope interface on the entire assembly. Thus, to expand the datasets related to antibody footprint on the virus assembly, we modeled the X-ray co-crystal structures of E-protein dimer antibody complexes on to the cryo-EM structures and resolved steric hindrance through energy minimization. Next, we computed the solvent accessible surface area (square Angstrom) of each E-protein residues in these various structures using DSSP software, which also permitted identification of the epitope footprint of the various antibodies on the entire

virus assembly. We employed an approach that we used previously to compute a network score that captured the structural constraints imposed on a residue due to its non-bonded interactions with the neighboring residues in the local environment. The solvent accessibility score and network scores were normalized based on highest network score of a residue in the aforementioned interfaces. The higher the network score, the more the constraints imposed by the environment of a residue on its ability to mutate. Subsequently, in order to investigate the structural correlates of potent neutralization of DENV antibodies, we examined the accessibility, conservation, mode of binding (e.g. occupancy) and binding affinity of a panel of different DENV antibodies (including 1A1D-2 (PDB: 2R6P), HM14c10 (PDB: 4CAU), 1F4 (PDB: 4C2I), 5J7 (PDB: 3J6U), B7 (PDB: 4UT6), Ab513 (PDB: 5AAW), 2D22 (PDB: 5A1Z)) and compared them with their reported PRNT50 values (Figure S1).

Predicting Quaternary Epitope Surface on Entire ZIKV Assembly

In order to investigate the neutralizing antibody epitope surfaces on ZIKV assembly, we built a 3D structure of ZIKV starting with the homology model of soluble E-protein. The procedure involved fitting the E-chain into the DENV density map (EMDB: EMD-5520). Specifically, the chains were fitted, one at a time, on the three E-protein molecules to form one-half of the raft. Then, the fitted chains were rotated and translated in a stepwise fashion using a steepest ascent optimization to orient the molecule within its local environment. After aligning the three chains in the density map, the remaining 59 copies in the symmetry-related orientations were generated using the 532 symmetry. Finally, the fitting of the 180 chains (60X3) within the density data was optimized using a global optimization program (Fit) in Chimera. Correlation coefficient was computed to determine the quality of fit of the atomic model in the EM map. The fitting of homology model of ZIKA E-protein on the EM map led to a correlation coefficient of 0.7432. Investigation of the rebuilt assembly showed a closed capsid without any major voids or steric overlaps. Interestingly, the rebuilt assembly contains far fewer (ϕ , ψ) outliers (0.61%) and steric overlaps (5 atoms per 1000) compared to published DENV cryoEM structures (Figure S6). As noted in Table S1, the homology model of ZIKV viral assembly was corroborated with the recently published ZIKV cryoEM structures (PDB structures PDB: 5H37, 5IRE, and 5IZ7).

Next, we correlated the normalized network and solvent accessibility scores and other antibody-based structural features (computed in the context of the whole viral assembly) with experimental observations such as neutralization potency (PRNT50 values) of an antibody that was known to bind to a specific epitope region. Similar to DENV, this analysis suggested a trend that highly potent neutralizing antibodies target epitope surfaces that span multiple chains on the entire E-protein assembly (Table S1). Furthermore, this analysis taken together with the sequence conservation of a residue in a given position permitted us to define thresholds for normalized network score (0.15 ± 0.02) and solvent accessible surface area (50 ± 5 square angstrom) for the prediction of quaternary epitope surfaces across the entire E-protein assembly. By using these thresholds and searching for clusters of residues that could constitute an epitope surface, we selected an epitope surface that contained the largest quantity of highly networked residues and had the largest median solvent accessible surface area. This region was proximal to the fusion loop and so we designated this quaternary epitope surface as FLEP. Given that FLEP is defined in the context of the E-protein assembly on the viral particle surface, it cannot be viewed in the context of a single domain structure, which is typically provided as an input into established antibody engineering and docking software.

Antibody Engineering

Our antibody engineering approach comprised of two steps: (1) scaffold selection and (2) paratope engineering. To facilitate scaffold selection, we searched >450 antigen-antibody co-crystals in PDB database for Ig scaffolds that target homologous fusion-loop epitope like structures. The homology to the E-protein fusion-loop epitope was defined using an RMSD cutoff of $1.5\text{\AA}$. Each selected scaffold was docked against the ZIKV assembly, where the alignment was guided by the fusion-loop structure. To increase the chances of selecting suitable scaffolds, we incorporated CDR diversity from naturally occurring antibody sequences by considering all the VH and VL/K sequences having the same canonical groups as the chosen scaffolds. The matching antibody sequences were found by searching over 8,718 human VH, 8,513 human VK and 6,779 human VL sequences from the NCBI GenBank database. Antibody scaffolds that had high surface complementarity ($Sc > 0.65$) to the fusion-loop region and displayed potential to make energetically favorable interactions with the side chains of the fusion-loop residues were given preference. Interactions with E-protein residues that have accessible surface area > 20%, network score > 0.25 and B-factor ($C-\alpha$) less than three standard deviations or less from the mean make were considered energetically favorable. In this overall structural context, Trp102 and Asn104 were considered critical from the epitope standpoint. In theory, the Fv of an antibody should lend itself to bind to an epitope on free E-protein in many different ways. However, binding to the envelope-embedded E-protein will be constrained by several steric factors: (1) spacing between the antigen, (2) spacing between the adjacently bound Fabs, and (3) multivalent interaction with E-protein. A combination of steric factors and epitope-paratope shape complementarity were employed to rank the antibody scaffolds. Scaffolds that had least constraints and were most permissive in terms of binding to E-protein without the steric factors described above were given higher preference. Three sets of scaffolds composed of different CDR canonical groups were included in the search: (1) L1:2/11A, L2:1/7A, L3:1/9A, H1:1/10A, H2:2/10A), (2) (L1:2/11A, L2:1/7A, L3: N/A, H1:1/10A, H2:1/9A), and (3) (L1:2/11A, L2:1/7A, L3:5/11A, H1:3/12A, H2:2/10A). Multiple antibody scaffolds (including mouse-derived pan-flavivirus 4G2, anti-EDE1 Dengue mAbs C8, C10 and anti-EDE2 Dengue mAb A11, anti-TDRD3 antibody and anti-HIV antibody PGT124) were used as starting templates for antibody engineering.

Having defined the antigen-antibody models, we explored favorable interactions between the CDR residues and remainder of the quaternary-epitope surface residues using amino acid interface (AIF) and evaluated them through experimentation to test their ability to bind to one or more ZIKV. Given the model of the antigen-antibody interaction, the contributions of the CDR residues to the binding

were evaluated and ranked based on their interface buried surface area. Then, CDR residues were altered through AIF one at a time starting with the residue that is most buried going all the way to the residue that is least buried. Loop insertions and deletions were attempted if they led to an increase in surface complementarity. At each step, the structure was minimized using CHARMM minimization in Discovery Studio. During minimization, residues that were within 4.5 Å² (in both antigen and antibody) from the position being modified were allowed to undergo both side chain and backbone changes whereas the remaining residues were kept fixed. The CDR change was accepted if the post-minimization CDR-antigen interaction energy was favorable when compared to the interaction energy before making the CDR change. Promising mutations from each interaction were used to remodel the antibody, dock against the antigen and generate a new antigen-antibody model using MLR as described before (Tharakaraman et al., 2013). The data from each iteration was used in remodeling the CDR loops, and subsequently to dock and refine the antigen-antibody model using the MLR metric (Tharakaraman et al., 2013). Certain CDR mutations had a detrimental effect on the interatomic network so different variants were tried or compensatory mutations were made in the spatially proximal CDR or framework regions. For example, the DENV E protein contains two N-linked glycosylation sites, at Asn-67 and Asn-153, with the former being in close proximity to the ZAb_FLEP binding domains. On the other hand, ZIKV does not possess the N67-linked glycosylation site. The engineering procedure included a framework mutation, S85K, to induce steric hindrance with the Asn-67 N-linked glycan of DENV E-protein and thereby impair binding to the DENV virus.

The engineered antibodies that satisfied the key epitope-paratope constraints while accounting for differences in accessibility and network scores of epitope residues (as these parameters vary across different icosahedral vertices on the spherical assembly) were taken forward and tested. The engineered ZAb_FLEP antibody buried a total of 1,089 Sq. Å² in the epitope-paratope interface, out of which the VH and the VL domains contribute approximately 46% and 54% of the buried area, respectively. Based on the structural model, ZAb_FLEP's footprint crosses over into the adjacent dimer at the 2-fold and 3-fold vertices, where the adjacent dimer contributes 101 Å² to the interface area at the 2-fold and 3-fold vertices. On the contrary, ZAb_FLEP showed restricted potential to interact at the 5-fold vertex due to probable steric hindrance from the adjacent dimer.

Expression and Purification of Antibodies

Antibodies were expressed in Freestyle 293 cells by transient transfection with Polyethylenimine (PEI) and purified by protein A chromatography (Robinson et al., 2015). The purified antibodies were quantified by IgG ELISA. Briefly 96-well plates were coated overnight at 4°C with appropriate antigen. The plates were washed and blocked with 1% BSA (Santa Cruz Biotechnologies). Serial dilution of antibodies were added to the plate and incubated for 2 hours at room temperature. Antigen bound IgG was detected using Rb2Hu IgG HRP Conjugated secondary antibody (Jackson ImmunoResearch) followed by TMB substrate (KPL) addition.

Antibody Affinity Measurements

Microtiter plates were coated with 0.05g of purified mouse 4G2 in carbonate buffer (pH 9.6) overnight at 4°C and blocked with 10% BSA for 2hrs at room temperature. Thereafter, 5x10⁴ pfu of DENV-1 (EDEN2402), DENV-2 (ST), DENV-3 (EDEN863), DENV-4 (EDEN2270) or ZIKV were added. After 1hr incubation at room temperature, serial two-fold dilutions of antibodies were added for 1hr, followed by goat anti-human IgG Fc cross-adsorbed HRP-conjugated anti-human IgG for 45min. Antibody binding was visualized by adding 3,3',5,5'-tetramethylbenzidine substrate and reaction was stopped after 10min with sulfuric acid. In between the different steps, plates were washed twice with PBST (PBS+0.05%Tween). Absorbance is read at 450nm using a plate reader (Robinson et al., 2015). The antibody affinity is obtained by measuring the mAb concentration that result in 50% maximal effective concentration of binding.

Plaque Reduction Neutralization Test

To assess if the monoclonal antibodies can neutralize DENV or ZIKV infection, we performed a plaque reduction neutralization test (PRNT), as described previously (Robinson et al., 2015). PRNT was performed on BHK-21 cells. Serial two-fold dilution of sera in RPMI maintenance media (MM) was incubated with 50pfu of DENV or ZIKV virus in equal volumes for 1hr before adding to BHK-21. After 1hr incubation at 37°C, media was aspirated and cells were overlaid with 1% methyl cellulose in MM. After 5 days at 37°C, cells were fixed with 20% formaldehyde and stained with 1% crystal violet. At least 18 two-fold dilutions (starting from 500μg/ml) of the mAb were tested, and dose-response trend lines were calculated by fitting a variable slope sigmoidal equation to calculate the mAb concentration that neutralizes 50% (PRNT50).

Protection Studies in A129 Mice

Mouse studies were conducted at the DUKE-NUS Medical School in Singapore after the approval from the Institutional Animal Care and Use Committee (IACUC). The protective and therapeutic efficacy of monoclonal antibodies against ZIKV were tested in adult A129 mice (8-11 weeks old). Mice were infected intraperitoneally (ip) with 5 x 10⁴ pfu of H/PF/2013 strain (French Polynesia). To assess protective or prophylactic efficacy of antibodies, mice were injected ip with antibodies (50μg) one day prior to infection. To assess therapeutic efficacy, mice were injected ip with antibodies (50μg) one day post-infection (pi). Efficacy of antibody was monitored by assessing mortality, weight loss and viremia reduction. Briefly, mouse blood was collected from facial vein on days 1-21 pi to measure serum viremia level by real-time PCR while mouse weight and survival were monitored daily until 30 days pi. To assess the protective efficacy of the antibody against vertical infection in pregnancy, 9-10 weeks old female A129 mice were housed with adult male A129 mice in same cages (1 female with 1 male) for 4 days. Female mice were infected intravenously (iv)

with 1×10^3 pfu of H/PF/2013 strain on day 10 (corresponding to embryo day 7–10). The mice were injected iv with 50 μ g of ZAb_FLEP or mouse isotype IgG one day pi. Blood was collected from facial vein on day 2 pi to measure viremia. Mice were euthanized by CO₂ inhalation on day 7 pi and blood was collected from the postcaval vein. Placenta and whole fetuses were harvested and snap-frozen in liquid nitrogen. Frozen samples were then homogenized by TissueLyser (Qiagen) in the presence of 500–1000 μ l of PBS, and the supernatants after centrifugation were used to measure tissue viral load. For the viral load measurement, total RNA was extracted from the homogenates using the TRIzol extraction method and 80 ng of RNA was used for real-time RT-PCR as described previously (Watanabe et al., 2015).

Virus Sample Preparation

Aedes albopictus C6/36 cells were used for amplification of ZIKA virus (ZIKV). The precipitation and purification were done following previously published virion preparation protocol (Tan and Lok, 2014). Briefly, virus was harvested from infected C6/36 cells 4 days post-infection by centrifuging virus-containing media at 9,000 g for 45 min. Virus was then precipitated with addition of 8% (w/v) polyethylene glycol (PEG) 8,000 in NTE buffer (10 mM Tris-HCl pH 8.0, 120 mM NaCl and 1 mM EDTA). Virus precipitate was pelleted by centrifugation at 14,000 g for 1 hr after overnight incubation. Pellet containing virus was then purified by 24% (w/v) sucrose cushion and 10–30% (w/v) potassium tartrate gradient. The virus band was visualized with a torch light in a linear potassium tartrate gradient and extracted with a syringe. It was then concentrated and buffer exchanged to NTE buffer by ultrafiltration using a 100 kDa molecular weight cut-off filter. The yield and purity of E protein were checked with SDS-PAGE using different amount of bovine serum albumin (BSA) as concentration standards.

Enzymatic Digestion of ZAb_FLEP

To obtain the Fab from ZAb_FLEP, papain digestion was carried out. Papain was firstly activated with L-cysteine. The activated papain was added to the digestion buffer (0.1 M Tris HCl pH 8.0, 2 mM EDTA) supplemented with L-cysteine. The reaction was incubated at 37°C for 7 hrs before it was stopped by addition of iodoacetamine to a final concentration of 20 mM. Fab 8 fragments were then purified with protein A affinity beads and gel filtration (Superdex 75 10/300 GL, GE Healthcare).

CryoEM Complex Preparation

To prepare ZIKV-Fab complexes for CryoEM, Fab and ZIKV virions were mixed with a E protein: Fab (ZAb_FLEP) molar ratio of 1:2 as described to ensure full occupancy (Zhang et al., 2016). The immune complex was allowed to form by incubation at 37°C for 30 min. The virus-Fab complex was then kept on ice for about an hour prior applying to cryoEM grids.

Cryo-Electron Microscopy

2 μ l of virus-Fab complex was applied onto glow-discharged 2-nm carbon coated holey grids (R1/2, Quantifoil GmbH, Germany) and incubated for 30 sec. Grids were manually blotted for 2 sec at RT and flash frozen in liquid N₂-cooled liquid ethane. Grids were loaded to an FEI Tecnai Arctica cryo-transmission electron microscope, operated at 200 kV and equipped with a back-thinned Falcon III direct electron detection device. A total of 304 micrographs were recorded in a movie mode as a set of 31 frames (total dose of 36 electrons per $\text{\AA}^2$) at a calibrated magnification of 109,375 resulting in a pixel size of 1.28 $\text{\AA}$ on the object scale and at defocus values from 0.4–1.5 μ m.

Image Processing

287 micrographs were selected for data processing after removing micrographs with significant astigmatism. Whole-image drift correction was carried out using motioncorr (Li et al., 2013). CTF estimation was performed with CTFFIND3 (Mindell and Grigorieff, 2003). Data was binned three times. Particles were picked from the micrographs using EMAN2 (Bell et al., 2016). Data was processed with RELION 1.4 (Scheres, 2012). In total, 4,256 particles were subjected to reference-free 2D classification to discard bad particles. 1,482 particles selected after 2D classifications were used for 3D classifications using a 60 $\text{\AA}$ filtered Zika EM map (EMDB: EMD-8116) (Sirohi et al., 2016). 925 particles selected after 3D refinement with icosahedral symmetry yielded an EM density map at 9.7 $\text{\AA}$ resolution according to the gold-standard FSC = 0.143 criterion.

Assessment of Predicted ZAb_FLEP:E-Protein Model Using EM Density Map

The 3.8 $\text{\AA}$ resolution cryoEM structure of ZIKV (EMDB: EMD-8116) (Sirohi et al., 2016) was fitted into the density map using Chimera (Pettersen et al., 2004). Following this, the asymmetric unit structure of ZIKV (5IRE) on the three E-protein molecules to form one-half of the raft. After aligning the three chains in the density map, the remaining 59 copies in the symmetry-related orientations were generated using the 532 symmetry. Having generated the viral assembly structure, the Fv of ZAb_FLEP was docked onto each of the 120 sites (180 minus 60 5-fold sites) guided by the predicted docking model, and its orientation relative to the Fab density was assessed in Chimera.

Escape Mutant Analysis

To evaluate propensity of escape mutant generation against ZAb_FLEP, we premixed ZIKV strain H/PF/2013 with 22.50 μ g/mL (EC90 of antibody that was predicted to neutralize the majority of the input virus), 500 μ g/mL IgG1 isotype control antibody. Antibody was allowed to opsonize virus at 37°C for 1 hour in DMEM maintenance media, before being used to infect Vero cells for 48–72 hours to

allow virus propagation to occur (1 passage). At the end of the 48–72 hour incubation, the supernatant containing virus was collected and half the volume was again pre-mixed with varying amounts of antibody, thus repeating the selection and propagation process. This propagation process was performed for a total of seven passages for each antibody combination. At each passage, aliquots of virus were frozen for plaque titer determination and subsequent sequencing of the virus by RT-PCR using primers designed to amplify the entire E gene, from the prM to NS1 gene. The resultant amplicon was run on an agarose gel, gel-extracted and sequenced. In the presence of control IgG1, wildtype virus was recovered in each passage. Sequencing of all detectable viruses was performed by first extracting viral RNA using the QIAamp Viral RNA Mini kit and then performing RT-PCR with primers designed to amplify the entire E gene (region between prM and NS1) using the One-Step Superscript III RT-PCR System with Platinum Taq. The resultant amplicon (~1,800 bp) was run on a 1% agarose gel, gel-extracted with the MinElute Gel Extraction Kit (Qiagen), and sequenced.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical Methods

Statistical significance was determined by student's *t* test between indicated groups using GraphPad Prism. Data presented represent the mean with error bars signifying the standard error of the mean.

DATA AND SOFTWARE AVAILABILITY

The informatics tool to determine the network scores from an X-ray crystal or cryoEM structure is made available online at <http://glycomics2.mit.edu/matlab/>. Protein residue solvent accessibility is determined using the DSSP software available at <http://swift.cmbi.ru.nl/gv/dssp/>. The extent of sequence conservation in alignments of E-protein (DENV/ZIKV) was measured using Jalview, which is available at <http://www.jalview.org/>. Computational docking between antibody scaffolds and E-protein epitope was enabled by ZDOCK (<http://zdock.umassmed.edu/>). Methods for predicting native antigen-antibody-like structures and affinity enhancing mutations have been described before (Tharakaraman et al., 2013). Cryo-EM density map of the virion complexed with Fab ZAb_FLEP has been deposited with the Electron Microscopy Data Bank: EMDB: EMD-7613.

Cell Host & Microbe, Volume 23

Supplemental Information

Rational Engineering and Characterization of an mAb that Neutralizes Zika Virus by Targeting a Mutationally Constrained Quaternary Epitope

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SUPPLEMENTARY FIGURES

Figure S1. Illustrative examples of serotype-specific and cross-reactive DENV neutralizing antibodies targeting different epitopes: (1) quaternary (HM14c10, 5J7, A11, C10, B7, 2D22, C8, 1F4), (2) domain epitopes (Ab513, 5H2, 2A10G6, 1A1D-2, 3E31), Related to STAR Methods. E106 is treated as a special case as it engages two adjacent EDIII domains bivalently, resulting in locking of E protein monomers. The theoretical apparent threshold was defined as a value exceeding the EC50 of the most potent single domain targeting antibody.

Figure S2. In vitro neutralization of ZV (isolates MR766 and PRVABC-59) by serotype-specific and cross-reactive antibodies, Related to STAR Methods. Data points represent averages of N=3 experiments with error bars representing standard deviation.

	ZV67	ZV48	Z23	C8	C10	Z3L1
MR766 EC50 (M)	1.83 E-09	1.25 E-07	6.94 E-08	2.03 E-09	7.27 E-10	3.56 E-09
PRVABC-59 EC50 (M)	1.59 E-09	2.35 E-07	4.39 E-08	1.61 E-09	6.78 E-10	2.63 E-09

Figure S4. Cryo-EM micrographs, Fourier Shell Correlation of the 3D reconstruction of ZIKV-ZAb_FLEP complex, Related to Figure 6. (A) Cryo-EM micrographs of purified ZIKV virions at higher (left) and lower (right) focuses, (B) 2D class averaging and (C) Fourier Shell Correlation (FSC) for the final EM density map.

A.

B.

C.

Figure S5. In vitro neutralization of ZIKV antibodies in FcγR-expressing THP1.2S cells, Related to Figure 2.

Figure S6. Backbone torsion angle (ϕ , ψ) outliers observed in the asymmetric unit of (A) DENV2 (PDB: 3J27, 3.6°) and (B) the rebuilt ZIKV viral assembly, Related to STAR Methods.

A.

B.

Table S1. Structural and neutralization properties of serotype-specific and cross-reactive ZIKV antibodies, Related to STAR Methods. To begin with, we built the 3D model of ZIKV assembly starting from the homology model of soluble E-protein in order to understand the various neutralizing antibody epitope surfaces (*Methods*). As more structural information became available (see below), they confirmed our structural homology model.

Antibody	Class	Subclass	Epitope	Source	Specificity	Neutralization EC50(M) (MR766)	Neutralization EC50(M) (PRVABC-59)	Predicted symmetry axes (occupancy)	Monomeric Affinity (M)	Inter-chain locking?	Median ASA of 2-fold hotspot residues ($\Delta > 0.15$, Å ²)	Median 2-fold Epitope Network Score
ZV48	Monomeric	Domain	EDIII (C-C' loop)	mouse	ZIKV	1.25E-07	2.35E-07	n/a	1.14 E-10 EC50 (EDIII)	No	40.957	0.167
ZV67	Monomeric	Domain	EDIII (lateral ridge)	mouse	ZIKV	1.83E-09	1.59E-09	2, 3 (120/180)	4.78 E-11 EC50 (EDIII)	No	53.311	0.140
Z23	Monomeric	Domain	EDIII (lateral ridge)	human	ZIKV	6.94E-08	4.39E-08	3, 5 (120/180)	4.414 E-07 k _o (sE)**	No	61.540	0.093
Z3L1	Monomeric	Multi-domain	EDI/EDII	human	ZIKV	3.56E-09	2.63E-09	2, 5 (120/180)	5.390 E-06 k _o (sE)**	Possible	73.086	0.125
C8	Quaternary	Intra-dimer	EDE	human	cross-reactive	2.03E-09	1.61E-09	2, 3, 5 (180/180)	n/a	Yes	58.420	0.152
C10	Quaternary	Intra-dimer	EDE	human	cross-reactive	7.27E-10	6.78E-10	2, 3, 5 (180/180)	n/a	Yes	51.142	0.158

Table S2. Comparison of *in vivo* models reported in ZIKV mAb studies, Related to Figure 3.

ZIKV antibodies	<i>In vivo</i> mouse model	Dosage employed	Prophylactic (D-)/Therapeutic (D+)?	Challenge strain and viral load	# of days survival was monitored
ZV-2, ZV-48, ZV-64, ZV-67	4- to 5-week-old WT C57BL/6 mice	250ug	D-1	African ZIKV strain (10 ⁵ FFU)	18
Z3L1, Z23	type I interferon (IFN) receptor knockout mice	10mg/kg (~250ug)	D+1	? (1 × 10 ⁶ pfu)	15
Z004, Z006	3-4 week old IFNR ^{-/-} mice	125ug	D-1, D+1	Puerto Rico (1.25 X 10 ⁵ PFU)	15
ZIKV-117	4–5-week-old male C57BL/6 mice with type I interferon knockout	100ug, 250ug	D+1, D+5	African (10 ³ FFU)	20
ZAb_FLEP	8-11 weeks old A129 mice	50ug	D-1, D+1	H/PF/2013 (10 ³ PFU)	30