CHARACTERISATION OF
HUMAN ANTIBODY RESPONSES TO
CHIKUNGUNYA VIRUS INFECTION

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ABSTRACT

Chikungunya virus (CHIKV), an alphavirus of the family *Togaviridae*, causes fever, rash and joint pain. There are three CHIKV genotypes: West African, Asian and East/Central/South African (ECSA). The latter two genotypes have been co-circulating and causing outbreaks in Malaysia. Although vaccines are still under development, a greater understanding of the human antibody responses to CHIKV infection is essential. The overall aim of the present study was to characterise human antibody responses to CHIKV. The antibody responses were studied in 102 serum samples collected during CHIKV outbreaks in Malaysia. The first objective of the study was to develop a panel of monoclonal antibodies targeting CHIKV E2 glycoprotein as immunological tools. The monoclonal antibody clone B-D2(C4) was chosen for use in subsequent serum neutralisation assay and development of immunoassays. For the second objective, the characteristics of cross-genotype immunity and epitopes were investigated. The neutralising capacity of late convalescent sera (ECSA and Asian) was analysed against representative clinical isolates as well as viruses rescued from infectious clones of ECSA and Asian CHIKV. Using whole virus antigen and recombinant E1 and E2 envelope glycoproteins, the antibody binding sites, epitopes and antibody titres were investigated using ELISA and western blotting. Both ECSA and Asian sera demonstrated stronger neutralising capacity against ECSA genotype, which corresponds to stronger epitope-antibody interaction. ECSA serum targeted conformational epitope sites in the E1-E2 glycoprotein, while E1-E211K, E2-I2T, E2-H5N, E2-G118S and E2-S194G were the key amino acids that enhanced cross-neutralising efficacy. As for Asian serum, the antibodies targeting E2 glycoprotein correlated with neutralising efficacy and I2T, H5N, G118S and S194G altered and improved the neutralisation efficacy. For the third objective, the pathogenic role of antibodies from immune sera was explored. Evidence for CHIKV antibody-dependent enhancement (ADE) was demonstrated in

K562 leukaemia cells, which express the Fc gamma receptor FcγRIIA (CD32) and supports active virus production. For the fourth objective, the neutralising role of IgM at different times post-infection was described and the independent contributions of IgM and IgG towards the neutralising capacity of human immune sera were examined. The differences in neutralising epitopes of IgM and IgG were investigated as well. Neutralising IgM starts to appear as early as day 4 of symptoms, and their appearance from day 6 is associated with a reduction in viraemia. IgM acts in a complementary manner with early IgG, but plays the main neutralising role up to a point between days 4 and 10 which varies between individuals. After this point, total neutralising capacity is attributable almost entirely to the robust neutralising IgG response. IgM preferentially binds and targets epitopes on the CHIKV surface E1-E2 glycoproteins, rather than individual E1 or E2. Overall, the findings from this study provide new knowledge in the immunoprotection mechanisms against co-circulating CHIKV genotypes. The findings have implications for effective design and development of vaccines, human monoclonal antibodies and diagnostic serological assays.

ABSTRAK

Virus chikungunya (CHIKV) adalah sejenis alphavirus daripada famili *Togaviridae* yang boleh menyebabkan demam, ruam dan sakit sendi. Terdapat tiga genotip CHIKV: Afrika Barat, Asia dan Timur/Tengah/Afrika Selatan (ECSA). Dua genotip terakhir sedang beredar bersama-sama dan menyebabkan wabak di Malaysia. Vaksin masih berada di peringkat pembangunan; namun begitu, pemahaman tentang tindak balas antibodi manusia kepada jangkitan CHIKV adalah penting untuk proses tersebut. Matlamat keseluruhan adalah untuk mencirikan balas antibodi manusia kepada jangkitan CHIKV. Sebanyak 102 sampel serum telah dikumpul daripada wabak berlaku di Malaysia dan digunakan untuk pencirian tindak balas antibodi. Objektif pertama kajian ini adalah untuk memperolehi satu panel antibodi monoklonal yang menyasarkan E2 glikoprotein CHIKV sebagai alat imunologi. Antibodi monoklonal klon B-D2 (C4) telah dipilih untuk asai serum peneutralan dan pembangunan imunoasai berikutnya. Bagi objektif kedua, ciri-ciri imuniti merentasi genotip dan epitop telah disiasat. Kapasiti peneutralan daripada sera pemulihan lewat (ECSA dan Asia) telah dianalisis terhadap isolat klinikal serta virus rekombinan ECSA dan Asia. Dengan menggunakan antigen virus keseluruhan dan rekombinan glikoprotein E1 dan E2, penyiasatan terhadap tapak ikatan antibodi, epitop dan titer antibodi telah dijalankan. Kedua-dua serum ECSA dan Asia menunjukkan kapasiti peneutralan lebih kuat terhadap virus genotip ECSA, selaras dengan interaksi yang kuat antara antibodi dengan epitop pada permukaan virus. Serum ECSA menyasarkan tapak epitop kesusukan pada glikoprotein E1-E2 dan E1-E211K, E2-I2T, E2-H5N, E2-G118S, E2-S194G merupakan asid amino utama yang telah mempertingkatkan keberkesanan kapasiti peneutralan. Bagi serum Asia, antibodi menyasarkan glikoprotein E2 berkait rapat dengan keberkesanan peneutralan dan perubahan asid amino I2T, H5N, G118S dan S194G menambahkan keberkesanan peneutralan. Bagi objektif ketiga, peranan patogenik antibodi daripada

serum imun telah diterokai. Bukti penggalakan bersandar antibodi (ADE) CHIKV telah ditunjukkan dalam sel leukemia K562, yang mengungkap reseptor Fc gamma, khususnya FcɣRIIA (CD32) dan menyokong pengeluaran virus aktif. Antibodi menyasarkan tapak ikatan di glikoprotein virus memainkan peranan penting untuk mengantara ADE. Bagi objektif keempat, peranan IgM kepada peneutralan CHIKV telah dijelaskan mengikut hari yang yang berlainan. Peranan IgM dan IgG terhadap kapasiti peneutralan juga telah diperiksa. Ini termasuk perbezaan tapak sasaran epitop peneutralan. Peneutralan IgM mula muncul seawal pada hari keempat selepas jangkitan, dan penampilan IgM pada hari keenam dikaitkan dengan pengurangan viremia. IgM dan IgG saling melengkapi dalam tempoh jangkitan yang awal, dan IgM boleh memainkan peranan peneutralan dominan sehingga hari kesepuluh, dengan variasi antara individu. Selepas melepasi tempoh tersebut, kapasiti peneutralan keseluruhan adalah sumbangan daripada IgG peneutralan yang teguh. IgM menyasarkan tapak epitop peneutralan utama pada permukaan glikoprotein E1-E2, berbanding dengan individu E1 dan E2. Secara keseluruhannya, kajian ini menyumbang kepada pengetahuan baru dalam mekanisme perlindungan imun terhadap genotip CHIKV yang beredar bersama-sama. Hasil kajian mempunyai implikasi terhadap rekabentuk vaksin yang berkesan, antibodi monoklonal manusia dan ujian serologi diagnostik.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

%	:	Percentage
°C	:	Degrees celcius
μg	:	Microgram
μl	:	Microlitre
ADE	:	Antibody-dependent enhancement
ANOVA	:	Analysis of variance
ATCC	:	American Type Culture Collection
BHK-21	:	Baby hamster kidney
bp	:	Base pair
BSA	:	Bovine serum albumin
CO_2	:	Carbon dioxide
CD	:	Cluster of differentiation
cDNA	:	Complementary deoxyribonucleic acid
CHIKV	:	Chikungunya virus
CMV	:	Cytomegalovirus
CPE	:	Cytopathic effect
DAB	:	3,3'-diaminobenzidine
DAPI	:	4',6-diamidino-2-phenylindole
DNA	:	Deoxyribonucleic acid
DPBS	:	Dulbecco's phosphate buffered-saline
DTT	:	Dithiothreitol
E1	:	Envelope 1
E2	:	Envelope 2
ECSA	:	East/Central/South African
EDTA	:	Ethylenediaminetetraacetic acid
ELISA	:	Enzyme-linked immunosorbent assay
EMEM	:	Eagle's Minimum Essential Medium
FBS	:	Foetal bovine serum
Fc	:	Fragment crystallizable
FcγR	:	Fragment crystallizable gamma receptor
FcγRIIA	:	Fragment crystallizable gamma receptor IIA
GM-CSF	:	Granulocyte-macrophage colony-stimulating factor
GMEM	:	Glasgow's Minimum Essential Medium
H_3PO_4	:	Phosphoric acid
HCS	:	High content screening
HEPES	:	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HRP	:	Horseradish peroxidase
icDNA	:	infectious clone DNA
IF	:	Immunofluorescence
IFN	:	Interferon
IgG	:	Immunoglobulin G
IgM	:	Immunoglobulin M
IL	:	Interleukin

IOL	:	Indian Ocean lineage
IPTG	:	Isopropyl β-D-1-thiogalactopyranoside
IRF	:	Interferon response factor
ISG	:	Interferon-stimulated gene
K562	:	Human erythroleukemic cell
KCl	:	Potassium chloride
kDa	:	Kilodalton
LB	:	Luria-Bertani
LiCl	:	Lithium chloride
mAbs	:	Monoclonal antibodies
MALDI	:	Matrix-assisted laser desorption/ionisation
MCP-1	:	Monocyte chemoattractant protein 1
MHC	:	Major histocompatibility complex
MIF	:	Macrophage migration inhibitory factor
ml	:	Mililitre
MOI	:	Multiplicity of infection
mRNA	:	Messenger ribonucleic acid
NaCl	:	Sodium chloride
NaH_2PO_4	:	Monosodium phosphate
NEAA	:	Non-essential amino acid
ng	:	Nanogram
NK	:	Natural killer
nsP	:	Non-structural protein
NT_{50}	:	Neutralising titre
OD	:	Optical density
ONNV	:	O'nyong-nyong virus
OPG	:	Osteoprotegerin
ORF	:	Open reading frame
PBMC	:	Peripheral blood mononuclear cell
PBST	:	Phosphate-buffered saline tween 20
PCR	:	Polymerase chain reaction
PDB	:	Protein Data Bank
RAW264.7	:	Mouse macrophage
rE1	:	Recombinant envelope 1
rE2	:	Recombinant envelope 2
RNA	:	Ribonucleic acid
RPMI	:	Roswell Park Memorial Institute medium
RRV	:	Ross River virus
RT-PCR	:	Reverse transcription polymerase chain reaction
S	:	Svedburg
SD	:	Standard deviation
SDM	:	Site-directed mutagenesis
SDS-PAGE	:	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	:	Standard error of the mean
Sf9	:	*Spodoptera frugiperda* cells

SFV	:	Semliki Forest virus
SG	:	Subgenomic promoter
TAE	:	Tris-acetate-EDTA buffer
TE	:	Tris-EDTA
TLR3	:	Toll-like receptor 3
TMB	:	3,3',5,5'-tetramethylbenzidine
UTR	:	Untranslated region
Vero	:	African green monkey cell
$\times$ g	:	Gravitational acceleration
X-gal	:	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
zsG	:	zsGreen

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Background

Chikungunya virus (CHIKV) is a re-emerging, mosquito-borne arbovirus which has caused unprecedented worldwide epidemics in recent years (Sam *et al.*, 2015b). There are three major CHIKV genotypes circulating: West African, East/ Central/ South African (ECSA) and Asian (Powers *et al.*, 2000). After the global outbreaks of ECSA between 2005 and 2010, the Asian genotype has re-emerged to cause large outbreaks in the Americas and the Pacific islands (Leparc-Goffart *et al.*, 2014; Pan American Health Organization, 2016) . Malaysia has experienced CHIKV outbreaks due to two different genotypes, Asian and ECSA. The endemic Asian CHIKV strain was responsible for small, geographically-restricted outbreaks in 1998 and 2006 (Lam *et al.*, 2001; AbuBakar *et al.*, 2007; Ayu *et al.*, 2010). An imported ECSA outbreak was reported in 2006 prior to an explosive nationwide outbreak which affected over 15,000 people across different states in 2008 (Noridah *et al.*, 2007; Sam *et al.*, 2009).

CHIKV is an alphavirus from the family *Togaviridae*. A CHIKV virion is 60-70nm in diameter, with a single-stranded positive RNA genome of approximately 11.8 kb in a capsid with a phospholipid envelope carrying glycoproteins E1 and E2. Its genome has 2 open reading frames encoding the non-structural (nsP1-nsP2-nsP3-nsP4) and structural polyproteins (C-E3-E2-6K-E1) (Khan *et al.*, 2002). The E1 and E2 glycoproteins form heterodimers which enable interaction with cellular receptors and fusion of the virion envelope with the cell membrane to initiate infection (Strauss and Strauss, 1994), while the capsid protein is required during virus assembly (Solignat *et al.*, 2009). These proteins are highly immunogenic, and most CHIKV-infected patients develop antibodies targeting the structural proteins (particularly E2) and, to a lesser extent, nsP3 (Sourisseau *et al.*, 2007b; Kam *et al.*, 2012a).

CHIKV infection is mainly characterised by abrupt fever, profound acute joint pain and erythematous maculopapular rashes (Sam and AbuBakar, 2006; Pialoux et al., 2007). Other less specific symptoms include nausea, abdominal pain and myalgia (Economopoulou et al., 2009). Viral loads of up to 10^9 viral RNA copies per ml occur during early infection, and viraemia may last for 5-7 days (Parola et al., 2006; Laurent et al., 2007). Interferon type I, particularly interferon-alpha (IFN-α) is induced during the viraemic period, and its concentrations correlate with viral loads (Schilte et al., 2010; Chow et al., 2011). Generally, IgM is detectable from day 3 to day 8 onwards after the onset of clinical symptoms, while convalescent IgG with neutralising activity is produced from day 4 (Suhrbier et al., 2012). CHIKV is usually a self-limiting disease, with rapid virus clearance within days; nevertheless, debilitating arthralgia that mainly affects the small joints may persist for longer periods (Miner et al., 2015). Among other immune factors, T cells may play a secondary role in suppressing infection (Poo et al., 2014b), although others have found that CD4+ T cells are more important in orchestrating joint inflammation (Nakaya et al., 2012; Teo et al., 2013).

Pre-clinical studies in mouse models have shown the importance of antibody-mediated immunity in controlling infection. CHIKV infection of Rag1$^{-/-}$ or Rag2$^{-/-}$ (lacking mature B and T lymphocytes) and μMT (B-cell deficient) mice resulted in persistent viraemia accompanied by joint inflammation (Hawman et al., 2013; Lum et al., 2013; Teo et al., 2013; Poo et al., 2014b; Seymour et al., 2015; Hawman et al., 2016). Passive transfer of CHIKV-specific antibodies into infected mice had both prophylactic and therapeutic effects (Couderc et al., 2009). Immune IgG from convalescent patients directly neutralises CHIKV, and may persist in immune individuals for life (Kam et al., 2012a; Nitatpattana et al., 2014). However, the functional role of specific immune (infection-induced) IgM against CHIKV is less well-characterised compared to immune IgG during acute and early convalescent phases of

infection in mice and humans (Kam *et al.*, 2012c; Lum *et al.*, 2013). Infection of athymic mice with the closely related alphavirus Semliki Forest virus revealed the role of IgM in clearing viraemia, but not virus localised in the brain (Amor *et al.*, 1996). Induction of a specific, neutralising IgM response by the flavivirus West Nile virus in mice reduces viraemia and dissemination into the brain and spinal cord (Diamond *et al.*, 2003). Similar observations were reported for rabies virus, influenza virus, vesicular stomatitis virus and smallpox vaccine, which demonstrated that immune IgM is important to confer protection, particularly in early stages before the IgG response (Hangartner *et al.*, 2006; Moyron-Quiroz *et al.*, 2009; Dorfmeier *et al.*, 2013; Skountzou *et al.*, 2014). A recent study in uninfected mice also demonstrated an unexpected role for natural antibodies, which are constantly secreted without specific stimulation as part of primary defence, in partially neutralising CHIKV (Lum *et al.*, 2013). Natural antibodies limit early viral and bacterial dissemination, enhance antigen trapping in secondary lymphoid organs and bridge innate and adaptive immunity (Ochsenbein *et al.*, 1999; Ochsenbein and Zinkernagel, 2000).

Phase I clinical trials have demonstrated the safety and efficacy of vaccination with virus-like particles using structural proteins derived from the West African genotype (Chang *et al.*, 2014), and a recombinant measles virus-based CHIKV vaccine derived from the ECSA genotype (Ramsauer *et al.*, 2015). Cross-reactivity can be achieved against heterogenous genotypes, by which CHIKV seropositive individuals infected with either ECSA or Asian CHIKV have cross-protection against both CHIKV genotypes (Sam *et al.*, 2009). However, the cross-neutralising efficacy of CHIKV-specific antibodies against Asian and ECSA genotypes, which are both currently circulating in Malaysia (Sam *et al.*, 2009), Brazil (Nunes *et al.*, 2015) and the Asian region (Weaver and Lecuit, 2015), is poorly understood. A distinct antigenic relationship has been established between West African and ECSA genotypes, in which

mice and hamsters immunised with the ECSA genotype had 4- to 8-fold differences in neutralising capacity when tested against a West African strain (Powers *et al.*, 2000). In a Singaporean cohort, CHIKV-immune sera exhibited differential antibody binding and neutralising capacity against isolates with a naturally occurring K252Q amino acid change in the E2 glycoprotein (Kam *et al.*, 2012a). Given the ability of CHIKV to rapidly spread across different parts of the world with displacement of one genotype with another, the understanding of cross-neutralising antibody and antigenic variation of different genotypes will have implications for continued outbreaks and the efficacy of vaccine candidates.

Strong immunogenicity of vaccine candidates has been well described in animal models, with the demonstration of protective efficacy (García-Arriaza *et al.*, 2014; Hallengärd *et al.*, 2014a; Hallengärd *et al.*, 2014b; Erasmus *et al.*, 2017; Roques *et al.*, 2017). However, in a recent study, single immunisation of mice with a CHIKV vaccine candidate did not produce high antibody titres, and unexpectedly, virus challenge led to disease manifestations with joint inflammation and swelling, raising the possibility of antibody-dependent enhancement (ADE) of infection (Hallengärd *et al.*, 2014a; Hallengärd *et al.*, 2014b). ADE has been widely studied in other arboviruses such as dengue virus, when weakly cross-reactive antibodies from primary infection bind to another serotype of virus during re-infection, resulting in disease enhancement and severe clinical outcome (Balsitis *et al.*, 2010; Zellweger *et al.*, 2010). Immune complexes interact with cells bearing Fc receptors, promoting internalisation of the virus and increasing infection. ADE may also engage an alternative pathway to alter and suppress cellular responses, which in turn favours virus replication as seen in DENV and RRV (Lidbury and Mahalingam, 2000; Mahalingam and Lidbury, 2002; Suhrbier and La Linn, 2003; Chareonsirisuthigul *et al.*, 2007; Tsai *et al.*, 2014). The significance of ADE induced by human CHIKV immune sera remains unknown. The phenomenon

of ADE is an important consideration in vaccine development as sub-neutralising or non-neutralising antibodies induced by vaccination could exacerbate rather than neutralise the infection (Thomas *et al.*, 2006; Lidbury and Mahalingam, 2014).

In this thesis, a combination of approaches was used to characterise and understand the human antibody responses to chikungunya virus. The effects of antigenic variation of ECSA and Asian CHIKV genotypes were investigated and evidence for antibody-dependent enhancement of CHIKV was shown. The last part of this study defined the neutralising role of immune IgM during early infection. The findings provide fundamental knowledge for vaccinology and antibody therapy.

1.2 Observations and hypotheses

Humoral immunity plays a pivotal role in the control of infection. Several issues need to be addressed to have a well-designed vaccine in terms of efficacy and safety. This includes the understanding of the level of cross-protective antibodies against different strains or genotypes that may be circulating. This is of particular relevance to Malaysia, where both Asian and ECSA genotypes of CHIKV co-circulate. The first hypothesis of this study is that there are key amino acids within the neutralising epitopes on the envelope glycoproteins of CHIKV which might affect the cross-protective efficacy of immune sera.

Another concern in vaccine safety is the potential induction of non-protective antibodies in vaccinees which could exacerbate disease upon exposure to the virus. Currently, it is not known whether an individual who acquires natural infection could induce a subset of antibodies that promote ADE. The second hypothesis in this study is that there may be a possible role for anti-CHIKV antibodies in mediating ADE.

Lastly, anti-CHIKV IgG is the main antibody isotype in providing life-long immunity, but the definitive role of IgM during early CHIKV infection is little studied. The third hypothesis is that IgM plays an important role in immune protection (particularly neutralising capacity) against CHIKV prior to appearance of IgG.

1.3 Aim

To characterise and understand the human antibody responses to chikungunya virus infection.

1.4 Objectives

  I.   To establish a panel of monoclonal antibodies targeting CHIKV E2 glycoprotein as an immunological tool.

 II.   To study the characteristics of cross-neutralising antibody efficacy and epitopes of antibodies to CHIKV in human immune sera.

III.   To study the possible pathogenic roles of CHIKV antibody in human immune sera.

 IV.   To describe the neutralising role of IgM during the early phase of chikungunya virus infection.

CHAPTER 2: LITERATURE REVIEW

2.1 Alphaviruses

The genus *Alphavirus*, along with *Rubivirus*, make up the family of *Togaviridae*. The *Alphavirus* genus has 29 species and can be classified antigenically into eight complexes: Eastern equine encephalitis (EEE), Venezuelan equine encephalitis (VEE), Western equine encephalitis (WEE), Trocara, Middleburg, Ndumu, Semliki Forest and Barmah Forest. CHIKV is clustered under the Semliki Forest virus complex which includes O'nyong-nyong virus (ONNV), Ross River virus (RRV), Semliki Forest virus (SFV), Getah virus, Bebaru virus and Mayaro virus, all of which share a range of serological cross-reactivity (Chanas *et al.*, 1976; Lewandowski, 2005; Griffin, 2007; Powers *et al.*, 2011).

Alphaviruses are widely distributed throughout the world and are transmitted by arthropod vectors to a vertebrate host in a classical transmission cycle. The needs for specific reservoir hosts, vectors and ecological conditions restrict individual species to a certain geographic distribution. Infection with alphaviruses causes a range of human and animal diseases, from severe polyarthritis to encephalitis. New World alphaviruses, including EEEV, VEEV and WEEV are mostly encephalitogenic, while the Old World alphaviruses, which include CHIKV, ONNV, SINV, RRV and SFV are generally arthritogenic. Despite the evolutionary divergence of alphaviruses into New and Old World groups, they share about 60% similarity in the non-structural proteins and 40% similarity in the least conserved of the structural proteins (Strauss and Strauss, 1994; Lewandowski, 2005). These differences are likely to affect the specificities of these viruses for vectors and hosts.

2.2 Chikungunya virus

CHIKV was first reported and isolated from the serum of a febrile human in Swahili village in southern Tanganyika (now Tanzania), East Africa in 1953, during an epidemic which was similar to dengue infection (Robinson, 1955). The name of chikungunya originates from the Makonde word "kungunyala" which means "to walk bent over" or "to become contorted", a locally applied name due to the distinctive intense joint pains suffered by infected patients (Lumsden, 1955). *Aedes aegypti* and *Aedes albopictus* are the main principal vectors in maintaining the human-mosquito transmission cycles.

A CHIKV virion is similar to other alphaviruses, and is characteristically spherical, 60-70 nm in diameter and composed of an outer phospholipid envelope glycoprotein and an RNA-containing nucleocapsid (Figure 2.1). The virion surface envelope consists of 80 trimeric spikes of heterodimer E1-E2 glycoproteins (80×3 heterodimers = 240 copies) and is arranged in an icosahedral symmetry with a triangulation number of four (T=4). Both viral envelope glycoproteins and lipid bilayer derived from host cell membrane, tightly enclose the particle core, which is an icosahedral nucleocapsid 40 nm in diameter. It is composed of 240 copies of the capsid protein and the genomic RNA (Lewandowski, 2005; Powers *et al.*, 2011). The interactions between cytoplasmic tails from heterodimers and capsid proteins at a 1:1 ratio maintain the regular spherical structure and cover the whole viral surface (Forsell *et al.*, 2000). The 3D structures of CHIKV E1-E2 heterodimers in mature and precursor forms andCHIKV virus-like particles (VLP) have been resolved by X-ray crystallography and high-resolution cryo-electron microscopy (Voss *et al.*, 2010; Sun *et al.*, 2013).
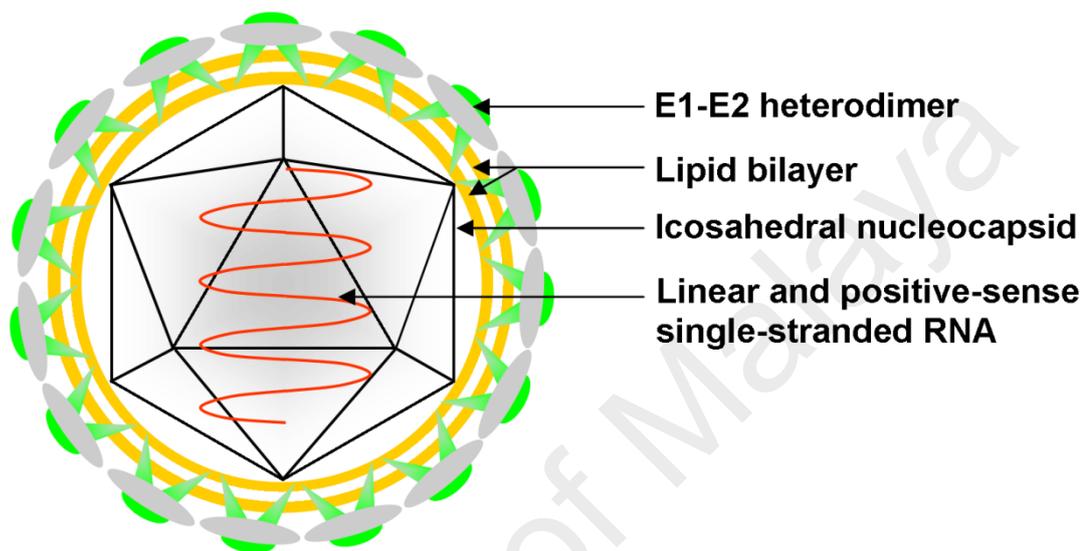
Figure 2.1: Schematic representation of an alphavirus virion. The nucleocapsid is enveloped by the host-derived lipid bilayer, which is embedded in the E1-E2 heterodimers envelope glycoproteins. The lipid bilayer is associated with cholesterol and sphingolipids (Wilkinson *et al.*, 2005; Umashankar *et al.*, 2008; Sousa *et al.*, 2011). The genomic RNA is encapsidated to form an icosahedral nucleocapsid. Figure adapted and modified from Lewandoski (2005) and Zhang *et al.* (2011).

The genomic RNA is linear, positive-sense and single-stranded. The size of the genome is approximately 11.8 kb with two open reading frames (ORF). Four non-structural proteins (nsP1, nsP2, nsP3 and nsP4) are translated from the genomic RNA from the first ORF, which covers nearly two-thirds of the genome. The second ORF, which is approximately one-third of the genome, encodes the five structural proteins (capsid, E3, E2, 6K and E1). The structural proteins are translated from a subgenomic (SG) mRNA using an internal SG promoter in the junction region of the genome (Levis *et al.*, 1990). The ORFs are flanked by the 5' untranslated region (UTR) with a 7-methylguanosine cap and the 3' UTR with a polyadenosine (poly-A) tail. The genomic order is 5' capped UTR-nsP1-nsP2-nsP3-nsP4-junction-C-E3-E2-6K-E1-3' UTR-poly (A) tail (Khan *et al.*, 2002).

CHIKV has three major genotypes circulating: West African, East/ Central/ South African (ECSA) and Asian (Powers *et al.*, 2000). A new Indian Ocean lineage (IOL) rapidly evolved within the ECSA genotype and is characterised by sequential genetic adaptations to *Ae. albopictus* (Schuffenecker *et al.*, 2006; Vazeille *et al.*, 2007; Tsetsarkin and Weaver, 2011; Tsetsarkin *et al.*, 2014). The most notable mutation, A226V in the E1 glycoprotein, favours replication in *Ae. albopictus* compared to *Ae. aegypti* (Tsetsarkin *et al.*, 2007). The improved vector competency and switching drove global outbreaks starting from 2005 and affected millions in immunologically naïve populations, especially in India and Southeast Asia (reviewed in Weaver and Lecuit, 2015). Unexpectedly, the Asian genotype which is endemic in the Asian region, has re-emerged to cause large outbreaks in the Americas and the Pacific Islands from late 2013 (Leparc-Goffart *et al.*, 2014).

2.3 Function of CHIKV proteins

The structural proteins of a virion comprise two major envelope glycoproteins (E1 and E2) and the capsid (Simizu *et al.*, 1984). These proteins are highly immunogenic and elicit strong antibody responses in infected hosts (Sourisseau *et al.*, 2007b). Non-structural proteins are expressed in the cell cytoplasm to facilitate viral RNA transcription and replication, RNA capping and polyprotein cleavage. These non-structural proteins interact with host factors or cellular proteins to regulate virus replication in cells (Das *et al.*, 2014a; Rathore *et al.*, 2014; Scholte *et al.*, 2015; Kim *et al.*, 2016). The formation of proper replication complexes or replicase requires the assembly of these individual four proteins, which later associate with the cell membrane (Thaa *et al.*, 2015).

2.3.1 Non-structural proteins

The nsP1 protein consists of 535 amino acids with a size of about 60 kDa. It serves as the viral RNA capping enzyme, and possesses guanine-7-methyltransferase and guanylyltransferase enzymatic activities in capping and cap methylation of newly synthesised positive-strand genomic and subgenomic RNAs (Ahola and Kääriäinen, 1995; Ahola *et al.*, 1997). The tight association of nsP1 with inner cellular membranes is mediated by palmitoylation of cysteine residues and direct interaction of the amphipathic binding peptide with anionic phospholipids. These characteristics provide crucial features to anchor the replication complexes during RNA replication (Peränen *et al.*, 1995; Laakkonen *et al.*, 1996; Ahola *et al.*, 1999; Salonen *et al.*, 2003). It is also involved in negative-strand RNA replicative intermediates (Wang *et al.*, 1991). Recently, Jones *et al.* (2013) reported that nsP1 antagonises the action of BST-2/tetherin and subsequently allows the virus to bud from the cell membrane, a process which is tethered by BST-2.

The nsP2 protein consists of 798 amino acids and has a size of about 90 kDa, making it the largest multifunctional, complex protein among the non-structural proteins. It has four enzymatic activities, acting as an RNA triphosphatase, nucleoside triphosphatase (NTPase), RNA helicase at the N-terminus, and protease at the C-terminus (Pastorino *et al.*, 2008; Karpe *et al.*, 2011; Das *et al.*, 2014b; Saisawang *et al.*, 2015). The helicase is responsible for unwinding the RNA duplex during RNA replication and transcription, which is modulated by a complete nsP2 structure or conformation and is fuelled by the interaction with NTPase (Das *et al.*, 2014b). The C-terminus of the cysteine protease is responsible for cleavage of the polyprotein at specific sites into intermediate and final component proteins. The coordination of polyprotein cleavage by nsP2 is necessary to regulate the replication of the viral genome in formation of negative sense replicase to positive sense replicase (Lulla *et al.*, 2012). The individual nsP2 can be located in the nucleus and the cytoplasm. It has multiple actions to shut off host transcription and translation, antagonise host defence (inhibition of interferon-induced JAK-STAT signalling) and induce cytotoxic effects (Garmashova *et al.*, 2007; Fros *et al.*, 2010; Akhrymuk *et al.*, 2012; Bouraï *et al.*, 2012; Fros *et al.*, 2013; Fros *et al.*, 2015b; reviewed in Fros and Pijlman, 2016).

The nsP3 consists of 523-530 amino acids with a size of about 57 or 58 kDa, and is a membrane-bound and hyperphosphorylated protein. It is comprised of a N-terminal macrodomain that binds to poly(ADP-ribose) and RNA molecules, the central zinc-binding domain, and a C-terminal hypervariable domain that varies in length and sequence (Peränen *et al.*, 1988; Li *et al.*, 1990; Malet *et al.*, 2009; Shin *et al.*, 2012). The role of CHIKV or alphavirus nsP3 was enigmatic until recently, when Fros *et al.* (2012) reported that it blocks stress granule assembly by sequestration of G3BP (Ras-GAP SH3 domain-binding protein). Formation of stress granules is part of antiviral activity which attenuates host translation in the presence of G3BP, a ubiquitously host-

expressed protein (Buchan and Parker, 2009). A G3BP homologue expressed in insects called Rasputin also interacts with nsP3 and has a significant effect on CHIKV infectivity in mosquitoes (Fros *et al.*, 2015a). The C-terminal hypervariable region of nsP3 has the potential to activate and exploit the phosphatidylinositol-3-kinase (PI3K)-Akt-mammalian target of rapamycin (mTOR) pathway, a prosurvival signalling cascade, to maintain efficient virus replication (Thaa *et al.*, 2015). In other alphaviruses, nsP3 is required for negative-strand RNA synthesis (Lemm *et al.*, 1994) and is the virulence determinant of pathogenesis in mice (Saul *et al.*, 2015).

The nsP4 protein consists of 611 amino acids with a size of about 68 kDa. It is an RNA-dependent RNA polymerase, and its catalytic activity is mapped to a conserved GDD motif at the C-terminal region (Kamer and Argos, 1984; Tomar *et al.*, 2006). The syntheses of positive and negative strands RNA are dependent on conformational changes and non-structural polyprotein processing (Shirako and Strauss, 1994). The nsP4 protein is not abundant in infected cells due to instability with a tyrosine residue at the N-terminus which leads to rapid degradation (Varshavsky, 1997). Furthermore, expression may be low due to the presence of a leaky opal stop codon preceding the nsP4 gene, leading to read-through codon efficiency of less than 20% (Strauss and Strauss, 1994). Despite being present in low amounts in infected cells, nsP4 has been demonstrated to suppress the serine51 phosphorylation of eukaryotic translation initiation factor, alpha subunit (elF2α) to overcome the host unfolded protein response (UPR) machinery, which is a natural host defence (Rathore *et al.*, 2013).

2.3.2 Structural proteins

The capsid protein consists of 261 amino acids with a size of about 30 kDa. It possesses an enzymatically active protein which catalyses autocleavage from the nascent structural protein. The N-terminus is rich in positive charge which is implicated in capsid-RNA binding to capsulate the genome during core assembly. The C-terminus is involved in formation of capsomeres to form the nucleocapsid and interacts with cytoplasmic tails of E2 glycoprotein during the budding process (Zhao *et al.*, 1994; Hong *et al.*, 2006; Solignat *et al.*, 2009).

E3 consists of 64 amino acids with a size of about 11 kDa, and is glycosylated at amino acid position 12. It is cleaved from pE2 or p62 by furin and is not associated with the mature virion (Simizu *et al.*, 1984; Ozden *et al.*, 2008). It serves as a signal sequence for translocation of the E3-E2-6K-E1 polyprotein into the endoplasmic reticulum and regulates the spike assembly (Snyder and Mukhopadhyay, 2012).

E2 consists of 423 amino acids with a size of about 50 kDa inclusive of two N-linked glycans at positions 263 and 345 (Sun *et al.*, 2013). It forms a heterodimer with the E1 glycoprotein and interacts with cellular receptors to mediate viral entry into the host cell. At neutral pH, the E2 covers much of the E1 glycoprotein to prevent exposure of the fusion loop. The E2 glycoprotein belongs to the immunoglobulin superfamily, with domain B exposed at the end of the β-ribbon connector and domain A in the middle (Voss *et al.*, 2010). Domains A and B have determinants of host tropism and epitopes for neutralising antibodies in alphaviruses, while domain C is in a less-exposed position and nearest to the viral membrane (Figure 2.2). Part of the β-ribbon is termed the "acid sensitive region" (ASR), as its conformation becomes disordered at low pH levels. The cysteine residues in the E2 glycoprotein maintain the conformation through formation of disulphide bonds and are important in virus assembly (Snyder *et al.*, 2012). The endo-domain region of E2 consists of a transmembrane helix and a cytoplasmic tail,

which function to anchor the glycoprotein in the lipid bilayer and to interact with the nucleocapsid, respectively.

The 6K protein consists of 61 amino acids and is cleaved by host signalase from the translated pE2-6K-E1 polyprotein. The exact function of 6K is poorly understood; however, the deletion of this region in SINV, SFV and RRV impair viral budding (Liljeström and Garoff, 1991; Sanz and Carrasco, 2001; Taylor *et al.*, 2016). RRV virions that lack the 6K proteins were more sensitive to pH and temperature changes. The 6K protein could form ion channels to alter the membrane permeability and potential of infected cells (Melton *et al.*, 2002). Metz *et al.* (2011) demonstrated that the 6K of CHIKV contains a native signal peptide, which is similar to E3 and translocates the E1 polyprotein to the endoplasmic reticulum for further processing.

E1 consists of 440 amino acids with a size of about 50 kDa inclusive of an N-linked glycosylation at position 141. It is a class II fusion protein which mediates fusion of the virion envelope with the cell membrane when the E1-E2 heterodimers dissociate at low pH (Kielian, 2010). It also has a role to convert the viral surface proteins into ion-permeable pores to allow ion exchanges and leads to physiological pH changes (Wengler *et al.*, 2003). The E1 ecto-domain consists of the three β-barrel domains I, II and III, and conformation is maintained by the presence of disulphide bonds (Voss *et al.*, 2010) (Figure 2.2). The endo-domain region of E1 is similar to E2, except the cytoplasmic tail does not interact with nucleocapsid (Mukhopadhyay *et al.*, 2006). Simizu *et al.* (1984) demonstrated that the E1 glycoprotein exhibits haemagglutinating activity.
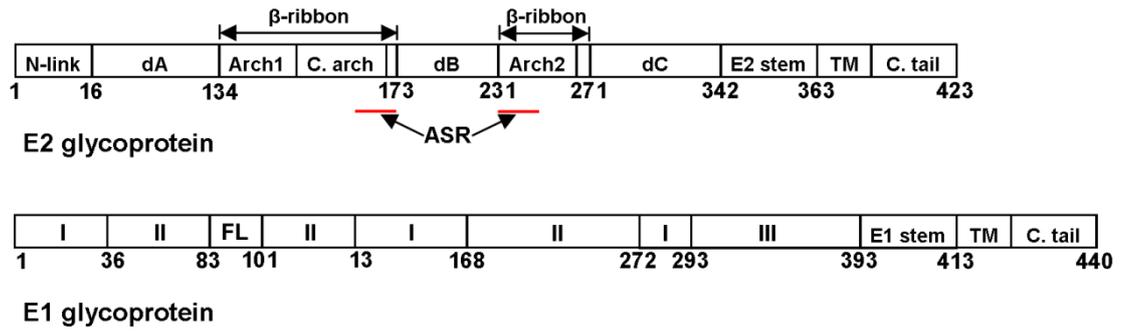
Figure 2.2: Organisation of different domains of E2 and E1 glycoproteins. The numbers refer to the amino acid positions demarcating the E1 and E2 domains. N, N-link; dA, domain A; C. arch, central arch; ASR, acid sensitive region; dB, domain B; dC, domain C; TM, transmembrane; C. tail, cytoplasmic tail; I, domain I; II, domain II; FL, fusion loop; III, domain III. The ASR region is indicated by red lines. The fusion loop is within the domain II region. Figure adapted and modified from Voss *et al.* (2010).

2.4 Replication cycle of CHIKV

The replication cycle of CHIKV is very similar to those of other alphaviruses. To initiate an infection, the E2 glycoprotein of CHIKV, particularly domain A, interacts and binds to host cellular receptor(s) (Sun *et al.*, 2013). The suggested CHIKV receptors are prohibitin (Wintachai *et al.*, 2012), phosphatidylserine-mediated virus entry-enhancing receptors (Moller-Tank *et al.*, 2013), glycosaminoglycans (Silva *et al.*, 2014) and ATP synthase β subunit (Fongsaran *et al.*, 2014). The subsequent event is virus entry or internalisation into cell cytoplasm via clathrin-mediated endocytosis, and the virus is delivered to endosomes (Sourisseau *et al.*, 2007b; reviewed in van Duijl-Richter *et al.*, 2015; Hoornweg *et al.*, 2016). The acidic environment in the endosome triggers an irreversible conformational rearrangement of viral glycoprotein. At low pH, the ASR of E2 will be disordered and the hydrophobic fusion loop of E1 will be uncapped by domain B of E2 and thus exposed (Kielian, 2010). The rearrangement or refolding of E1 glycoprotein triggers membrane fusion in the early endosome by forming fusogenic homotrimers and fusion pores and results in the release of nucleocapsid (Bernard *et al.*, 2010; reviewed in Kielian *et al.*, 2010). Finally, the interaction of nucleocapsid with ribosomes facilitates the disassembly that releases the genomic viral RNA (Wengler *et al.*, 1992). The initial exposure of the virion in the endosome could have a secondary effect in priming nucleocapsid disassembly (reviewed in Leung *et al.*, 2011) (Figure 2.3).

The positive 49S genomic RNA serves as mRNA for non-structural polyprotein synthesis. The 5' ORF is translated into a P123 polyprotein or P1234 polyprotein by read-through of an opal codon located between nsP3 and nsP4. The syntheses of negative-strand and new positive-strand RNA are mutually exclusive events, and the organisation of replication complexes determines the types of RNA synthesis (Lulla *et al.*, 2012). The formation of replication complexes is characterised by the presence of

spherules, which are located near to the cell membrane (Utt *et al.*, 2016). The nsP4 is *cis* cleaved out from P1234 by nsP2, producing a negative strand replicase. It binds to genomic RNA as a template to synthesise full-length negative-strand RNA and forms dsRNA with its template. A short-lived intermediate replication complex is formed when another *cis* cleavage occurs between the nsP1 and nsP2 regions of P123. As infection proceeds, nsP2 and nsP3 are cleaved from P23 in a *trans* manner to form a stable replication complex with nsP1 and nsP4. The sequential cleavages produce a positive strand replicase to synthesise exclusively two types of positive strand RNA, 49S genomic RNA and 26S subgenomic RNA (Figure 2.3).

The positive 26S subgenomic RNA serves as mRNA for the second ORF, leading to synthesis of structural proteins. This RNA is transcribed from negative sense RNA; the promoter is located in the junction sequence between non-structural and structural gene regions. The second ORF translates into the C-pE2-6K-E1 polyprotein precursor. The autocatalytic protease of capsid cleaves itself from the nascent precursor and proceeds with RNA packaging to form the nucleocapsid with the newly synthesised 49S genomic RNA. The exposed N-terminus of pE2-6K-E1 contains a signal sequence for translocating the polyprotein into the rough endoplasmic reticulum for glycoprotein synthesis. Host signalase cleaves the precursor structural protein into pE2, 6K and E1. In the Golgi apparatus, pE2 and E1 form heterodimers, followed by furin cleavage to form E2 and E3. The final cleavage forms the mature E2-E1 heterodimers, which are transported to the cell membrane by protein cargo. The E2 cytoplasmic tail interacts with capsid in the participation of virus budding. The newly released virus acquires a phospholipid bilayer from the host cell membrane which is embedded within the envelope glycoproteins in trimeric spikes (Figures 2.1 and 2.3).
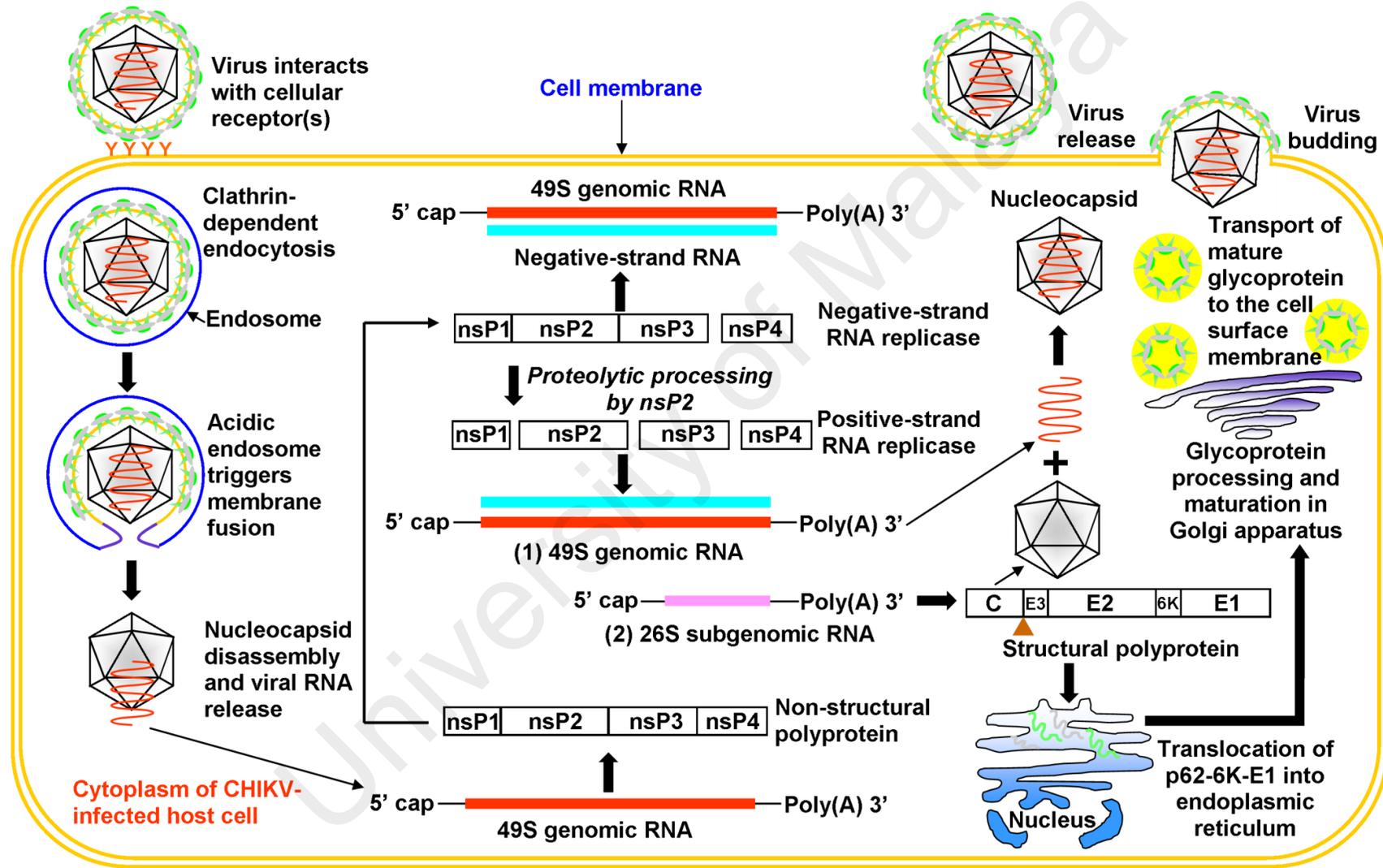
Figure 2.3: Replication cycle of CHIKV from virus binding to budding and release.

Figure 2.3, continued: Replication cycle of CHIKV from virus binding to budding and release. CHIKV first binds to the cellular receptor, followed by clathrin-dependent endocytosis into the cell cytoplasm. The acidic compartment within the endosome triggers conformational changes of E1-E2 heterodimers, which subsequently fuse with endosomes. The disassembled nucleocapsid releases the genomic viral RNA, and this is followed by production of non-structural proteins which are necessary for RNA replication by forming replication complexes (replicase). Transcription of 26S subgenomic RNA from negative-strand RNA leads to the production of structural proteins that are required for virion structure formation. The capsid protein encapsulates the newly synthesised 49S genomic RNA to form the nucleocapsid, while new envelope proteins undergoes a series of processing and maturation steps. The nucleocapsid interacts with mature envelope glycoproteins on the cell membrane, before budding of the complete virion. Figure adapted and modified from Jose *et al.* (2009) and Schwartz and Albert (2010).

2.5 Epidemiology

CHIKV was first isolated in Tanzania in 1952. The sylvatic and enzoonotic transmission cycle maintains the CHIKV transmission and circulation between mosquitoes (*Ae. africanus, Ae. furcifer-taylori, Ae. luteocephalus, Ae. neoafricanus* and *Culex sp.*) and animal reservoirs, in particular monkeys (reviewed in Rougeron *et al.*, 2015; Weaver and Forrester, 2015). Humans become the accidental host when occasional spillover infections occur and subsequently initiate and amplify the transmission cycle with mosquitoes (*Ae.aedes aegypti* and *Ae. albopictus*). Since the 1950s, small and sporadic epidemic were documented in Africa until the first CHIKV case outside of Africa was reported in Thailand (Hammon *et al.*, 1960; reviewed in Weaver and Lecuit, 2015). The isolated strain belonged to the Asian genotype and for the next few decades, this genotype was confined to Asia, primarily Southeast Asian countries (reviewed in Ng and Hapuarachchi, 2010; Tsetsarkin *et al.*, 2011).

The global outbreaks in 2005 were caused by the ECSA genotype, and initially spread from Kenya to the Indian Ocean islands and India, and subsequently to other Asian countries, affecting millions of people (Figure 2.4) (reviewed in Tsetsarkin *et al.*, 2016). Two different lineages - Indian and Indian Ocean (IOL) evolved within the ECSA genotype. Most of the IOL strains have acquired an E1-A226V mutation, which likely arose in La Réunion (Schuffenecker *et al.*, 2006; de Lamballerie *et al.*, 2008b). This mutation increased adaptation to *Ae.albopictus*, which helped to expand the scale of the epidemics (Tsetsarkin *et al.*, 2007). The movement of air travellers from affected regions further expanded the magnitude of the epidemic by introducing the virus into Europe, Asia and the USA (Parola *et al.*, 2006; Lanciotti *et al.*, 2007; Rezza *et al.*, 2007; Panning *et al.*, 2008; Huang *et al.*, 2009; Grandadam *et al.*, 2011).

Further massive outbreaks were reported in 2013, which were due to the Asian genotype, and have resulted in more than 1 million reported cases (Petersen and Powers,

2016). The re-emergence of Asian genotype was reported mainly in Indonesia and the Philippines, and related strains were subsequently seen in Oceania, including New Caledonia and Yap, Federated States of Micronesia, before reaching the Caribbean and Americas (Figure 2.4) (Lanciotti and Valadere, 2014; Leparc-Goffart *et al.*, 2014; Roth *et al.*, 2014; Pan American Health Organization, 2016). In 2014, Brazil reported co-circulation of ECSA and Asian genotypes (Nunes *et al.*, 2015; Teixeira *et al.*, 2015; Rodrigues Faria *et al.*, 2016). Duplication in 3' UTR was shown to favour the replication of certain Asian strains in mosquito cell lines (Stapleford *et al.*, 2016). A comprehensive genome-scale phylogenetic study suggests that CHIKV has currently diversified and evolved to expand its geographic areas (Chen *et al.*, 2016).

In Malaysia, no clinical case of CHIKV infection was reported until 1998. However, before this, seroprevalence studies had been conducted to investigate the presence of antibodies in animals and human in Peninsular and East Malaysia against Sindbis, Getah, chikungunya, Japanese encephalitis and dengue viruses (Bowen *et al.*, 1975; Marchette *et al.*, 1978; Marchette *et al.*, 1980). Seropositivity towards CHIKV was reported in human populations at the Malaysia-Thailand border and in East Malaysia, suggesting that CHIKV was endemic in these regions. CHIKV has been isolated from non-human primates (*Macaca fascularis*) in Malaysia (Apandi *et al.*, 2010), but their role in maintaining a sylvatic cycle is likely minimal (Sam *et al.*, 2015a).

The first CHIKV outbreak was reported in and confined to Klang city, Selangor state, which is 25 kilometres from Kuala Lumpur (Lam *et al.*, 2001). The Asian genotype was found to be responsible for this first small outbreak in December 1998, which affected 51 persons (Hasebe *et al.*, 2002). Another Asian CHIKV outbreak occurred in Bagan Panchor, Perak state with at least 200 people affected in 2006 (Kumarasamy *et al.*, 2006; AbuBakar *et al.*, 2007; Ayu *et al.*, 2010). Although the second outbreak coincided with the ongoing ECSA epidemics reported in India and the Indian Ocean since 2005, the

causative strains were from the Asian genotype, and it was suggested that the Asian genotype was endemic and circulating at low levels following the first outbreak. In late 2006, CHIKV cases due to ECSA genotype were reported for the first time in Perak state, which was highly likely imported from India (Noridah *et al.*, 2007; Soon *et al.*, 2007; Chem *et al.*, 2010). The limited sequences reported did not include the E1-226 position, but the strains were closely related to Indian strains which carried E1-226A. Starting from 2008, an unprecedented nationwide outbreak was reported spreading from Johor state in the south, which affected over 10,000 people across different states (Figure 2.5) (Sam *et al.*, 2009; Chua, 2010). Strains of the ECSA genotype carrying the A226V mutation were identified to be responsible and were closely related to Indian strains from 2007. The epidemic ECSA strain replicated better in Malaysian *Ae. albopictus* compared to the endemic Asian genotype (Sam *et al.*, 2012). This may explain the explosive and extensive outbreak in 2008-2010 compared to the restricted outbreaks in 1998 and 2006, as the A226V mutation in the E1 glycoprotein enhances virus dissemination where *Ae. albopictus* predominates. Both Asian and ECSA genotypes are now circulating in Malaysia.
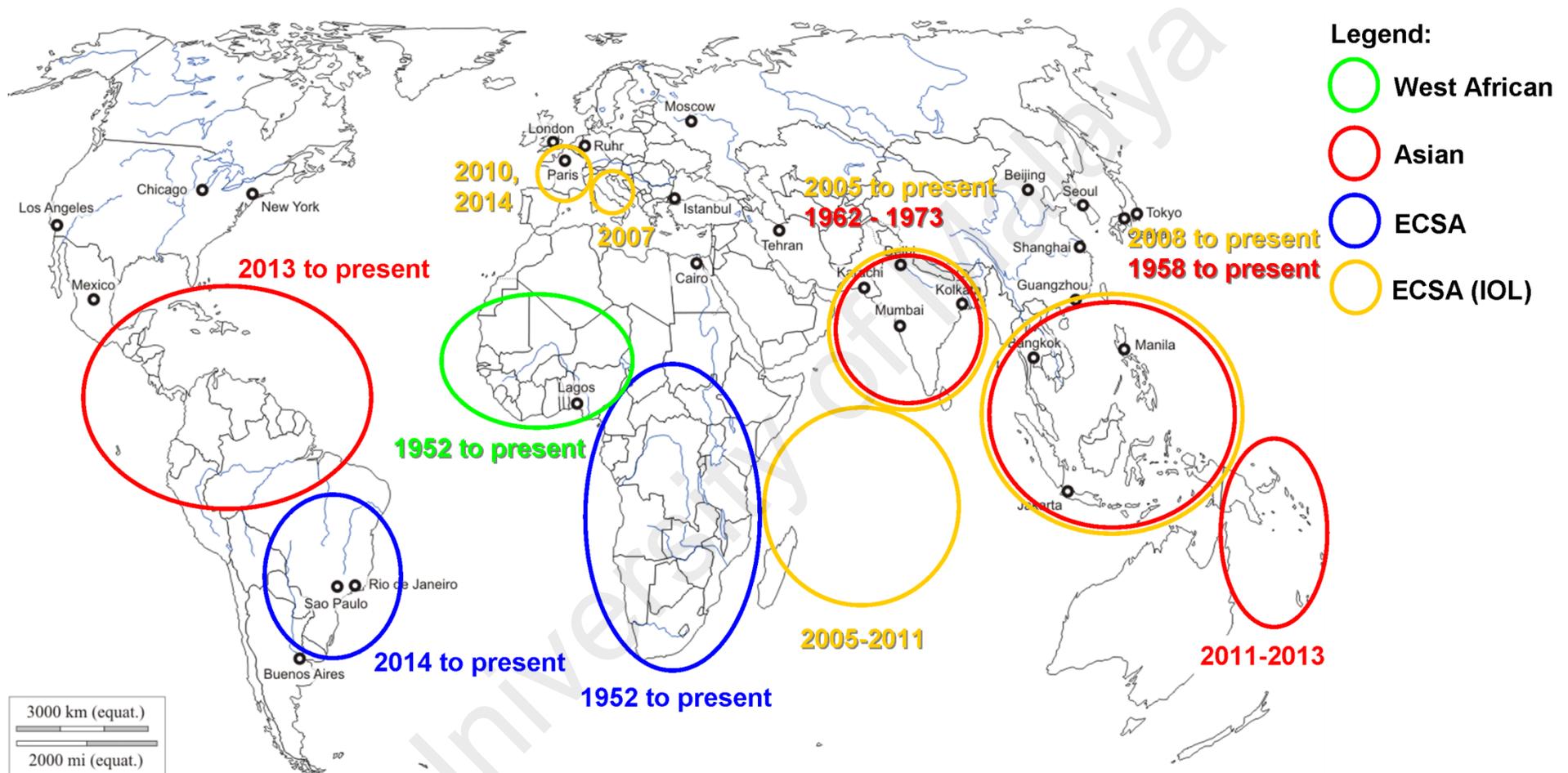
Figure 2.4: Geographical distribution of CHIKV outbreaks with different genotypes circulating. The map was retrieved from http://www.d-maps.com and modified from Horwood and Buchy (2015), Musso *et al.* (2015), Weaver and Lecuit (2015), Amraoui and Failloux (2016).
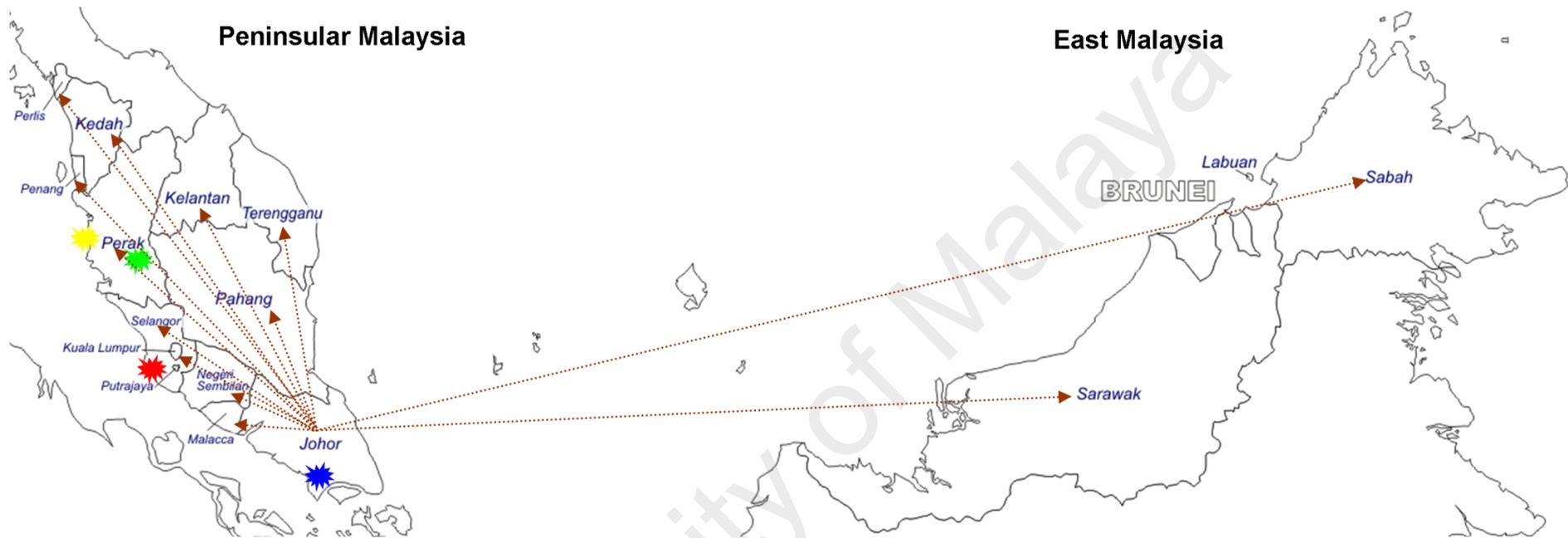
Legend:

⭐ Dec 1998   - Klang, Selangor state (Asian)

⭐ Mar 2006   - Bagan Panchor, Perak state (Asian)

⭐ Aug and Dec 2006   - Batu Gajah and Ipoh, Perak state (ECSA)

⭐ April 2008-2010   - Johor state and nationwide outbreak (ECSA)

Figure 2.5: Geographical distribution of CHIKV outbreaks in Malaysia. The map was retrieved from http://www.d-maps.com and modified accordingly.

2.6 Clinical manifestations

In Malaysia, other tropical infections such as dengue and leptospirosis share similar clinical presentations with CHIKV which can lead to misdiagnosis and impact clinical management. Co-infection with another arbovirus is possible, and interestingly, triple co-infections of Zika, CHIKV and dengue were reported in South America (Chahar *et al.*, 2009; Edwards *et al.*, 2016; Sardi *et al.*, 2016; Villamil-Gómez *et al.*, 2016a; Villamil-Gómez *et al.*, 2016b). Empirical diagnosis based on comparative clinical features aids in subsequent diagnostic decision making (Laoprasopwattana *et al.*, 2012; Lee *et al.*, 2012; Mohd Zim *et al.*, 2013).

The classical symptoms of CHIKV infection are sudden fever, profound arthralgia, myalgia and erythematous maculopapular rash (Sam and AbuBakar, 2006; reviewed in Schwartz and Albert, 2010; reviewed in Suhrbier *et al.*, 2012). Those symptoms appear after an incubation period of 2–10 days and are accompanied by viraemia. Generally, CHIKV is self-limiting and the acute symptoms resolve within 7–10 days, but the clinical manifestations are variable in infants, children and adults (Gasque *et al.*, 2015). Blistering or vesiculobullous and peeling lesions are observed in infants less than one year old (Valamparampil *et al.*, 2009; Robin *et al.*, 2010). Children get milder arthralgia and less frequently develop chronic arthralgia (Sebastian *et al.*, 2009; Ritz *et al.*, 2015; Simarmata *et al.*, 2016). Adults can either fully recover or experience a chronic phase with persistent arthralgia, which may last for months or years (Essackjee *et al.*, 2013; Mohd Zim *et al.*, 2013; Schilte *et al.*, 2013). The chronic arthralgia may restrict movement around the peripheral joints (wrists, ankles, phalanges) and larger joints (knee) (Taubitz *et al.*, 2007; Borgherini *et al.*, 2008; Staikowsky *et al.*, 2008; Staikowsky *et al.*, 2009; de Andrade *et al.*, 2010; Miner *et al.*, 2015). In some severe cases, patients experience bone or joint erosions and destructive arthritis resembling rheumatoid arthritis (RA) (Bouquillard and Combe, 2009; Malvy *et al.*, 2009;

Chaaithanya *et al.*, 2014); however, these studies did not exclude patients with RA who acquired CHIKV infection. For patients with chronic symptoms, quality of life may greatly impaired physically (discomfort and pain) and mentally (emotional) (de Andrade *et al.*, 2010).

Other common and less specific acute symptoms include weakness, nausea, abdominal pain, headache, diarrhoea, vomiting, dizziness, oedema, retro-orbital pain, photophobia and conjunctivitis. Severe manifestations such as multi-organ failure, shock, haemorrhage, severe purpuric lesions, meningoencephalitis, acute hepatitis and fatal cases have been documented (Economopoulou *et al.*, 2009; Chua *et al.*, 2010; Sam *et al.*, 2010; Cardona-Ospina *et al.*, 2015; Torres *et al.*, 2015). The presence of underlying co-morbid conditions such as diabetes, heart disease, hypertension and respiratory disease heighten the risk for disease severity and may contribute to deaths associated with acute CHIKV. Recently, there have been increased reports of neurological disease associated with CHIKV infection, such as encephalitis and Guillain-Barré syndrome (Chandak *et al.*, 2009; Lebrun *et al.*, 2009; Chusri *et al.*, 2011; Nelson *et al.*, 2014; Gérardin *et al.*, 2016).

Apart from symptomatic infected cases, asymptomatic individuals with subclinical infection might potentially contribute to virus transmission. This can occur through blood transfusion, necessitating precautionary steps such as symptomatic or laboratory screening of all donors, particularly during outbreaks (Appassakij *et al.*, 2013; Gallian *et al.*, 2014; Gay *et al.*, 2016; Simmons *et al.*, 2016).

2.7 Disease pathogenesis

Humans acquire arbovirus infection after intradermal inoculation from infected female mosquitoes, along with salivary molecules (Wichit *et al.*, 2016). CHIKV is delivered into blood capillaries at the dermis layer of skin by the proboscis of a mosquito. CHIKV can infect skin resident cells such as melanocytes (Gasque and Jaffar-Bandjee, 2015), keratinocytes (Puiprom *et al.*, 2013; Bernard *et al.*, 2015), skin fibroblasts (Thon-Hon *et al.*, 2012; Ekchariyawat *et al.*, 2015) and macrophages (Sourisseau *et al.*, 2007a). The presence of salivary components from mosquito or CHIKV or both, alters the immune response of these resident cells to permit local viral replication (Puiprom *et al.*, 2013; reviewed in Briant *et al.*, 2014; Ekchariyawat *et al.*, 2015). This can be achieved by suppresing IL-8 expression to reduce migration or recruitment of immune cells such as monocytes/macrophages and neutrophils. The virus disseminates from the initial replication site and drains to lymph nodes for further amplification (reviewed in Lum and Ng, 2015). Free virus or infected blood monocytes which serve as primary cellular vehicles, migrate through blood and lymphatic systems to secondary infection sites (Sourisseau *et al.*, 2007b; Her *et al.*, 2010). The endothelial, epithelial, fibroblast, satellite and osteoblast cells in peripheral organs such as spleen, muscles, liver, joint, eye and brain may be infected, leading to clinical symptoms (Ozden *et al.*, 2007; Sourisseau *et al.*, 2007b; Couderc *et al.*, 2008; Ganesan *et al.*, 2008; Couderc *et al.*, 2012; Noret *et al.*, 2012). This viraemic phase of the disease, in which virus remains in blood circulation for 2–10 days, enables onward active virus transmission through bites by non-infected mosquitoes.

CHIKV tropism favours non-haematopoietic cells such as endothelial, epithelial and fibroblast cells (Sourisseau *et al.*, 2007b; Salvador *et al.*, 2009; Wikan *et al.*, 2012). Most haematopoietic cells are refractory to CHIKV infection, except for monocytes and macrophages (Sourisseau *et al.*, 2007b; Her *et al.*, 2010; Ruiz Silva *et al.*, 2016). These

two immune cell types, along with NK and T cells, have been implicated in mediating joint pathology, as demonstrated in animal studies and patients with chronic arthralgia. The analyses of synovial biopsies showed infiltrations of monocytes/macrophages (CD14, CD18), NK (CD56) and T (CD4) cells with high levels of MCP-1, IL-6 and IL-8, along with virus antigen and RNA (Hoarau *et al.*, 2010). Similar observations were noted in infected mice and non-human primates with infiltrations of monocytes/macrophages in the inflamed joints (Gardner *et al.*, 2010; Labadie *et al.*, 2010).

Various independent studies have defined the role of immune cells and mediators in pathogenesis with the use of knock-out mice and treatments. Development of joint pathology was reduced in MHCII$^{-/-}$ and CD4$^{-/-}$ mice (Nakaya *et al.*, 2012; Teo *et al.*, 2013). The use of fingolimod in CHIKV-infected mice reduced the infiltrations of CD4+ cells to joints with low level secretions of IFN-ɣ (Teo *et al.*, 2017), which could likely reduce inflammation attributed to NK and T cells in the absence of granzyme A secretion, an identified serine protease which drives inflammation (Wilson *et al.*, 2017). CD4+ cells comprise regulatory T (Treg) cells and conventional T helper (Th) cells (reviewed in Vignali *et al.*, 2008; reviewed in Corthay, 2009). The activation of Treg cells may suppress any potential adverse activities of Th cells, and thus suppress pathogen-induced immunopathology. A complementary study to expand regulatory T cells suppressed the activation of effector CD4 cells, which protected mice against CHIKV-induced pathology (Lee *et al.*, 2015). The depletion of NK cells in mice with anti-asialo GM1 antibody led to reduced disease score (Teo *et al.*, 2015). The depletion of macrophages by clodronate liposome and inhibition of MCP-1 synthesis by bindarit had significant effects in alleviating joint pathology (Gardner *et al.*, 2010; Chen *et al.*, 2015b). High levels of MCP-1 (also known as CCL2) were detected during the acute phase of CHIKV infection accompanied by increased monocyte infiltration at the

inflammation sites as demonstrated in mice (Gardner *et al.*, 2010; Labadie *et al.*, 2010; Poo *et al.*, 2014a). Although MCP-1 mediates monocyte/macrophage infiltrations to drive joint pathology, CHIKV infection in CCR2$^{-/-}$ mice lacking in MCP-1 receptor developed more severe disease outcome with cartilage damage (Poo *et al.*, 2014a). The cellular infiltrates in joints were dominated by neutrophils, rather than monocytes, which in turn led to dysregulation of immune and inflammatory mediator pathways. High levels of IL-6 were found in patients with high viral load and patients with chronic arthralgia (Hoarau *et al.*, 2010; Chow *et al.*, 2011). The major source and role of this pleiotropic cytokine remains unclear. A potential source of IL-6 was identified as CHIKV-infected primary osteoblasts, which subsequently alter the ratio of RANKL: OPG (receptor activator of nuclear factor kappa-B ligand: osteoprotegerin) (Her *et al.*, 2012; Noret *et al.*, 2012). IL-6 production follows an autocrine loop pattern, further disrupts the ratio of RANKL: OPG and contributes to bone loss with the occurrence of arthralgia (reviewed in Chen *et al.*, 2015a). The use of neutralising IL-6 antibody in RRV-infected mice maintained a normal ratio of RANKL: OPG and abrogated RRV-induced joint pathology (Chen *et al.*, 2014). IL-6 has been implicated in the pathophysiology of rheumatoid arthritis (Srirangan and Choy, 2010). Moreover, CHIKV-induced arthritis and rheumatoid arthritis share high levels of similarities in gene expression signatures, inflammatory processes and disease outcome (Nakaya *et al.*, 2012).

Direct evidence of innate components in mediating pathogenesis has been elucidated from CHIKV-infected patients. The gene expression of innate components such as myeloid arginase 1 (Arg1), CD74 (MIF) and pentraxin 3 (PTX3) are upregulated in patients' PBMCs during the acute phase of infection, and peaked with viral load and disease severity. Arg1-expressing myeloid cells inhibited activation and function of T cells, with suppression of IFN-ɣ and CD69 expression in mice (Burrack *et al.*, 2015).

Furthermore, Arg1 was highly expressed in the infiltrating macrophages present in inflamed joints of infected mice (Stoermer *et al.*, 2012). CD74 interacts with macrophage migration inhibitory factor (MIF) and regulates inflammatory monocyte/macrophage recruitment (Leng *et al.*, 2003). CHIKV-infected CD74^{-/-} mice resulted in reduced tissue inflammation, accompanied by reduced capacity to recruit inflammatory monocytes and NK cells without disrupting the expression of pro-inflammatory cytokines and chemokines (Herrero *et al.*, 2013). PTX3 is involved in complement pathway and inflammation (Jaillon *et al.*, 2007). Overexpression of PTX3 in mammalian cells enhanced RRV infectivity, while RRV-infected PTX3^{-/-} mice exhibited reduced disease scores and delayed monocyte infiltrations (Foo *et al.*, 2015). The pathogenesis studies in mice show that joint inflammation and pathology are due to immune mechanisms and dysregulation rather than direct damage by the virus itself.

The role of host age has been investigated to compare the spectrum of clinical outcomes and immunological profiles. High levels of TGF-β in older humans and mice impact the antibody responses with imbalance in pro and anti-inflammatory mediators, leading to chronic joint pathology (Uhrlaub *et al.*, 2016). Another study in aged non-human primates demonstrated the reduced capacities of T and B cell responses (Messaoudi *et al.*, 2013). These data support the clinical findings that the elderly are more susceptible to chronic disease, likely due to dysregulation of immune responses (Hoarau *et al.*, 2010; Chow *et al.*, 2011; Chopra *et al.*, 2012).

2.8 Immune controls of CHIKV

Poo *et al.* (2014b) conducted a comprehensive and comparative study to define different immune factors in controlling acute and chronic CHIKV infection. The interplay between innate and adaptive immune responses is necessary for disease control. However, CHIKV non-structural proteins can antagonise the action of innate immune controls as mentioned in section 2.3.1.

2.8.1 Innate immune responses

Innate immune responses can be mediated by both haematopoietic and non-haematopoietic cells (reviewed in Gasque *et al.*, 2015). Type 1 interferon (IFN), IFN-α and -β exert antiviral responses and involve transcription of related interferon-stimulating genes (ISGs) (Sourisseau *et al.*, 2007b; Schilte *et al.*, 2010; Teng *et al.*, 2012). Retinoic acid-inducible gene I (RIG-1)-like receptors, melanoma differentiation-associated gene 5 (MDA5) and Toll-like receptor 3 (TLR3) act as pathogen recognition receptors (PRR) to initiate the induction of type 1 IFN upon recognition of pathogen-associated molecular motifs (PAMPs), which are the ssRNA and dsRNA of CHIKV (Schilte *et al.*, 2010; Her *et al.*, 2015; Sanchez David *et al.*, 2016). The functional PRR signalling requires different adaptor proteins such as IFN promoter stimulator 1 (IPS-1, also known as CARDIF, MAVS or VISA) with RIG-1 and MDA-5 (White *et al.*, 2011) and TIR domain-containing adaptor-inducing interferon-β (TRIF) with TLR3 (Her *et al.*, 2015). Downstream, this leads to upregulation of interferon response factor 3 (IRF3) and IRF7, which subsequently induces the production of type I IFN. Double knock-out IRF3/7$^{-/-}$ mice succumbed to CHIKV infection earlier in the absence of IFN, accompanied by induction of haemorrhagic shock (Rudd *et al.*, 2012; Schilte *et al.*, 2012). Studies have shown the importance of IFN-α/β in controlling CHIKV infection

in IFN-α/βR$^{-/-}$ 129s/v, STAT129 and A129 mice (Couderc *et al.*, 2008; Partidos *et al.*, 2011; Gardner *et al.*, 2012).

Apart from IRF3 and IRF7, other important ISGs have been reported that mediate antiviral activity against CHIKV. These include viperin (Rsad2) (Teng *et al.*, 2012), tetherin (BST-2) (Jones *et al.*, 2013), ISG15 (Werneke *et al.*, 2011), protein kinase R (PKR) (White *et al.*, 2011), 2'5'-oligoadenylate synthase 3 (OAS3) (Bréhin *et al.*, 2009), ZAP (Gläsker *et al.*, 2014), PARP12L (Atasheva *et al.*, 2012), IFIT1 (Reynaud *et al.*, 2015) and IFITM3 (Poddar *et al.*, 2016). In-depth characterisations of other ISGs identified by high throughput screening are in progress (Schoggins and Rice, 2011; reviewed in Schoggins *et al.*, 2011; reviewed in Long and Heise, 2015). Gene expression study of PBMCs from a Singaporean cohort demonstrated significant elevated transcripts of viperin and TLR3, suggesting the combination effects of innate immune responses provide substantial protection against progressing to severe pathological conditions (Teng *et al.*, 2012; Her *et al.*, 2015). The findings are further supported by the mouse models, as TLR3$^{-/-}$ and Rsad2$^{-/-}$ mice suffered severe joint pathology and higher viral load compared to wild-type mice with similar genetic background.

Multiple studies have been conducted to characterise the immune mediator profiles at different phases of disease from different geographic cohorts. The acute phase of infection is dominated by proinflammatory cytokines and chemokines. High levels of IFN-α are associated with a higher viral load. The expression of IL-6 or MCP-1 and high viral load has a positive correlation (Chow *et al.*, 2011; Reddy *et al.*, 2014). High-grade fever and severe joint pain are associated with upregulations of IL-1β, IL-6 and TNF-α (Ng *et al.*, 2009; Hoarau *et al.*, 2010; Chow *et al.*, 2011). High levels of IL-1β, IL-6 and RANTES are associated with disease severity, and may be useful indicators for disease monitoring (Ng *et al.*, 2009). Persistent arthralgia is associated with high

levels of IL-6 and GM-CSF (Hoarau *et al.*, 2010; Chow *et al.*, 2011). MCP-1 can mediate either pathology or protection and its expression by monocytes is dependent on interaction with other leukocytes (Ruiz Silva *et al.*, 2016). Teng *et al.* (2015) conducted a systematic meta-analysis of immune signatures in CHIKV-infected patients. This includes 14 comparative studies from Gabon, India, Thailand, Singapore, Italy, La Réunion, Sri Lanka and Thailand, which resulted in 20 identified immune signatures. IFN-α is an important biomarker for CHIKV infection. While most published findings are from adults, the immune mediator profiles in children provide different perspectives in understanding the host response. The levels of IL-18, IFN-α2 and IL-2Ra are higher in CHIKV-infected children, while the absence of joint pain manifestation is associated with high levels of GM-CSF (Simarmata *et al.*, 2016). The latter unexpected finding suggests that the role of GM-CSF might be crucial in regulating a balance of host response and clinical outcome, while differences in basal levels of cytokines across ages might influence disease outcome (Biancotto *et al.*, 2013; Kleiner *et al.*, 2013).

Monocytes/macrophages, NK cells and dendritic cells have been implicated in the acute phase of infection. The recruitment of monocytes/macrophages to the site of inflammation with a high level of MCP-1 appears to be critical for preventing excessive pathology, as described in section 2.7. Activation of NK cells and plasmacytoid dendritic cells (pDCs) has been reported from CHIKV-infected patients (Hoarau *et al.*, 2010; Petitdemange *et al.*, 2011).

2.8.2 Adaptive immune responses

Innate immune responses provide rapid and first line defence against viruses, while adaptive immune responses confer long-term, sustainable and complementary protection. Humoral and cell-mediated responses comprise the adaptive immune responses, which are mediated by B and T cells, respectively. These cells act in a highly specific manner and mount effective responses against viral infection.

2.8.2.1 B cells and antibodies

B cells and CHIKV-specific antibodies are important in controlling infection and virus elimination. Persistent viraemia and RNA were detected in Rag1$^{-/-}$ or Rag2$^{-/-}$ (lacking mature T and B lymphocytes) and µMT (B cell-deficient) mice with joint inflammation up to 1 year (Hawman *et al.*, 2013; Lum *et al.*, 2013; Teo *et al.*, 2013; Poo *et al.*, 2014b; Seymour *et al.*, 2015; Hawman *et al.*, 2016). During early infection, IgM production is detected, followed by IgG seroconversion (reviewed in Schwartz and Albert, 2010). In immune individuals, neutralising antibodies can be detected for up to 21 months, with IgG3 dominating the anti-CHIKV IgG responses (Kam *et al.*, 2012a; Nitatpattana *et al.*, 2014; Verma *et al.*, 2014). A high viral load at day 4 post-infection has a significant impact on the magnitude of antibody response, as the early production of neutralising antibodies (IgG) is driven in the presence of high IL-6 (Kam *et al.*, 2012c). High titres of neutralising antibodies (IgG) target the linear epitopes on the E2 glycoprotein, which have been mapped from immune individuals and experimentally-infected mice and non-human primates (Kam *et al.*, 2012b; Lum *et al.*, 2013; Kam *et al.*, 2014). Notably, a well-characterised E2EP3 linear epitope was highly recognised by the antibodies collected from immune individuals and animals. Administration of purified immune human plasma provides protection to infected animals with interferon-alpha receptor knock out (IFNAR$^{-/-}$) and neonatal mice (Couderc *et al.*, 2009). Several mAbs

of mouse or human origin have been developed, which neutralise CHIKV infection (Warter *et al.*, 2011; Fric *et al.*, 2013; Goh *et al.*, 2013; Pal *et al.*, 2013; Selvarajah *et al.*, 2013; Fong *et al.*, 2014; Fox *et al.*, 2015; Smith *et al.*, 2015). These findings suggest that the vaccine development and antibody therapy are highly feasible to provide antibody-mediated protection by active and passive immunisation, respectively. However, only the IgG isotype and its subclasses have been extensively studied in CHIKV infection, while the role of IgM during early CHIKV infection in humans remains to be explored. The role of antiviral IgM has been defined in mouse models for other alphaviruses (Amor *et al.*, 1996; reviewed in Fazakerley, 2002; Metcalf and Griffin, 2011).

2.8.2.2 T cells

In contrast to B cells, limited studies are focused on T cell responses in infected individuals. During the acute phase of infection, lymphopenia has been observed (Borgherini *et al.*, 2007; Staikowsky *et al.*, 2009). CD8+ cells predominate the early stage of infection together with the strong innate immune response, followed by the engagement of CD4+ cells at a later stage (Wauquier *et al.*, 2011). Hoarau *et al.* (2010) reported similar findings on the activation of CD4+ and CD8+ cells in conjunction with the elevated response from NK cells. An *ex vivo* T-cell proliferation assay with peptide pools demonstrated that CD8+ cells mounted strong IFN-ɣ responses compared to CD4+ cells, and was mainly directed against the C-terminus of E2 glycoprotein (Hoarau *et al.*, 2013). In mouse studies, aside from antibodies, CD4+ cells may exert secondary functions in controlling the viraemia, despite their presence contributing to joint pathology (Teo *et al.*, 2013; Poo *et al.*, 2014b).

2.9 Diagnostics

As the clinical manifestations of CHIKV share similar clinical features to other endemic tropical diseases (dengue, typhus, leptospirosis, typhoid, malaria) (reviewed in Sam *et al.*, 2015b), differential laboratory diagnosis is crucial to determine clinical management and to public health control measures and surveillance. The diagnostic methods will depend on the timing of sample collection, which will highly determine the assay sensitivity and specificity. During the viraemic phase of infection, infectious virus or antigen can be isolated or detected from blood serum at peak levels of up to 10^9 viral RNA copies per ml (Laurent *et al.*, 2007); in some cases with neurological complications, CHIKV RNA can be detected in cerebrospinal fluid. RNA can also be found in tissue samples (brain and liver) from biopsy and placenta (Grivard *et al.*, 2007; Chandak *et al.*, 2009; Kashyap *et al.*, 2010; Bandeira *et al.*, 2016). Numerous diagnostic methods have been described, which greatly improve the reliability of assays and shorten the time frame for diagnosis. This is highly beneficial to some countries with CHIKV circulating in resource-limited settings. Since the emergence of Zika virus in South America in late 2013 (reviewed in Weaver *et al.*, 2016), multiplex real-time RT-PCR have been developed which allow simultaneous detections of dengue, CHIKV and Zika virus in epidemic regions where all three co-circulate (Pabbaraju *et al.*, 2016; Waggoner *et al.*, 2016).

After 4-5 days of illness, serological assays become more important, as the host starts to produce CHIKV-specific IgM first, followed by IgG. The presence of antibodies in blood reduces viraemia, which reduces the sensitivity of molecular assays. Many serological CHIKV assays have been commercially developed and some have apparently good sensitivity and specificity compared to reference IgM assays (Johnson *et al.*, 2016; Prince *et al.*, 2016). However, performances in clinical settings have been weak, with most demonstrating poor sensitivity in early samples, especially for IgM

detection in ELISA or immunochromatographic tests using recombinant CHIKV proteins (Rianthavorn *et al.*, 2010; Yap *et al.*, 2010; Blacksell *et al.*, 2011; Kosasih *et al.*, 2012; Prat *et al.*, 2014; Burdino *et al.*, 2016; Johnson *et al.*, 2016). Recently, an Eilat virus-based chimera was developed for serological diagnosis with the replacement of the structural protein region with CHIKV proteins (Erasmus *et al.*, 2015). A large difference in sensitivity of IgM assays was described during two separate outbreaks of different CHIKV clades in Singapore (Yap *et al.*, 2010). This suggests that the antigen selected for a diagnostic kit plays a critical role in determining the assay sensitivity, especially as no prior study of IgM binding sites has been carried out. Other more laborious and specific tests such as haemagglutination-inhibition (HI) test and neutralisation assay can detect CHIKV-specific antibodies. However, the presence of CHIKV-specific IgM or IgG is indistinguishable by HI as well as neutralisation, but the latter assay would be more specific to rule out cross-reactivity with other alphaviruses.

Deciding on the various options for laboratory CHIKV diagnosis would depend on the availability of laboratory facilities, financial and human resources, magnitude of the outbreak, and timing of sample collection. Dried-blood spots instead of frozen serum samples are cheaper and easier to collect for confirmatory laboratory tests without significant loss of sensitivity to detect RNA and CHIKV antibodies (Andriamandimby *et al.*, 2013; Smit *et al.*, 2014; Matheus *et al.*, 2015). Yet, the need for a simple, rapid, affordable and reliable test for use in developing countries remains unfulfilled (Sam *et al.*, 2011).

2.10 Treatment

There is no specific and effective treatment for CHIKV infection; rather the management is supportive to relieve symptoms experienced by patients. Analgesics and non-steroid anti-inflammatory drugs such as paracetamol and meloxicam may be prescribed (Staikowsky *et al.*, 2008; Chopra *et al.*, 2014), while patients are advised to increase fluid intake. In some patients with post-chikungunya rheumatic disorders, the use of disease-modifying anti-rheumatic drugs such as methotrexate, hydroxychloroquine, sulphasalazine, and tumour necrosis factor-α blockers have greatly improved the clinical outcomes (Brighton, 1984; Bouquillard and Combe, 2009; Ganu and Ganu, 2011; Javelle *et al.*, 2015; Blettery *et al.*, 2016). De Lamballerie *et al.* (2008) and Chopra *et al.* (2004) reported that the use of chloroquine during acute infection did not decrease viral load or improve clinical symptoms, and was therefore unjustified. Hence, the use of chloroquine at different phases of infection remains to be defined. There are case reports that other drugs such as ribavirin and colchicine may improve chronic symptoms, but these certainly require further investigation with more patients (Ravichandran and Manian, 2008; Redel, 2016). For patients with neurological complications, corticosteroid treatment may improve the disease prognosis (Chandak *et al.*, 2009), but need careful evaluation due to potentially serious side effects (Mylonas *et al.*, 2004). A proof-of-concept immunotherapy study was performed in CHIKV-infected mice, in which neutralising antibodies from human immune plasma provided prophylactic and therapeutic efficacy (Couderc *et al.*, 2009). Numerous neutralising monoclonal antibodies have been generated from mouse and human origin (Table 2.1). Those antibodies act by different mechanisms such as blocking virus entry, fusion and egression. The use of immunotherapy could be appropriate for high risk populations such as pregnant women, neonates and immunocompromised patients.

Table 2.1: Prophylactic and therapeutic monoclonal antibodies against CHIKV which were examined in mouse models.

Origin	Clone	Target epitope	References
Human	5F10; 8B10[1]	E2 (5F10), E2 and/or E1 (8B10)	(Warter *et al.*, 2011; Fric *et al.*, 2013)
	C9[2]	E2	(Selvarajah *et al.*, 2013)
	IM-CKV063[2]	E2	(Fong *et al.*, 2014)
	2B4; 2H1; 4N12; 9D14; 4J21[3]; 5M16[3]	E2, except for 4N12 which targets an unknown site.	(Smith *et al.*, 2015)
Mouse	CHK-102; CHK-152; CHK-166; CHK-263	E2, except CHK-166 which targets E1.	(Pal *et al.*, 2013)[4]
	1.3A2; 4.6F5	E2	(Goh *et al.*, 2013)[5]
	CHK-187, CHK-265	E2	(Fox *et al.*, 2015)[6]

[1,2,3] The mechanism of neutralisation was described in Porta *et al.* (2015), Jin *et al.* (2015), Long *et al.* (2015), respectively.

[4] The mechanism of neutralisation was described in the same reference.

[5] The mechanism of neutralisation was not described.

[6] The mechanism of neutralisation was described in the same reference and the mAb clones were obtained from Pal *et al.* (2013). Both clones exhibited broadly neutralising activity against MAYV and ONNV.

2.11 Vaccine development

Vaccines are the conventional way to provide medical intervention to healthy individuals who are immunological naïve to CHIKV, apart from vector controls to break the virus transmission cycle. Although no licensed CHIKV vaccine is available yet, many vaccine candidates have been continually developed since the CHIKV expansion in the last decade. Preclinical vaccine candidates in different formats or platforms have been described such as an immunogenic neutralising peptide, recombinant subunit proteins, virus-like particles (VLP), chemical- or UV-inactivated, live-attenuated, chimeric virus vectors and DNA-based vectors, which are derived from different genotypes or strains (reviewed in Schwameis *et al.*, 2016; reviewed in Smalley *et al.*, 2016). Different animal models such as mice from different genetic backgrounds and non-human primates have been recruited in immunisation, virus challenge and cross-protection studies.

At this moment, only live-attenuated (TSI-GSD-218), formalin-inactivated, recombinant measles virus (MV-CHIK) and VLP (VRC-CHKVLP059-00-VP) vaccine candidates have been evaluated in human clinical trials up to Phase II (reviewed in Schwameis *et al.*, 2016; reviewed in Smalley *et al.*, 2016). Although the strong immunogenicity of vaccines serves as a major advantage, several drawbacks have been discussed such as unexpected or unacceptable adverse events, unstable attenuation, cost, dosage and safety issues in immunocompromised individuals. The other concern is the cross-protection efficacy against different genotypes which are circulating around the world or even within a country. Interestingly, a VLP vaccine derived from West African genotype (37997 strain) elicited broad neutralising antibody responses in humans to all three genotypes (Goo *et al.*, 2016) . The neutralisation potency against its homologous and heterologous genotypes (including a Malaysian strain from 2008, similar to the ECSA strain used in this thesis) was almost equal, except for lower neutralisation

potency against a Nigeria strain from the West African genotype. It is not known whether VLP or other vaccines derived from an ECSA or Asian strain would have similar cross-protection efficacy, as these are the two most important currently circulating genotypes.

The general vaccine candidates follow an active immunisation strategy, which involves the activation of different immune cells. A modified version of passive immunisation has been described by delivering synthetic plasmids which encode biologically active anti-CHIKV envelope mAb into mice (Muthumani *et al.*, 2016). This technique provides rapid immune protection. Genetic engineering knowledge has been applied to attenuate wild type virus infectivity by recoding the codon, resulting in alteration of genetic characteristics without changing the amino acid sequences (Nougairede *et al.*, 2013). However, the efficacy of attenuation remains to be defined in an animal model.

Although antibodies generally provide protective immunity, in certain circumstances antibodies can mediate a pathogenic role, and this is known as antibody-dependent enhancement (ADE) (Takada and Kawaoka, 2003). ADE has been widely reported in infections of the flavivirus dengue virus (DENV) and the closely related alphavirus Ross River virus (RRV) (Linn *et al.*, 1996; Halstead *et al.*, 2010). ADE, which was initially described in dengue, is caused by the presence of cross-reactive and non-neutralising antibodies to heterologous dengue virus (Halstead and O'Rourke, 1977), or by sub-neutralising concentration of antibody. Fc receptor-bearing cells such as blood monocytes, macrophages and dendritic cells interact with virus-antibody complexes, and this mediates increased virus uptake into target cells at optimal binding between complexes and receptors (Takada and Kawaoka, 2003; Chan *et al.*, 2015; Taylor *et al.*, 2015). This dramatically increases viral replication and production. An enhancement of disease with foot swelling was reported by Hallengärd *et al.* (2014a, 2014b) when

vaccine-primed mice with 10^4 antibody titres were challenged with CHIKV. These findings serve as a warning for future vaccine trials. However, it is not known whether CHIKV-specific antibodies from human immune sera can mediate ADE. This knowledge will be greatly beneficial in vaccine development to ensure that protective immunity can be achieved safely.

CHAPTER 3: METHODOLOGY

3.1 Cell culture

Vero cells (African green monkey kidney, ATCC no. CCL-81, passage number 10 to 30), BHK-21 cells (baby hamster kidney, ATCC no. CCL-10, passage number 15 to 30), K562 (human erythroleukemia, ATCC no. CCL-243, passage number 2 to 10) were used to propagate viruses in this study. Vero cells were procured from ATCC. BHK-21 cells and K562 cells were kindly provided by Prof. Peter Liljeström (Karolinska Institutet) and Prof. Cheah Swee Hung (Dept of Physiology, University of Malaya), respectively.

For recombinant protein expression in insect cells, Sf9 cells (*Spodoptera frugiperda*) and TriExSf9 cells were procured from Merck Millipore.

Vero cells were maintained in Eagle's minimal essential medium (EMEM) supplemented with 10% foetal bovine serum (FBS) and $1 \times$ non-essential amino acids (NEAA). BHK-21 cells were maintained in 5% FBS Glasgow minimal essential medium (GMEM) supplemented with 10% tryptose phosphate broth and 20 mM HEPES. Roswell Park Memorial Institute (RPMI) medium was used to culture K562 cells in the presence of 10% FBS. All the media were supplemented with 5 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. Virus-infected cells were maintained in media containing 2% FBS. Cells used in this study were grown in 75 cm^2 or 25 cm^2 tissue culture flasks (TPP, Switzerland), 6-well and 24-well tissue culture plates (BD, USA), and 96-well CellCarrier-96 optic black plates (PerkinElmer, EU). All the FBS was obtained from Bovogen Biologicals (Australia) and heat-inactivated. The media and supplements were from Hyclone (China), Life Technologies (USA) and Sigma-Aldrich (USA). The insect cells, Sf9 and TriExSf9, were maintained in commercial BacVector Insect cell medium and TriEx cell medium (Merck Millipore), respectively.

Vero, BHK-21, Sf9 and TriExSf9 cells are adherent cells, while the K562 cells were grown in a semi-adherent way. To improve the attachment of K562 cells, flasks or 96-well plates were coated with poly-L-lysine in advance for 1 hour at 37°C, followed by rinsing with $1 \times$ Dulbecco's PBS (DPBS) before the cells were seeded. The cells were passaged upon reaching 80% confluency and trypsinised with 0.12 % trypsin-EDTA for Vero cells and 0.05% trypsin-EDTA for BHK-21 cells. K562, Sf9 and TriExSf9 cells were detached by sloughing. All the cells were maintained in 75 cm^2 flasks with 10 ml growth media, except for K562, which was maintained with 20 ml growth media.

Hybridomas were maintained in 20 ml growth media (10% FBS RPMI) supplemented with similar concentrations of L-glutamine, NEAA, penicillin and streptomycin. The cells were detached by sloughing. To expand the hybridomas, the cells were cultured in a CELLine bioreactor CL350 (Integra Biosciences, Switzerland) with 20% ultra-low IgG FBS RPMI medium (Life Technologies) in the cell compartment and 1% FBS (Biochrom, Germany) in the medium compartment. The cells were sub-passaged every 7 days, then seeded at a total density of 1.5×10^6 viable cells back into the cell compartment. The medium containing the monoclonal antibody was pre-cleared by centrifugation in a 15 ml tube and kept at -80°C until further purification.

All the cell culture and media preparations were done under aseptic conditions in a Class II, type A2 biological safety cabinet (ESCO, USA). Vero, BHK-21, K562 and hybridomas were grown in filter-capped flasks and incubated at 37°C, in the presence of humidified 5% CO_2. The insect cells were grown in vent-capped flasks and incubated at 28°C in the absence of CO_2.

For cell cryopreservation, a cell density of $1–3 \times 10^6$ cells for Vero, BHK-21, K562, hybridoma or $3–3.5 \times 10^6$ cells for Sf9 and TriExSf9 were mixed in a final 5% (for mammalian cells) or 10% (for insect cells) DMSO cryopreservation media with 10% FBS media in cryovials. The vials were placed on ice, and subsequently placed in a

freezing container containing isopropanol overnight, before being transferred into liquid nitrogen storage.

To revive frozen cell lines, cryovials were retrieved from liquid nitrogen storage and immediately thawed in a 37°C water bath. The thawed cells were immediately transferred into a 15 ml tube pre-filled with 10 ml media, drop-by-drop to dilute the toxic effects of DMSO. The tube was spun at $150 \times g$ for 5 minutes and the supernatant was discarded. The cell pellet was resuspended in 10–20 ml fresh medium gently and transferred to a 75 cm^2 flask. The cells were incubated at the conditions mentioned above.

3.2 Virological assays

This section describes the virus stock propagation, purification and techniques to titrate virus and its infectivity.

3.2.1 Virus isolates and stocks

Two clinical isolates and two rescued viruses from infectious clones of CHIKV were used in this study. The clinical isolates used, which have been previously characterised (Sam *et al.*, 2012) were MY/06/37348, an Asian genotype strain isolated from a patient in Bagan Panchor in 2006 (accession number FN295483) and MY/08/065, an ECSA virus isolated from a patient in Kuala Lumpur in 2008 (accession number FN295485). Both isolates had been passaged two times in Vero cells before further propagated in BHK-21 cells. The infection was performed at a multiplicity of infection (MOI) of 0.1 plaque forming units/cell. The virus suspension was allowed to adsorb onto confluent BHK-21 cells in a 75 cm^2 flask at 37°C for 1 hour, before being replacing with medium containing 2% FBS. Upon reaching 70% cytopathic effect (CPE) with cells rounding and clumping, the virus supernatant was harvested, pre-cleared by centrifugation at 4,000 x g for 10 minutes, aliquoted and stored at -80°C. The third virus passage (P3) of the clinical isolates was used for subsequent work. MY/06/37348 (Asian genotype) was used mainly in the second objective of this study, while MY/08/065 (ECSA genotype) was used mainly in the first, second and fourth objectives of this study.

Two viruses were rescued from their respective DNA-launched infectious clones (icDNA), which were derived from ECSA and Asian genotypes of CHIKV. The infectious clones were provided by Prof. Andres Merits (University of Tartu). The ECSA molecular clone was named "ICRES1", while the Asian molecular clone was designated as "CAR" (section 3.3.5). The viruses were rescued from icDNA by electroporation. Stocks of rescued viruses (P0) were harvested and titrated by plaque

assay on BHK-21 cells. To obtain P1 stocks, confluent BHK-21 cells grown in 75 cm^2 flasks were infected with P0 stocks at an MOI of 1 plaque forming unit/cell and maintained in 2% FBS GMEM. P1 stocks were harvested after 24 or 48 hours and titrated by plaque assay. Both infectious clones were used in the second, third and fourth objectives of the study.

3.2.2 Purification of virus using sucrose-cushion ultra-centrifugation

The protocol was kindly provided by Dr Lisa F.P.Ng from Singapore Immunology Network, A*STAR. The viral supernatant was collected and filtered using a 0.2 µm bottle-top filter (Nalgene, USA). A sterile ultra-centrifuge tube was filled with 4 ml of 20% sucrose-TNE buffer (50 mM Tris-HCl, 100 mM NaCl and 0.5 mM EDTA at pH 7.4). The pre-cleared virus supernatant (32 ml) was gently layered on the top of the cushion. The tubes were balanced carefully prior to centrifugation at 125,000 × g for 4 hours at 4°C. All the supernatant was discarded and the virus pellet was recovered with 1× TE buffer (10 mM Tris-HCl and 1 mM EDTA at pH 8.0) and stored at -80°C. For preparation of whole virus antigens used for indirect IgG and western blot, the virus pellet was treated with 1% Triton X-100 in TE buffer for 30 minutes on ice, clarified by centrifugation at 20,000 × g, and stored in 50% glycerol at -20°C.

3.2.3 Indirect immunofluoresence assay

BHK cells were seeded at 1×10^4 cells per well in the chamber slide (Lab-Tek, USA) prior to infection. The cells were infected at an MOI of 0.1 per well. At 24 hours post-infection, the cells were fixed with 0.4% paraformaldehyde and permeabilised with 0.25% Triton-X 100. The slide was pre-incubated with Image-IT FX Signal Enhancer (Life Technologies) for 1 hour and subsequently probed with 5 µg/ml monoclonal antibody, followed by Alexa Fluor 488 anti-mouse IgG (Life Technologies) as the secondary

antibody at 1:200 dilution. Cell nuclei were counter-stained with DAPI. The slide was mounted and observed under a Nikon Eclipse TE2000-E fluorescence microscope at 20× objective magnification and the images were acquired with Nikon Digital Sight DS-Ri1 (Japan) and NIS-Elements AR Imaging Software (Nikon, version 4.0).

3.2.4 Virus rescue from DNA-launched icDNA

The protocol used was previously published by Saul *et al.* (2015). Five micrograms of each DNA-launched icDNA was electroporated into $3.5–4 \times 10^6$ BHK-21 cells using a Gene Pulser Xcell electroporation system (Bio-Rad, USA) under exponential decay pulse type at the following conditions: 220 V, 975 µF, one pulse in a 4 mm cuvette, with a total volume of 250 µl of cell suspension accompanied with plasmid and 50 µg of salmon sperm DNA carrier (Life Technologies) in 10% FBS GMEM (in the absence of penicillin/streptomycin). After electroporation, the cells were immediately recovered with the addition of media. A small aliquot (120 µl) of electroporation mixture was transferred out for the infectious centre assay and the rest was transferred into 75 cm^2 flasks in 10% FBS GMEM. After incubation for 24 hours at 37°C, P0 rescued viruses stocks were harvested and titrated by plaque assay.

3.2.5 Plaque assay

The clarified virus supernatant was serially diluted ten-fold in serum-free MEM (containing 20 mM HEPES, 0.2% BSA, 5 mM L-glutamine) and transferred to 6-well plates containing pre-seeded confluent BHK-21 cells (approximately 8×10^5 cells per well). The plate was incubated at 37°C for 1 hour with intermittent rocking every 15 minutes. The cells were overlaid with 2 ml of plaque medium (3 parts of 2% FBS GMEM mixed with 2 parts of 2% carboxymethylcellulose) in each well. After 3–4 days incubation, the cells were fixed and stained with crystal violet (0.5% crystal violet, 20%

methanol). The plaque forming unit per millilitre (pfu/ml) was calculated with the following formula: (number of plaques x dilution factor)/ volume of inoculum (ml).

3.2.6 Infectious centre assay

Fresh 10% FBS GMEM (1080 µl) was added to the aliquot of electroporated BHK-21 cells, which was then serially ten-fold diluted and transferred to 6-well plates containing pre-seeded, uninfected BHK-21 cells (6×10^5 cells per well). After 2–3 hours of incubation at 37°C, cells were overlaid with 2 ml of plaque medium in each well. After 3–4 days incubation, the plates were fixed, stained and scored visually. Plaques were counted and specific infectivity was expressed as the number of plaque-forming units per 1 µg of electroporated DNA.

3.3 Molecular cloning and assays

This section describes the construction of expression cassettes and infectious clones.

3.3.1 Bacteria host and preparation of competent cells

TOF10F' (Invitrogen) and XL-10 Gold (Agilent) served as cloning hosts for site-directed mutagenesis, icDNA, replicons and expression cassettes propagation (pIEX-5 and pGEM-T vectors). BL21(DE3) (Novagen) is an expression host used with an expression vector, pET-52b(+) (Merck). Vectors carrying the antibiotic selection marker were transformed into bacteria by the calcium chloride method. To prepare competent cells, the bacteria were streaked on a Luria-Bertani (LB) (BD) plate with antibiotic supplements (12.5 µg/ml tetracycline for TOP10F', 25 µg/ml chloramphenicol plus 12.5 µg/ml tetracycline for XL-10 Gold) or on a plain plate for BL21(DE3). The bacterial plates were incubated at 37°C overnight. A single colony was then inoculated into 5 ml LB broth with a similar amount of antibiotics and grown overnight. One part of overnight culture was added to 100 parts of fresh LB broth and incubated at 37°C with continuous orbital shaking until the OD_{600} reached between 0.6 and 0.8, at mid-log phase. The bacteria culture was collected into 50 ml tubes and centrifuged at $4,000 \times g$ for 10 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in 25 ml ice-cold 0.1 M $CaCl_2$ with gentle pipetting. The tube was incubated on ice for at least 1 hour to reach competency. After 1 hour, the tube was spun at $4,000 \times g$ for 10 minutes at 4°C. The previous $CaCl_2$ was discarded and the competent cells were resuspended in fresh ice-cold $CaCl_2$ containing 15% glycerol (10 ml), aliquoted into sterile 1.5 ml tubes and kept at -80°C.

3.3.2 Vectors

The bacteria expression vector, pET-52b(+) was used in recombinant protein expression in bacteria. The vector was transformed into BL21(DE3) and the bacteria were grown in the presence of ampicillin at a final concentration of 100 µg/ml.

The insect expression vectors, pIEX-5 and pIE-neo (Novagen) were used in recombinant protein expression in insect cells. The vectors were transformed into TOP10F' and the bacteria were grown in the presence of ampicillin at a final concentration of 100 µg/ml.

CHIKV DNA-launched icDNA (ICRES1 and CAR) were transformed into XL-10 Gold and the bacteria were grown in the presence of kanamycin at a final concentration of 50 µg/ml.

SP6 promoter based icDNAs and replicons were transformed into XL-10 Gold and the bacteria were grown in the presence of ampicillin at a final concentration of 100 µg/ml.

The cloning vector, pGEM-T (Promega, USA) was used in site-directed mutagenesis. The vector was transformed into XL-10 Gold and the bacteria were grown in the presence of ampicillin at a final concentration of 100 µg/ml. Blue-white screening was used to select the clone carrying the constructs, with ampicillin plates pre-overlaid with 100 µl of 100 mM IPTG and 50 µl of 50 mg/ml X-gal.

pUC-CC56 and pSK-CAR-XN contained parts of genes from DNA-launched icDNA CAR. These vectors were transformed into XL-10 Gold and the bacteria were grown in the presence of kanamycin and ampicillin, respectively.

3.3.3 Construction of expression cassettes

Viral RNA was extracted from clinical isolates (Asian MY/06/37348 and ECSA MY/08/065) with the QIAamp Viral RNA kit (Qiagen, Germany). cDNA was synthesised using reverse-transcription with SuperScriptIII Reverse Transcriptase (Invitrogen, USA). For cDNA synthesis in a 20 µl volume, 8.5 µl ultra-pure H$_2$O, 2 µl 10 mM dNTP, 0.5 µl random primers and 2 µl viral RNA were mixed together in a PCR tube and incubated at 65°C for 5 minutes. The tube was placed immediately on ice for 3 minutes. The cDNA was synthesised with 1 µl of 200 U Superscript III Reverse Transcriptase (Invitrogen), 1 µl of 0.1 M DTT (Invitrogen), 1 µl of 40 U RNaseOUT (Invitrogen) and 4 µl of 5 × first strand buffer (Invitrogen). The reaction was incubated at 25°C for 15 minutes, 50°C for 60 minutes, and finally at 70°C for 15 minutes to inactivate the enzyme. The final cDNA product was kept at -20°C.

The genes were amplified using Q5 high-fidelity DNA polymerase (NEB, USA). In a 50 µl reaction mixture, it comprised 10 µl 5 × Q5 reaction buffer, 1 µl 10 mM dNTP, 1 µl 30 µM forward primer, 1 µl 30 µM reverse primer, 1 µl cDNA template, 35.5 µl ultra-pure H$_2$O, and 0.5 µl Q5 high-fidelity DNA polymerase. The PCR was performed with initial denaturation at 98°C for 30 seconds, 30 cycles of denaturation at 98°C for 10 seconds, annealing at 55 or 60°C for 30 seconds and extension at 72°C for which 1 kb of template was extended for 0.5 second. The cycle ended with a final extension at 72°C for 5 minutes. The amplicons were resolved with 1.2% agarose gel in 1 × Tris-acetate-EDTA buffer with pre-stained GelRed (Biotium, USA). The gel was viewed under ultraviolet illumination using a BioSpectrum AC Imaging system with a Biochemi HR Camera (UVP, USA). The band was excised and gel-purified with either Expin Combo GP (GeneAll, Korea) or DNA Clean & Concentrator-5 (Zymo Research, USA). The purified amplicons were subjected to restriction enzyme digestion (NEB or Thermo Scientific, USA) and cleaned up as mentioned above.

For construction of bacteria expression cassette, the whole E2 gene (from amino acids 1-423) was cloned into a pET-52b(+) vector at *Bam*H1 and *Not*1 restriction sites (Table 3.1).

For production of native recombinant proteins of E1 (rE1, from amino acids 1-412) and E2 (rE2, from amino acids 1-362), the transmembrane regions and cytoplasmic tails of the glycoproteins were not included in the expression cassette, to ensure solubility of the recombinant proteins. The genes were amplified with designed primers (Table 3.1). The amplicons were ligated into a pIEX-5 vector directionally at *Bam*H1 and *Not*1 restriction sites.

For construction of the fusion E1-E2 cassette, two separate PCR reactions were performed independently. The first amplification reaction of the E2 region (amino acids 1-362) and the second amplification reaction of the E1 region (amino acids 1-412) were performed for either Asian or ECSA genotypes using the primer sets in Table 3.1. The reverse primer in the first reaction and forward primer in the second reaction have an integrated sequence corresponding to a short peptide linker and 8 × His-tag. Both PCR reactions were cleaned up, and another round of overlapping PCR was performed by mixing both cleaned up amplicons in a reaction, with the forward primer from the first PCR reaction and the reverse primer from the second PCR reaction. The final amplicons were cleaned up and ligated into a pIEX-5 vector directionally at *Bam*H1 and *Not*1 restriction sites.

Table 3.1: Primers used for construction of expression cassettes.

Gene[1]	Sequences with cloning sites integration (5' to 3') and underlined restriction sites (*Bam*H1 and *Not*1)[2]		Vector
rE2-ECSA (aa 1-423)	E2W-ECSA-F	GCGGGATCCTAGCACCAAGGACAACTTCAAT	pET-52b(+)
	E2W-ECSA-R	GCGCGGCCGCCGCTTTAGCTGTTCTGATGCA	
rE1-ECSA (aa 1-412)	E1-ECSA-F	GCGGGATCCTTACGAACACGTAACAGTGATC	pIEX-5
	E1-ECSA-R	GCGCGGCCGCCTTCTGCACCCATGACATCGC	
rE2-ECSA (aa 1-362)	E2-ECSA-F	GCGGGATCCTAGCACCAAGGACAACTTCAAT	pIEX-5
	E2-ECSA-R	GCGCGGCCGCCAGCTCATAATAATACAGAAT	
rE2-Asian (aa1-362)	E2-A-F	GCGGGATCCTAGTATTAAGGACCACTTCAAT	pIEX-5
	E2-A-R	Similar to E2-ECSA-R	
Fusion E1-E2 glycoproteins (rE1, aa 1-412; rE2, aa 1-362) 1. rE2-E1-Asian 2. Hybrid rE2_Asian-E1_ECSA 3. Hybrid rE2_ECSA-E1_Asian 4. rE2-E1-ECSA	First PCR 1. E2-A-F/E2-ECSA-F 2. E2-His8-R Second PCR 1. E1-His8-F 2. E1-A-R	ATGGTGATGGTGATGGTGGTGAGAACCGCCGCCCAGCTCATAATAATA CACCATCACCATCACCATCACGGCGGTGGTGGCTACGAACACGTAACA Identical to E1-ECSA-R	pIEX-5

[1] aa, amino acids

[2] F, forward primer; R, reverse primer.

3.3.4 Transformation and plasmids extraction

The frozen competent cells in tubes (section 3.3.1) were thawed on ice. The ligation mixture was added and cells were further incubated on ice for 30 minutes. The transformation mix was heat-shocked exactly at 42°C for 1 minute and immediately placed on ice for another 3 minutes. One ml of LB broth was added to aid recovery from the heat shock process. Next, the tubes were incubated at 37°C for 1 hour and the cell pellet was collected by centrifugation at 1,000 × g for 10 minutes. The supernatant was discarded and the transformed bacteria were layered onto agar plates with appropriate antibiotic supplements (100 µg/ml for ampicillin or 50 µg/ml for kanamycin). The plate was incubated at 37°C overnight.

For plasmid extraction, a single colony of transformed bacteria was inoculated into LB broth with antibiotic supplements and allowed to grow for about 16 hours. The cell pellet was collected by centrifugation at 4,000 × g for 10 minutes. The plasmids were extracted using different kits. For sequencing and site-directed mutagenesis, the plasmids were prepared with Hybrid-Q plasmid (GeneAll). As for the plasmids for transfection into insects and mammalian cells, the endotoxin-free plasmids were prepared with PureLink HiPure Plasmid Miniprep Kit (Life Technologies) in ultra-pure H_2O or Exfection plasmid LE (GeneAll). The plasmid yield was quantitated with a Take-3 Micro-Volume plate and the reading was acquired with an Epoch ELISA reader (Biotek Instruments, USA).

3.3.5 Chikungunya-based vector systems - infectious clones (icDNA) and replicons

The construction of chikungunya-based vector systems was generated in collaboration with Professor Andres Merits from Institute of Technology, Tartu, Estonia. Prof. Merits designed all the vector systems and assembled the constructs in Estonia. All the clones were verified and tested for virological assay by the student and sent to Malaysia. The use of vector systems involves genetic manipulation to alter amino acid residues and to test the effect of changes.

All the chikungunya-based vector systems belonging to the Asian genotype were engineered by gene synthesis and assembled by the restriction enzyme approach based on the consensus sequence for strain 3462, isolated in Yap State in 2013 (accession no. KJ451623). However, the protein coding regions in the non-structural and structural proteins were changed to be identical to isolate CNR20235 from the Caribbean outbreak, which was isolated in Saint Martin Island in 2013 (http://www.european-virus-archive.com/virus/chikungunya-virus-strain-h20235stmartin2013). The Asian CHIKV clone is termed the CAR clone.

DNA-launched icDNAs are the plasmid vectors capable of producing infectious viruses and were constructed under the control of the human cytomegalovirus (CMV) immediate-early promoter. The CHIKV infectious clone ICRES1derived from the ECSA genotype was based on LR2006-OPY1, isolated in Reunion Island in 2006, and has been described previously (Hallengärd *et al.*, 2014a). Both molecular clones ICRES1 and CAR have the ZsGreen gene incorporated as a reporter and duplication of the subgenomic promoter (Figure 3.1).

For construction of the DNA-launched chimeric viruses, a subcloning vector (pGEM-T, Promega) containing the structural genes and 3' UTR from icDNA ICRES1 was constructed, flanked by *Pme*1 and *Not*1 restriction sites (template: pGEM-T-ICRES1-PN). The ectodomain regions of envelope glycoprotein genes E1 (amino acids

1-381) and E2 (amino acids 1-341) in the ICRES1 backbone were replaced with those of Semliki Forest virus (SFV) E1 (amino acids 1-381) and E2 (amino acids 1-340) from DNA-launched icDNA SFV6 (Ferguson *et al.*, 2015) using NEBuilder HiFi DNA Assembly Master Mix (NEB) with self-designed primers (Figures 3.1 and 3.2, Table 3.2). Similar work was performed to replace the E2 domain A and domain B of CHIKV with those of SFV (Figure 3.1). These chimeric viruses were used to test the neutralising epitopes on the envelope proteins and its domains. The sequences of all the constructs were verified by control restrictions and sequence analysis using self-designed primers (Table 3.3, Appendix A). A "hybrid CAR" was constructed by replacing the structural region (C-E3-E2-6K-E1) of ICRES1 with that of CAR using pCMV-ICRES1-2SG-zsGreen as the main backbone (Figure 3.1). Infectious centre assay was performed on all the viruses rescued from DNA-launched icDNA to ensure comparable virus infectivity between wild type and mutants. The specific infectivities and virus titres of all the constructs are shown in Table 3.4. The rescued viruses from DNA-launched icDNA were used for studies of neutralising epitopes and antibody-dependent enhancement.

SP6 RNA polymerase-driven infectious clones are similar to DNA-launched clones, except that their vectors have an SP6 phage promoter instead of a CMV promoter. RNA was required to be transcribed *in vitro* in the presence of a linearised DNA template (Figure 3.1), and was electroporated into K562 as stated in section 3.3.7. SP6 RNA polymerase-driven icDNA derived from the ECSA genotype was kindly provided by the Merits lab (Pohjala *et al.*, 2011). DNA-launched and SP6 RNA polymerase-driven icDNA are full-length replication-competent vectors.

CHIKV replicons are replication-defective vectors which lack the structural region in the genome. Only the non-structural proteins of virus are expressed from non-replicating RNA transcribed from a vector, under the control of SP6 promoter (Figure

3.1). The structural genes were replaced by a zsGreen reporter gene. The SP6 RNA polymerase-driven replicon derived from the ECSA genotype was kindly provided by the Merits lab (Gläsker *et al.*, 2013).
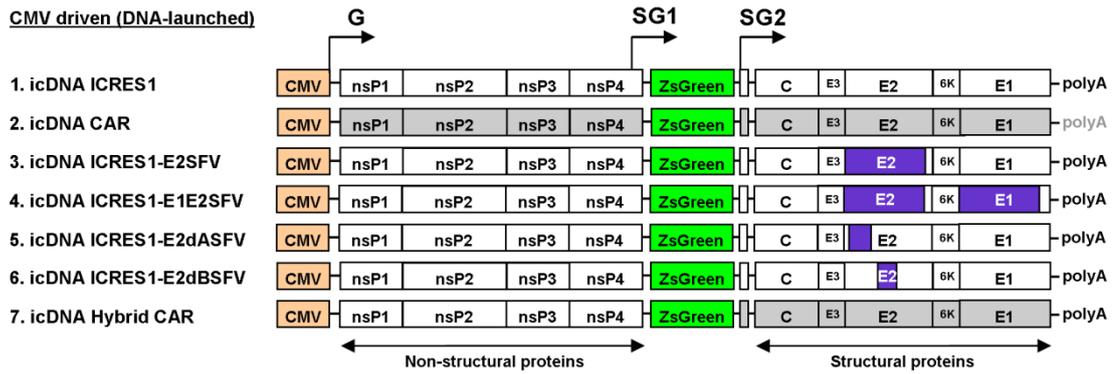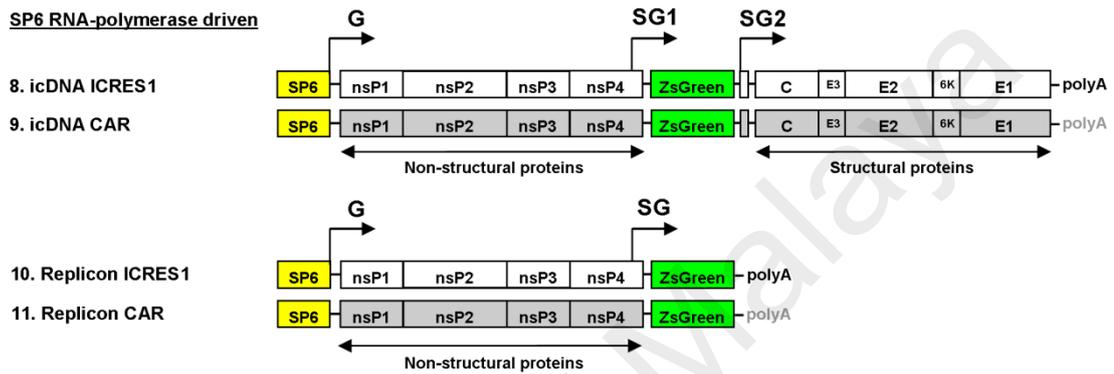
Figure 3.1: Schematic diagram showing the different constructs of chikungunya-based vector systems used in this study. G, genomic promoter; SG, subgenomic promoter. White boxes represent sequences of CHIKV of the ECSA genotype; Grey boxes represent sequences of CHIKV of the Asian genotype; purple boxes represent Semliki Forest virus sequences.
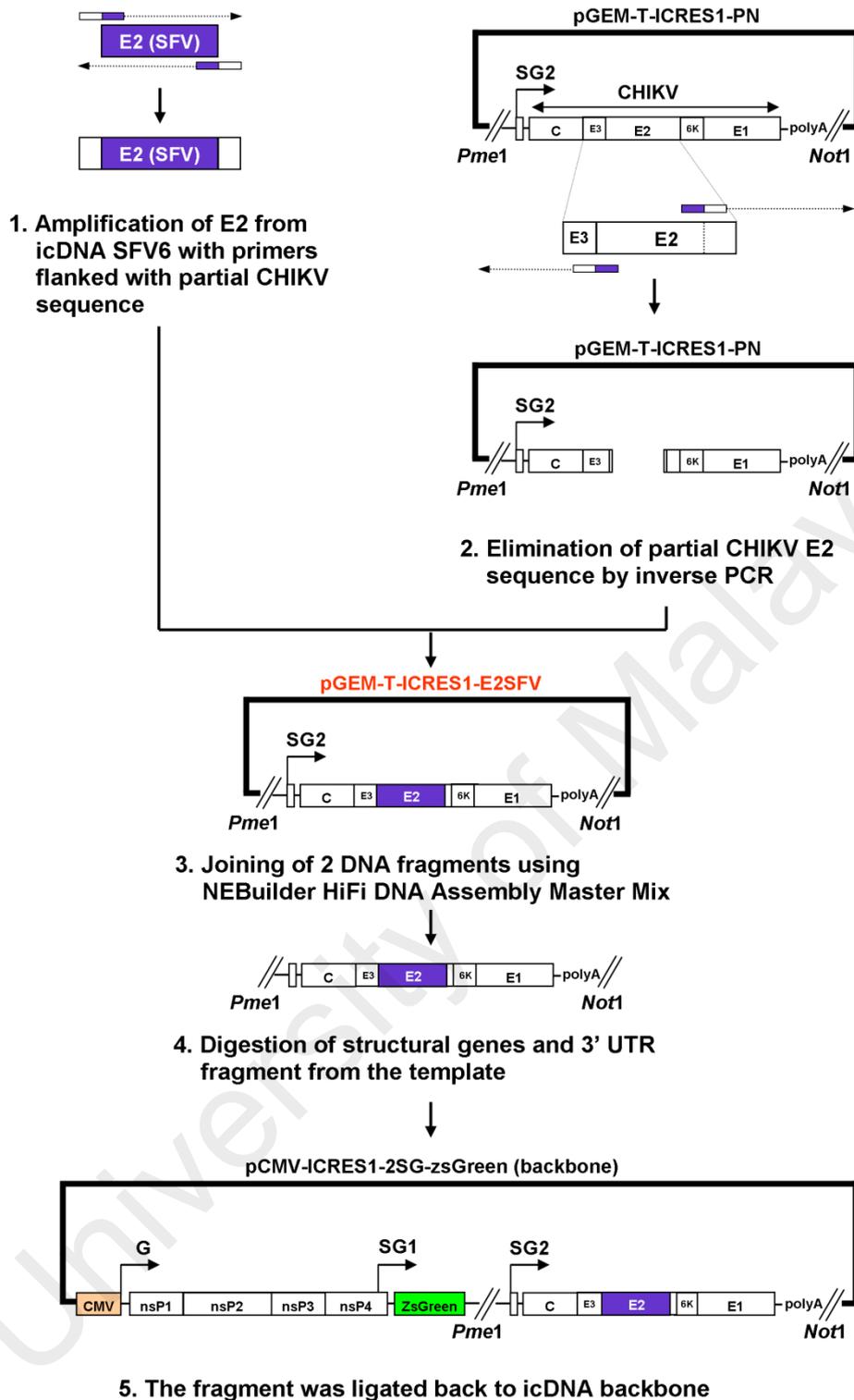
Figure 3.2: Schematic flow showing the construction of a chimeric virus in which the E2 gene of CHIKV is replaced with the E2 gene of SFV. White boxes represent sequences of CHIKV of the ECSA genotype; purple boxes represent Semliki Forest virus sequences.

Table 3.2: Overlapping primers used for construction of chimeric viruses.

Construct[1]	Primer[2]	5'-Sequence-3'	Note
ICRES1-E2SFV	E2-SFV-AA1-F	CGCCAGCGACGCAGCGTGTCGCAACACTT	To amplify the E2 SFV from icDNA SFV6
	E2-SFV-AA340-R	GGCTGTACCGTTTGTAGATAATTGAGACCAAAGCCTCACTGG	
	invICRES1-E2-F	CCAGTGAGGCTTTGGTCTCAATTATCTACAAACGGTACAGCC	To eliminate the partial E2 CHIKV sequence from pGEM-T-ICRES1-PN
	invICRES1-E2-R	AAGTGTTGCGACACGCTGCGTCGCTGGCG	
ICRES1-E1E2SFV	E1-SFV-AA1-F	ACTGTGAGCGCGTACGAACATTCG	To amplify the E1 SFV from icDNA SFV6
	E1-SFV-AA381-R	GTTGACTATGTGGTCCTTCGGG	
	invICRES1-E1-F	CCCCCGAAGGACCACATAGTCAACTAC	To eliminate the partial E1 CHIKV sequence from pGEM-T-ICRES1-E2SFV
	invICRES1-E1-R	TGTTCGTACGCGCTCACAGTGTGGGCA	
ICRES1-E2dASFV	E2dA-SFV-F	AAGCCACAAGACCATACATCGCGTACTGCGCCGA	To amplify the E2 domain A SFV from icDNA SFV6
	E2dA-SFV-R	TTCCCGACCTATCACAGGAGGGTCATGATGATATTG	
	invICRES1-dA-F	CAATATCATCATGACCCTCCTGTGATAGGTCGGGAA	To eliminate the partial E2 domain A CHIKV sequence from pGEM-T-ICRES1-PN
	invICRES1-dA-R	TCGGCGCAGTACGCGATGTATGGTCTTGTGGCTT	

[1] No viable virus rescued from icDNA ICRES1-E1SFV.

[2] F, forward primer; R, reverse primer; dA, domain A; dB, domain B; AA, amino acid; ICRES1, ECSA virus from icDNA; CAR, Asian virus from icDNA.

Table 3.2, continued: Overlapping primers used for construction of chimeric viruses.

Construct[1]	Primer[2]	5'-Sequence-3'	Note
ICRES1-E2dBSFV	E2dB-SFV-FP	ATAGAGGTACACATGCCCCCAGATACGCCGGACAGG	To amplify the E2 domain B SFV from icDNA SFV6
	E2dB-SFV-RP	TACTGCCACTTTTTGTGATTCGTCACTGAGACGTGGCACT	
	invICRES1-dB-F	AGTGCCACGTCTCAGTGACGAATCACAAAAAGTGGCAGTA	To eliminate the partial E2 domain B CHIKV sequence from pGEM-T-ICRES1-PN
	invICRES1-dA-R	CCTGTCCGGCGTATCTGGGGGCATGTGTACCTCTAT	
Hybrid CAR	Fragment-CAR-C-F	TGGACTCCGCGCCCTACTATCCAAGTCATCAG	To amplify the structural region CAR from icDNA CAR
	Fragment-CAR-E1-R	CGTGATCTTCTGCACCCATGACATCGCCGTAGC	
	invPCR-ICRES1-E1-F	TGGGTGCAGAAGATCACGGGAGGTGTGGGA	To eliminate the whole structural region ICRES1 from pGEM-T-ICRES1-PN
	invPCR-ICRES1-C-R	CTGATGACTTGGATAGTAGGGCGCGGAGTCCA	

[1] No viable virus rescued from icDNA ICRES1-E1SFV.

[2] F, forward primer; R, reverse primer; dA, domain A; dB, domain B; AA, amino acid; E1, envelope glycoprotein 1; C, capsid; ICRES1, ECSA virus from icDNA; CAR, Asian virus from icDNA.

Table 3.3: Sequencing primers for construction of chimeric viruses.

Primer	5'-Sequence-3' (forward primer)	Template (pGEM-T-ICRES1-PN)		
		Replacement with E2SFV	**Replacement with E1E2SFV**	**Replacement with E2dASFV, E2dBSFV**
T7	TAATACGACTCACTATAGGG	✓	✓	✓
E15F	TCGAAGTCAAGCACGAAGG	✓	✓	✓
E16F	TGCTTGAGGACAACGTCATGAG	✓	✓	✓
E17F	AGTCCGGCAACGTAAAGATCAC			✓
SFV17F	AATCTGGCAATGTAAAGATCAC	✓	✓	
E18F	TATCAGTGGCCACGTTCATACT	✓	✓	✓
E19F	ACAAAACCGTCATCCCGTCTC	✓		✓
SFV19F	ACAAGACGGTCGTCCCGTCGC		✓	
E20F	CGGCGCAGGAAGACCAGGAC	✓		✓
SFV20F	CGGATCTGGGCAACCAGGGC		✓	
E21F	AGCAGGCACTAACTTGACAATTAAGTATGAAG	✓	✓	✓
SP6	ATTTAGGTGACACTATAG	✓	✓	✓

Table 3.4: Viruses rescued after electroporation of DNA-launched icDNA CHIKV.

Backbone	Construct	Specific infectivity (pfu/1 µg of DNA)	Virus titre (pfu/ml), P0, 24 hour	Virus titre (pfu/ml), P1, 24 hour	Rescued virus used in objective	Remarks
pCMV-ICRES1		5.07×10^4	N.D.	N.D.		No extra subgenomic promoter and fluorescent marker, ECSA genotype
pCMV-ICRES1-2SG-zsGreen	ICRES1	3.73×10^4	6.00×10^8	6.33×10^8	2, 3, 4	Wild type
	ICRES1	-	-	4.33×10^9	2	Sucrose cushion-purified in TE buffer. For competitive peptide blocking assay
	ICRES1-E1SFV	-	-	-		Non-viable
	ICRES1-E2SFV	4.27×10^4	1.00×10^7	8.00×10^7 (48 h)	2, 3, 4	The E2 gene of ICRES1 is replaced with SFV
	ICRES1-E1E2SFV	5.60×10^4	1.20×10^8	2.03×10^8 (48 h)	2, 3, 4	The E1 and E2 genes of ICRES1 are replaced with SFV
	ICRES1-E2dASFV	9.60×10^4	7.67×10^7	1.40×10^8 (48 h)	3	The E2 domain A of ICRES is replaced with SFV
	ICRES1-E2dBSFV	3.20×10^4	4.00×10^8	7.33×10^8 (48 h)	3	The E2 domain B of ICRES is replaced with SFV
	Hybrid CAR	6.93×10^4	1.33×10^8	7.00×10^8	3	The structural proteins (C-E3-E2-6K-E1) of ICRES are replaced with CAR
pCMV-CAR		1.32×10^4	N.D.	N.D.		No extra subgenomic promoter and fluorescent marker, Asian genotype

N.D., not determined; SG, duplicated subgenomic promoter; dA, domain A; dB, domain B; ICRES1, ECSA virus from icDNA; CAR, Asian virus from icDNA; SFV, Semliki Forest virus.

Table 3.4, continued: Viruses rescued after electroporation of DNA-launched icDNA CHIKV.

Backbone	Construct	Specific infectivity (pfu/1 μg of DNA)	Virus titre (pfu/ml), P0, 24 hour	Virus titre (pfu/ml), P1, 24 hour	Rescued virus used in objective	Remarks
pCMV-CAR-2SG-zsGreen	CAR	7.20×10^3	2.03×10^7	1.37×10^8	2, 3	Wild type
	CAR-E1-A145T	2.31×10^4	2.43×10^7	8.67×10^7	2	Mutation A145T in E1
	CAR-E1-E211K	1.97×10^4	1.17×10^7	2.47×10^8	2	Mutation E211K in E1
	CAR-E1-A226V	9.33×10^3	1.70×10^7	1.33×10^8	2	Mutation A226V in E1
	CAR-E1-M269V	1.03×10^4	1.60×10^7	1.37×10^8	2	Mutation M269V in E1
	CAR-E2-I2T	6.40×10^3	6.83×10^6	5.00×10^8	2	Mutation I2T in E2
	CAR-E2-H5N	7.33×10^3	3.50×10^6	5.00×10^8	2	Mutation H5N in E2
	CAR-E2-I2T-H5N	9.60×10^3	1.52×10^6	2.28×10^8	2	Mutation I2T and H5N in E2
	CAR-E2-G118S	7.47×10^3	1.00×10^6	5.00×10^8	2	Mutation G118S in E2
	CAR-E2-R149K	1.01×10^4	1.83×10^6	2.35×10^8	2	Mutation R149K in E2
	CAR-E2-S194G	1.35×10^4	2.10×10^7	1.23×10^8	2	Mutation S194G in E2
pCMV-SFV6	SFV6	N.D.	1.23×10^9	3.68×10^8	2	

N.D., not determined; ; SG, duplicated subgenomic promoter; dA, domain A; dB, domain B; ICRES1, ECSA virus from icDNA; CAR, Asian virus from icDNA; SFV, Semliki Forest virus.

3.3.6 Site-directed mutagenesis (SDM)

PCR-based SDM was performed on the rE2-Asian, fusion E2-E1 in pIEX-5 vector to identify the key amino acids that increase antibody binding capacity. Subsequently, SDM was performed on the CAR construct to study the effects of point mutations on the neutralising epitopes. All SDM was performed using Q5 High-Fidelity DNA polymerase with designed primers (Table 3.5). The primers were designed using an online website (http://www.genomics.agilent.com/primerDesignProgram.jsp) to facilitate the generation of point mutations. To minimise PCR errors which might be deleterious to virus rescued from icDNA, E1 gene and 3' UTR of icDNA (template: pSK-CAR-XN, between *Xho*1 and *Not*1) were subcloned into a pSK vector to facilitate SDM. The original synthesised gene construct (initial building fragment for icDNA CAR) which contains part of the E2 gene fragment (template: pUC-CC56, between *Age*1 and *Xho*1) was used as a template in SDM. A modified version of PCR was carried out for this SDM. For each template in single SDM, two single primer reactions were set up in parallel (Figure 3.3). Both tubes contained the PCR master mix with template, but one tube contained the forward primer and the other contained the reverse primer. The amplications were performed according to the manufacturer's instructions, except the annealing temperature was set at 55°C. At the end of amplication, both mixtures were pooled into a single tube and 0.5 µl of *Dpn*1 was added to digest the parental template for 30 minutes. Next, the mixture was denatured and slowly cooled to allow reannealing of PCR products following the parameters: 95°C for 5 minutes; 90°C for 1 minute; 80°C for 1 minute; 70°C, 60°C, 50°C, and 40°C for 30 seconds each; and cooling at 4°C. Another round of *Dpn*1 digestion was carried out under similar conditions before the master mix was ready to be transformed into bacteria. The correct clones were identified and sequenced using self-designed primers (Table 3.6). Plasmids were extracted as described in section 3.3.4.

For the manipulation of the icDNA, once a confirmed mutation was present in the subclone construct of pSK-CAR-XN or pUC-CC56, the vector was digested accordingly and the fragment was inserted back into the icDNA backbone.

Table 3.5: Primers used for mutagenesis of CHIKV E1 and E2 proteins and CHIKV infectious clones.

Mutation		5'-Sequence-3'	Template		
			rE2-E1-Asian (antibody binding)	rE2-Asian (antibody binding)	CAR (DNA-launched infectious clone)
E1-S72N	F	GCAGAGTGTAAGGACAAGAACCTACCTGATTACAGC	✓		
	R	GCTGTAATCAGGTAGGTTCTTGTCCTTACACTCTGC			
E1-T98A	F	CCTACTGCTTCTGCGACGCCGAAAATACGCAATTG	✓		
	R	CAATTGCGTATTTTCGGCGTCGCAGAAGCAGTAGG			
E1-A145T	F	CGTTTGCATAAGCAGTTACAGTGATATTATTTCCTTGGTAAAGG	✓		✓
	R	CCTTTACCAAGGAAATAATATCACTGTAACTGCTTATGCAAACG			
E1-E211K	F	CGCACGCCTGAGAGCAAAGACGTCTATGCTA	✓		✓
	R	TAGCATAGACGTCTTTGCTCTCAGGCGTGCG			
E1-S225A	F	CCGTACCCGCGGCCGGTCTCTGCAG	✓		
	R	CTGCAGAGACCGGCCGCGGGTACGG			
E1-A226V	F	CAGAGACCGTCCGTGGGTACGGTGCAC	✓		✓
	R	GTGCACCGTACCCACGGACGGTCTCTG			
E1-M269V	F	CCCGGTAAGAGCGGTGAACTGCGCCGT	✓		✓
	R	ACGGCGCAGTTCACCGCTCTTACCGGG			
E2-I2T	F	GCTCGCGGATCCTAGTACTAAGGACCACTTCAATG		✓	
	R	CATTGAAGTGGTCCTTAGTACTAGGATCCGCGAGC			
E2-H5N	F	CGGATCCTAGTATTAAGGACAACTTCAATGTCTATAAAGCC		✓	✓
	R	GGCTTTATAGACATTGAAGTTGTCCTTAATACTAGGATCCG			
E2-G118S	F	CGGTGGGGGTTCACTGATAGTAGAAAGATCAGTCAC		✓	✓
	R	GTGACTGATCTTTCTACTATCAGTGAACCCCACCG			

F, forward primer; R, reverse primer

Table 3.5, continued: Primers used for mutagenesis of CHIKV E1 and E2 proteins and CHIKV infectious clones.

Mutation	5'-Sequence-3'		Template		
			rE2-E1-Asian (antibody binding)	rE2-Asian (antibody binding)	CAR (DNA-launched infectious clone)
E2-R149K	F	CGACCGCAGCACGGTAAGGAACTACCTTGC		✓	✓
	R	GCAAGGTAGTTCCTTACCGTGCTGCGGTCG			
E2-A157V	F	GCAGCACGTACGTGCAGAGCACCGC		✓	
	R	GCGGTGCTCTGCACGTACGTGCTGC			
E2-A164T	F	GCAGAGCACCGCTGCAACTACCGAGGAGAT		✓	
	R	ATCTCCTCGGTAGTTGCAGCGGTGCTCTGC			
E2-S194G	F	GTAAAGATCACAGTCAATGGTCAGACGGTGCGGTAC		✓	✓
	R	GTACCGCACCGTCTGACCATTGACTGTGATCTTTAC			
E2-S207N	F	AGTGCAATTGTGGTGACTCAAATGAAGGATTAACCACTAC		✓	
	R	GTAGTGGTTAATCCTTCATTTGAGTCACCACAATTGCACT			
E2-S248L	F	TCCGGTCCCCTAATTCAGCATTGCGCGGGAC		✓	
	R	GTCCCGCGCAATGCTGAATTAGGGGACCGGA			
E2-K252Q	F	GGAATGTGAACTTTTCCCTGCCGGTCCCCGGATTCAG		✓	
	R	CTGAATCCGGGGACCGGCAGGGAAAAGTTCACATTCC			
E2-V255I	F	CCGGGGACCGGAAAGGAAAAATTCACATTCCATTTC		✓	
	R	GAAATGGAATGTGAATTTTTCCTTTCCGGTCCCCGG			
E2-I2T	F	GCCAGCGGCGCAGTACTAAGGACCACTTCAA			✓
	R	TTGAAGTGGTCCTTAGTACTGCGCCGCTGGC			
E2-I2T-H5N	F	CGGCGCAGTACTAAGGACAACTTCAATGTCTATAAAG			✓
	R	CTTTATAGACATTGAAGTTGTCCTTAGTACTGCGCCG			

F, forward primer; R, reverse primer

1. DNA template

2. Two reactions of single primer PCR (designed primer with point mutation)

3. Generation of mutant single DNA strand

4. Both reaction tubes from the same template were pooled into a single tube

5. Digestion of methylated DNA template with *Dpn*1

6. Re-annealing of 2 complementary DNA strands
7. Repeat step 5 for another round and followed by re-annealing step

Bacteria transformation

Figure 3.3: Workflow of site-directed mutagenesis.

Table 3.6: Sequencing primers for site-directed mutagenesis .

Primer	5'-Sequence-3' (forward primer)	Template	
		pUC-CC56	pSK-CAR-XN
A14F	AGATGGCAACGAACAGGGC	✓	
A15F	TCGAAGTCAAGCATGAAGG	✓	
A16F	TGCTTGAAGACAATGTCATGAG	✓	
A17F	AGTCCGGCAATGTAAAGATCAC	✓	
A19F	ATAAAACCGTTATCCCGTCTC		✓
A20F	CGGCGCAGGAAGACCAGGAC		✓
A21F	AGCAGGCACTAACTTGACAACTAGGTATGAAG		✓
M13F-pUC(-40)	GTTTTCCCAGTCACGAC		✓

3.3.7 *In vitro* RNA transcription

This section describes the steps involved in *in vitro* RNA transcription from linearised DNA, RNA quantification and electroporation. The 5' capped RNA was electroporated into K562 and BHK-21 cells.

Six micrograms of each endotoxin-free SP6-driven plasmid (icDNAs and replicons) was subjected to linearisation with *Not*1 restriction enzyme for 2 hours. The linearised template was cleaned up with DNA Clean & Concentrator-5 (Zymo Research, USA) and the final product was eluted in ultra-pure H_2O. The full-length RNA transcript was transcribed using the SP6 mMESSAGE mMACHINE kit (Ambion, USA) with 0.5 or 1.0 µg linearised template in a 10 µl or 20 µl volume of reaction for 2 hours at 37°C, according to the manufacturer's instructions. At the end of incubation, an equal volume of ultra-pure H_2O was added and the mixture was kept on ice until further RNA quantification. An extra *in vitro* transcription reaction was prepared which served as an RNA reference and was cleaned up by lithium chloride (LiCl) precipitation. Briefly, 30 µl ultra-pure H_2O and 30 µl LiCl were added to 20 µl transcribed RNA sample, which was mixed by vortexing and stored at -20°C for 30 minutes. The tube was spun at $20,000 \times g$ at 4°C for 15 minutes. The supernatant was discarded carefully and the pellet was rinsed with 1 ml chilled 70% ethanol. The tube was spun again for 5 minutes and the ethanol was discarded. The pellet was air-dried and resuspended in 50 µl ultra-pure H_2O. RNA yield was quantitated with a Take-3 Micro-Volume plate and the reading was acquired with an Epoch ELISA reader.

RNA quantification was performed with a semi-quantitative method by non-denaturing agarose gel electrophoresis. The band intensity/volume of the samples was compared to reference bands with known amounts of RNA. The transcribed RNA samples cannot be cleaned up with conventional RNA purification methods because the 5' capped will be lost after purification and defective RNA is non-functional. One part

of transcribed RNA was diluted with 3 or 4 parts of ultra-pure H_2O. All the RNA samples and RNA references were mixed with equal volumes of gel loading buffer II (Ambion, USA) prior to loading on 0.8% agarose gel, which was prepared in $1 \times$ TAE buffer and pre-stained with GelRed. Two microlitres of each diluted RNA sample and six different amounts of known RNA references (1.8, 1.5, 1.2, 0.9, 0.6, 0.3 µg) were loaded. Electrophoresis was carried out at 200 V for 30 minutes. The agarose gel was visualised under UV illumination with a BioSpectrum AC imaging system (UVP). The imaged gel was analysed with VisionWorks LS, UVP, version 8.0.1. A standard plot was generated based on the band volumes quantitated from the software and the actual concentration of RNA samples was determined in µg/µl. All the RNA samples were kept at -80°C.

For RNA electroporation in K562 cells, an optimised protocol was adapted from a previous study (Van Tendeloo *et al.*, 2001). Briefly, K562 cells at mid-log phase were washed 2 times with serum-free RPMI medium, prior to resuspension in Opti-MEM 1 Reduced Serum Media (Gibco, USA) to a final cell count of 1×10^7. In a 4 mm cuvette, 250 µl of cell suspension was mixed with 10 µg RNA samples, followed by electroporation under exponential decay pulse type at 300 V, 150 µF, and one pulse using Gene Pulser Xcell electroporation system (Bio-Rad, USA). The electroporated cells were immediately put on ice for 5 minutes and the recovered cells were incubated in a 25 cm^2 flask with 10 ml growth media.

To verify the integrity of RNA, a parallel electroporation procedure was carried out in BHK-21 cells. Five micrograms of each RNA sample was electroporated into 5×10^6 cells in 800 µl ice-cold PBS under square wave pulse type with the following conditions: 240 V, 25 msec, 3 sec interval, and 2 pulses. One ml of growth media was immediately added and the mixture was allowed to recover at room temperature for 5 minutes. The recovered cells were incubated in a 75 cm^2 flask with 10 ml growth media.

3.4 Protein assays

This section describes the recombinant protein production in bacterial and insect cells and related techniques.

3.4.1 Protein expression in BL21(DE3)

The bacteria expression system pET-52b(+) carrying the whole E2 insert was transformed into BL21(DE3) (Novagen). A single colony was inoculated into 5 ml LB broth with ampicillin and the culture was grown overnight at 37°C with constant orbital shaking at 200 rpm. The next day, 1 part of overnight culture was added to 100 parts of fresh LB broth containing ampicillin in a 500 ml conical flask. The large culture was grown for approximately 2 hour 15 minutes until the OD_{600} reached 0.6. Sterile IPTG was added to a final concentration of 1 mM and incubation was continued for another 4 hours. At the end of incubation, the cell pellet was collected by centrifugation at 4000 × g for 10 minutes. The supernatant was discarded and the cell pellet was kept at -20°C.

3.4.2 Protein expression in Sf9 and TriExSf9

For stable clone generation, Sf9 cells were seeded at a density of 2×10^6 cells in 5 ml in a 25 cm^2 flask. To set up the transfection, 3.2 µg plasmid DNA (pIEX-5 carrying the E1 or E2), 0.8 µg pIE1-neo vector and 32 µl Cellfectin II reagent (Invitrogen) were added to 500 µl of Sf9 growth medium in a 1.5 ml sterile tube. The transfection complex was incubated for 30 minutes at room temperature before addition into the flask. After 24 hours, G418 sulphate (Gibco, Life Technologies) was added to a final concentration of 500 µg/ml and the cells were incubated for another 2–3 days. The media was replaced every 5 days in the presence of 5% heat-inactivated FBS to boost the growth of stable clones for another 2–3 weeks. Stable clones expressing rE1 and rE2 were maintained under selection with G418 sulphate at 1000 µg/ml. The stable clones

were expanded in flasks and the recombinant proteins secreted from stables clones were collected in a sterile 50 ml tube and kept at 4°C.

For transient protein expression of E2 mutants and fusion E1-E2 glycoprotein, 1.6×10^6 cells were seeded in a 6-well plate in 2 ml of TriEx Insect Cell Medium. To set up the transfection, 2 µg of plasmid and 5 µl TransIT-Insect transfection reagent were added to the same tube containing 600 µl of medium. The transfection complex was allowed to form for 30 minutes at room temperature before distributed onto the cells. The supernatant was harvested after 48 hours and kept at 4°C.

3.4.3 Protein purification

All the recombinant proteins contained fusion His-tag which facilitates purification. The protein from bacteria cell pellets was purified under denaturing conditions. The frozen cell pellet from 50 ml culture was thawed at room temperature and resuspended in 10 ml native binding buffer (50 mM NaH$_2$PO4, 300 mM NaCl and pH 8.0) and sonicated using a Branson Sonifier 250 (USA) with a constant power output at about 10–20% for 10 minutes on an ice-bath. The bacteria suspension was centrifuged at $14,000 \times g$ in a fixed-angled rotor for 10 minutes at 4°C. The cell pellet was resuspended in 8 ml denatured binding buffer (8 M urea, 50 mM NaH$_2$PO$_4$, 300 mM NaCl, 10 mM imidazole and pH 8.0) and allowed to redissolve overnight at 4°C. The clarified lysate was loaded into a polypropylene column (Qiagen) containing activated Profinity IMAC resins (Bio-Rad) and the binding was performed for 1 hour at 4°C. The unbound fraction was allowed to flow out from the column and the column was rinsed with denatured washing buffer (8 M urea, 50 mM NaH$_2$PO$_4$, 300 mM NaCl, 20 mM imidazole and pH 8.0). The bound proteins on the resin were eluted with elution buffer (8 M urea, 50 mM NaH$_2$PO$_4$, 300 mM NaCl, 250 mM imidazole and pH 8.0), aliquoted

and kept at -20°C. The bacterially-expressed recombinant E2 glycoprotein was used for mouse monoclonal antibodies production.

For the purification of native secreted proteins (rE1, rE2) from insect cells, the supernatant was collected in a 50 ml tube and spun at 4000 × g for 10 minutes to remove cell debris, and the media was filtered through a 0.45 µm pore size membrane (Sartorius, Germany). The media was mixed with native binding buffer (50 mM NaH₂PO4, 300 mM NaCl, pH 8.0) at 1:2 ratio (50 ml media: 100 ml binding buffer) without adjusting the pH. The whole mixture was incubated with 1 ml activated Profinity IMAC resin at 4°C with constant stirring for 1 hour. The resin was collected in a polypropylene column. The column was washed 5 times with 10 ml binding buffer, eluted with 5 ml native elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0) and kept at 4°C. The eluates were concentrated with an Amicon centrifugal unit (Merck Millipore) and the buffer was exchanged with sodium phosphate buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0). The proteins were stored at -20°C in 50% glycerol and were used for western blot as described in section 3.4.4 and ELISA (indirect IgG) in section 3.5.5.2. For competitive protein blocking assay, the proteins were filter-sterilised and kept at 4°C.

3.4.4 Western blot

To detect the reactivity of mAbs, the bacterially-expressed purified rE2 protein (25 µg) and sucrose purified CHIKV (80 µg) were loaded onto 12% SDS-PAGE and electro-transferred to a PVDF membrane (Millipore). The membrane was blocked with 5% skimmed milk in 0.05% PBST, assembled onto a Mini-Protean II multi screen apparatus (Bio-Rad) and probed with each monoclonal antibody at 5 µg/ml or CHIKV anti-E2 polyclonal antibody at 1:100 dilution. Goat anti-mouse IgG-HRP (Merck Millipore) was diluted at 1:2000. Colorimetric change was developed with metal

enhanced DAB (Thermo Scientific). Images were acquired with a GS-800 densitometer (Bio-Rad).

To detect the reactivity of human immune sera against CHIKV, the native proteins (rE1, rE2, fusion E1-E2, mutant E2) were resolved with 12% SDS-PAGE under either reducing or non-reducing conditions and electro-transferred onto a nitrocellulose membrane (GE). The membrane was blocked with 10% skimmed milk in 0.05% PBST. The immunoreactivity of recombinant proteins was evaluated with pools of CHIKV immune sera applied at indicated dilutions in the blocking buffer. The bound antigen-antibody complex was detected by rabbit anti-human IgG-HRP (DakoCytomation, Denmark, cat. no. P0214) at 1:5000 dilution in 1% BSA-0.05% PBST. The membrane was visualised by chemiluminescence (Bio-Rad) and images were acquired with a BioSpectrum AC imaging system (UVP). Mouse anti-His tag antibody (Merck Millipore) was included as a loading control. Mouse anti-E2 CHIKV monoclonal antibody (clone: B-D2 (C4); EIEVHMPPDT), which was generated from this study, was included as a control.

For the detection of anti-CHIKV IgM in western blot, the pooled sera were treated with RIDA RF-Absorbens (R-Biopharm AG, Germany) in 1% BSA-PBS prior to blotting. The immunoreactivity of recombinant CHIKV proteins and virus antigen were evaluated at 1:100 or 1:400 dilutions. The bound antigen-antibody complex was detected by goat anti-human IgM-HRP (KPL, USA, cat. no. 474-1003) at 1:5000 dilution in 1% BSA-0.05% PBST.

3.4.5 Mass-spectrometry (MS)

The bacterially-expressed recombinant E2 protein band was excised from the Coomassie-colloidal blue G-250 stained gels. The gel plugs were destained in 100 mM

ammonium bicarbonate. In-gel digestion of the protein was performed by incubation with sequencing grade modified trypsin (Promega) at 37°C for 2 hours. The digested peptides were eluted by 50% acetonitrile and 0.1% trifluoroacetic acid, mixed with α-Cyano-4-hydroxycinnamic acid in a 1:1 ratio and spotted onto an Opti-TOF 384-well insert plate. The peptide mass fingerprinting were generated in a 4800 Plus MALDI-TOF/TOF analyser (Applied Biosystems, USA). All the peptide mass fingerprinting generated from matrix-assisted laser desorption/ionisation-time of flight (MALDI-ToF) and MS/MS ions from MALDI-ToF/ToF was searched through the MASCOT database for identification (http://www.matrixscience.com/search_form_select.html).

3.5 Immunological assays

This section describes the techniques to produce mouse monoclonal antibodies and to study the antibody responses of CHIKV immune individuals.

3.5.1 Production and purification of mouse monoclonal antibodies

To prepare the antigen for immunisation, the bacterially-expressed recombinant E2 protein was resolved with 12% SDS-PAGE and visualised by chilled 0.1 M KCl. The protein band was excised, homogenised in PBS, and boiled for 2 minutes. Following approval from the Animal Care and Use Committee (ACUC) of University Malaya (ref. no. MP/14/07/2010/JICS(R)), five BALB/c mice aged 6–8 weeks were primed subcutaneously with 50 µg protein mixed with incomplete Freund's adjuvant. Eight boosters were given every 2 weeks and blood was collected prior to administration of booster. The antibody titre was monitored by ELISA. The blood from immunised mice was collected and served as a positive control. The immunised mouse with the highest antibody titre was sacrificed; the spleen was harvested and fused with myeloma cells (X63, ATCC no. CRL1580) to generate hybridomas secreting monoclonal antibodies. Selection of stable, positive clones and isolation of single clone was performed on the high-throughput automated clone selection system, ClonePix FL (Molecular Devices, USA). Cell fusion, clone screening and selection were performed by InnoBiologics (Nilai, Malaysia). Some parental clones which retained reactivity were sub-cloned by limiting dilution. Desired clones were expanded in a bioreactor as described in section 3.1.

For the antibody purification, the pooled culture supernatant from the bioreactor was clarified and buffer exchange was performed with dialysis against antibody binding buffer (20 mM NaH_2PO_4, pH 7.0) using a Snakeskin dialysis bag (Thermo Scientific) overnight. The dialysed antibody in the tube was retrieved with a syringe and clarified

by centrifugation at 4000 × g for 20 minutes. The crude lysate was passed through a Protein G column (GE) followed by rinsing the column with 5 volumes of binding buffer. The antibody was eluted with 5 ml 0.2 M glycine-HCl, pH 2.7 and immediately neutralised with 1 ml of 1 M Tris-HCl at pH 9.0. The eluted, purified mouse monoclonal antibodies were dialysed against 1× DPBS, concentrated by an Amicon Centrifugal Filter (Merck Millipore), filter-sterilised and aliquoted. The concentration of each antibody was quantitated by Bradford assay. The isotype of monoclonal antibodies were determined with the Rapid ELISA Mouse mAb Isotyping Kit (Thermo Scientific).

3.5.2 Ethical approval and CHIKV immune sera panels

The use of human immune sera in this study was approved by the Medical Ethics Committee of the University Malaya Medical Centre (reference no. 800.70 and 20157-147). Informed consent for retrospective studies of archived and anonymised samples was not required in agreement with the policy of University of Malaya.

This study used two panels of serum samples. Panel A comprised 27 samples collected from patients attending University Malaya Medical Centre, Kuala Lumpur, during the 2008-2010 outbreak of CHIKV of East Central/ South African (ECSA) genotype. These were acute samples collected from viraemic patients between day 1 and day 9 after disease onset. Viral loads had been quantified by real-time PCR targeting the E1 region in a previous study (Chiam *et al.*, 2013). In this study, for all but 4 of the samples in this panel, only neutralising titres of total antibodies (and not IgM and IgG separately) were determined due to limited sample volumes. This panel provided information regarding the relationship between viraemia and the appearance of neutralising antibodies.

For panel B, the neutralising titres of IgM and IgG were determined separately in 79 samples to study the relative contributions to total neutralising activity. Of these 79 samples, 39 serum samples were from the same 2008–2010 outbreak and known to contain neutralising anti-CHIKV IgM and/or IgG. These samples were collected from patients attending University Malaya Medical Centre 4 days to 6 months after symptoms, and included 4 samples from panel A for which sufficient serum volumes were available for additional analysis. A further 40 samples were collected from patients 11−14 months after an Asian CHIKV outbreak in Bagan Panchor, Perak state, in 2006. The samples were categorised into 3 groups by the duration between sample collection and time of acute disease onset: 4−20 days (panel B1, n=16), 1−6 months (panel B2, n=23) and 11−14 months (panel B3, n=40).

Serum samples from 15 healthy controls with no past infection of CHIKV were included as negative controls, confirmed by the absence of antibodies by serum neutralisation assay. This made a total of 117 sera used in this study. The use of different serum panels for different objectives is depicted in Figure 3.4.

Immune sera with known time of disease onset and viral load, 2008–09

Immune sera with known time of disease onset, 2006 and 2008–10 (n=79)

Panel A, 1–9 days, 2008–09 (n=27)

Panel B1, 4–20 days, 2008–09 (n=16 including 4 from panel A)

Panel B2, 1–6 months, 2008–10 (n=23)

Panel B3, 11–14 months, 2006 (n=40)

Objective 4

Objective 4

Objective 2, 3, 4
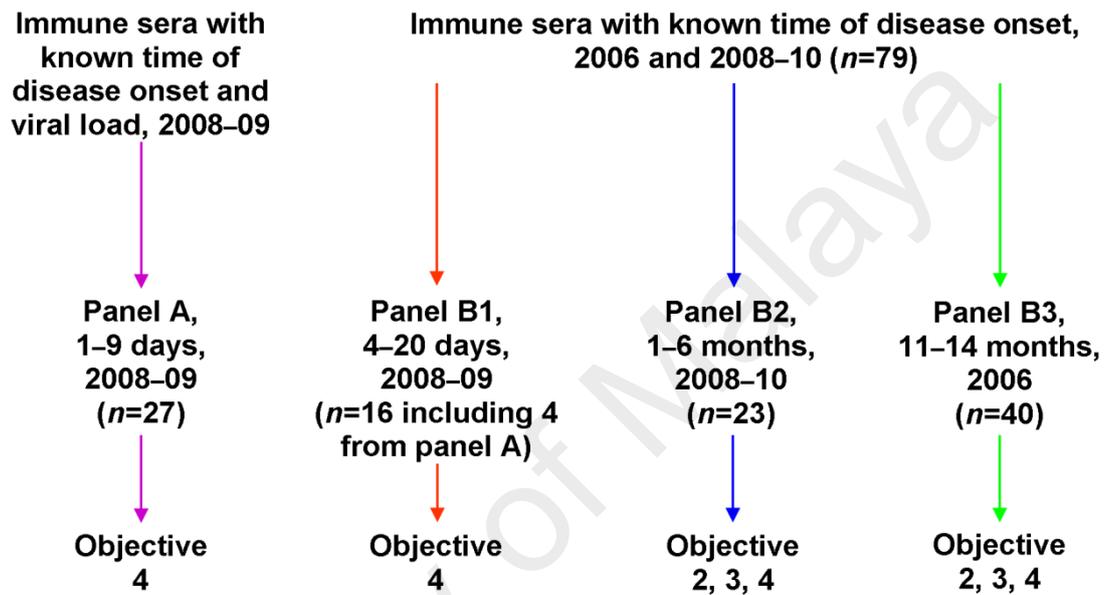
Objective 2, 3, 4

Figure 3.4: The use of different serum panels for different objectives.

3.5.3 Rabbit polyclonal antibodies production against linear epitopes of E2 glycoprotein

To investigate the effect of sequence variation of neutralising epitopes in ECSA and Asian genotypes, polyclonal rabbit anti-LP1 (STKDNFNVYKATRPY), anti-LP1A (SIKDHFNVYKATRPY) and anti-LP47 (NHKKWQYNSPLVPRN) were produced commercially (GenScript). LP1 is similar to E2EP3, an immunogenic peptide from an ECSA virus, which was previously reported to elicit neutralising antibodies (Kam *et al.*, 2012b). LP1A is the corresponding variant peptide with the Asian genotype sequence. The LP47 peptide sequence is conserved in both genotypes. Neutralisation assay was performed with purified antibody at 25 µg/ml against the rescued viruses.

3.5.4 Serum neutralisation assays

A modified version of neutralisation assay was performed as previously described using BHK-21 cells (Kam *et al.*, 2012c). CHIKV immune sera were heat-inactivated and serially diluted 2-fold (1:100 up to 1:6400 dilution) in $1 \times$ DPBS. The diluted sera (200 µl) were mixed with CHIKV pre-diluted with 2% FBS GMEM in equal volumes, and cells were infected at a final MOI of 10 per well in triplicate. The virus-antibody mixture was incubated for 2 hours at 37°C before inoculation into 10^4 cells in a 96-well CellCarrier-96 optic black plate and further incubation for 1.5 hours at 37°C. The inocula were decanted and 2 % FBS GMEM was added. The plate was fixed with 4% paraformaldehyde after 6 hours of incubation at 37°C, permeabilised with 0.25% Triton X-100 for 10 minutes, and immunostained using CHIKV monoclonal antibody B-D2(C4) at 1 µg/ml followed by rabbit anti-mouse IgG-FITC (Thermo Fisher Scientific, USA, cat. no. 31561) at 1:100 dilution. Cell nuclei were counter-stained with DAPI. Fluorescence intensity was analysed with a Cellomics High Content Screening ArrayScan VTI (Thermo Fisher) at $5 \times$ magnification. Percentage of infectivity was

calculated according to the following equation: % infectivity = (mean average fluorescence intensity from serum sample/mean average fluorescence intensity from virus control) $\times$ 100. The neutralising titre (NT_{50}) was expressed as the serum dilution that reduced infectivity by 50% using non-linear regression fitting in GraphPad Prism 5. For non-converged regressions with no sigmoidal curve, the neutralising titre was set to 1.

For neutralisation assay using rescued viruses, diluted sera were mixed with viruses pre-diluted with 2% FBS GMEM (with infection performed at an MOI of 50), followed by the steps described above. The plates were fixed after 7 hours of incubation at 37°C. The plates were only counter-stained with DAPI prior to acquisition of ZsGreen fluorescence.

To investigate the cross-reactivity of CHIKV sera against another alphavirus, SFV was rescued from icDNA SFV6 as described in section 3.2.4. Diluted sera (1:25 and 1:100 dilutions) were mixed with SFV pre-diluted with 2% FBS GMEM (with infection performed at an MOI of 10), followed by the steps described above. The plates were fixed after 6 hours of incubation at 37°C, and stained with mouse anti-alphavirus monoclonal antibody (Santa Cruz, USA, cat. no. sc-58088) at 1:100 dilution.

To assess the IgM neutralisation activity, the human IgG antibody from heat-inactivated sera was first precipitated with RIDA RF-Absorbens (R-Biopharm). The absorption buffer was prepared in 1:10 dilution in 1 $\times$ DPBS and serum was diluted 2-fold with absorption buffer from 1:100 to 1:1600. The IgG antibody from samples was precipitated at 37°C for 30 minutes prior to mixing with CHIKV. The cells were rinsed with 1 $\times$ DPBS at the end of cell-virus mixture incubation prior to replenishment with maintenance medium.

For determination of neutralising activity solely due to IgG, the heat-inactivated sera were treated with 0.1M dithiothreitol (DTT) (Life Technologies) to a final concentration

of 5 mM, to inactivate IgM, and were incubated at 37°C for 1 hour prior to dilution from 1:100 to 1:6400. In parallel with antibody-dependent assay comparison, neutralisation was performed with immune sera serially diluted 4-fold from 1:50 to 1:204,800 in $1 \times$ DPBS.

3.5.5 Enzyme-linked immunosorbent assay (ELISA)

3.5.5.1 Epitope mapping of monoclonal antibodies

Eighty-four biotinylated synthetic peptides (Mimotopes, Australia) (Appendix B) covering the E2 glycoprotein sequence from amino acids 1–423 were generated, with 15-mer peptides each with a 10-mer overlap based on the CHIKV MY/08/065 sequence (GenBank accession number FN295485). The peptides were dissolved in DMSO, further diluted to a working concentration of approximately 15 µg/ml in PBS, and incubated in streptavidin-coated 96-well flat-bottomed Maxisorp microplates (Nunc, Denmark). Supernatants from different hybridoma clones were harvested and incubated on peptide-coated plates for 1 hour at room temperature. The plates were rinsed 4 times with 0.05% PBST and bound antigen-antibody complexes were detected by HRP-conjugated goat anti-mouse IgG Fc (Merck Millipore, cat. no. AP127P) at 1:10,000 dilution in 1% BSA-PBST. The plates were rinsed before addition of TMB substrate (KPL, USA), and the optical density (OD) was measured with an Epoch plate reader (BioTek, USA).

3.5.5.2 Binding activity of monoclonal antibodies

For study of mAbs binding, 96-well flat-bottomed Maxisorp microplates were coated with 4 µg/ml sucrose-purified CHIKV antigen or 1 µg/ml bacterially-expressed purified recombinant E2 protein in 0.05 M carbonate–bicarbonate buffer (pH 9.6) and incubated at 4°C overnight. The plates were blocked with 3% BSA-0.05 % PBST. Serially-diluted

mouse serum or purified monoclonal antibodies were added to the plates and incubated at 37°C for 1 hour. Bound antigen-antibody complex was detected by incubation with TMB substrate (KPL) for 10 minutes before the reaction was terminated by 1 M H_3PO_4. The absorbance was acquired at 450 nm with 630 nm as the reference wavelength using an Epoch ELISA reader.

3.5.5.3 Indirect IgG for CHIKV immune sera

All incubation steps were performed at 37°C for 1 hour, using 1% BSA-0.05% PBST as the diluent for serum and antibodies. The 96-well flat-bottomed Maxisorp microplates were washed 4 times with 0.05% PBST after each incubation step. To determine the relative level of anti-E2 antibodies, the plates were coated with 250 ng of virus antigen or 100 ng of rE2 in 0.05 M carbonate-bicarbonate buffer (pH 9.6). The antigens were normalised with monoclonal antibody B-D2(C4) to determine the relative level of anti-E2 antibodies, to ensure similar antigen binding sites between rE2 and whole virus antigens. The plate was blocked with 3% BSA in 0.05% PBST. The sera were tested at the indicated dilutions or 2-fold serial dilutions from 1:512 to 1:1,048,000 or 1:640 to 1:655,000. The IgG end-point titre was determined as the reciprocal of the highest dilution that produced an OD reading of three times greater than that of the negative control. Rabbit anti-human IgG-HRP at 1:5000 dilution was added to detect the bound antibodies. The plates were developed with TMB substrate as described in section 3.5.5.1. The cut-off value was established as the OD obtained from healthy controls sera plus three standard deviations (SD). The relative level of anti-rE2 antibodies was calculated with the following formula: (end point titre for rE2/end point titre for whole virus antigen) × 100.

3.5.5.4 IgM capture ELISA for CHIKV immune sera

Formalin-treated virus supernatant was utilised as antigen for the capture IgM assay. Formalin (37%) (Merck, Germany, cat. no. 1040032500) was added to a final concentration of 0.75%, with constant rotation at 4°C for 24 hours. This source of antigen was used within 3 days when kept at 4°C.

All the incubation steps were performed at 37°C for 1 hour, using 1% BSA-0.05% PBST as diluents for serum and antibodies. The plates were washed 4 times with 0.05% PBST after each incubation step, and 6 times after antigen, monoclonal and secondary antibody incubation steps.

The 96-well flat-bottomed Maxisorp microplate was coated with rabbit anti-human IgM (DakoCytomation, Denmark, cat. no. A0425) to a final concentration of 2.8 µg/ml and blocked with 3% BSA-0.05% PBST. Sera were diluted at 1:200 and added. Antigen was then added, and this was either 10^6 pfu per well of formalin-treated virus supernatant diluted in 1% BSA-PBS, or purified recombinant rE1 or rE2 glycoprotein diluted in 1% BSA-PBST to a final concentration of 20 µg/ml. Anti-CHIKV antibody was diluted in 1% BSA-PBST to a final concentration of 1 µg/ml and added to the plate, which was then incubated for 30 min. For the ELISA using rE2 and formalin-treated virus as antigens, anti-E2 monoclonal antibody B-D2(C4) was the antibody used; for the ELISA using rE2 as antigen, anti-alphavirus antibody (Santa Cruz Biotechnology, USA, cat. no. sc-58088) detecting E1 was used (Kumar *et al.*, 2014). Goat anti-mouse IgG-HRP (Bio-Rad, USA, cat. no. 170-6516) was added at 1:20,000 dilution, followed by 30 min incubation. The plates were developed with TMB substrate as described in section 3.5.5.1. The cut-off value was established as the OD obtained from healthy controls sera plus three SD.

3.5.5.5 Peptide-based ELISA for CHIKV immune sera

Fifty-nine biotinylated synthetic peptides (Table 3.7) covering the E2 glycoprotein sequence from amino acids 1-362 were used to screen CHIKV immune sera for binding to linear epitopes. All the peptides were amidated at the C-terminus. Similar steps were performed as described in section 3.5.5.3 except that the plates were washed 6 times after incubation with human sera and secondary antibody. The 96-well flat-bottomed Maxisorp microplates were coated with 20 µg/ml streptavidin (NEB) and blocked with 5% BSA-PBST. The dissolved peptides in DMSO were further diluted to a working concentration of approximately 150 µg/ml in 1% BSA-PBST. CHIKV immune sera and healthy control sera were diluted at 1:1000, and screened against peptides in duplicate. The peptides with the highest OD reading from 2 adjacent overlapping synthetic peptides were considered as identified B-cell epitopes.

Table 3.7: The fifty-nine overlapping peptides used for the peptide-based ELISA cover the CHIKV E2 glycoprotein sequence from amino acids 1 to 362, based on the CHIKV MY/08/065 sequence (accession no. FN295485).

Binding region	Peptide annotation	Sequence[1]
N-link	LP1	SGSGSTKDNFNVYKATRPY
Domain A	LP4	SGSGLAHCPDCGEGHSCHS
	LP5	SGSGDCGEGHSCHSPVALE
	LP6	SGSGHSCHSPVALERIRNE
	LP7	SGSGPVALERIRNEATDGT
	LP8	SGSGRIRNEATDGTLKIQV
	LP9	SGSGATDGTLKIQVSLQIG
	LP10	SGSGLKIQVSLQIGIKTDD
	LP11	SGSGSLQIGIKTDDSHDWT
	LP12	SGSGIKTDDSHDWTKLRYM
	LP13	SGSGSHDWTKLRYMDNHMP
	LP14	SGSGKLRYMDNHMPADAER
	LP15	SGSGDNHMPADAERAGLFV
	LP16	SGSGADAERAGLFVRTSAP
	LP17	SGSGAGLFVRTSAPCTITG
	LP18	SGSGRTSAPCTITGTMGHF
	LP19	SGSGCTITGTMGHFILARC
	LP20	SGSGTMGHFILARCPKGET
	LP21	SGSGILARCPKGETLTVGF
	LP22	SGSGPKGETLTVGFTDSRK
	LP23	SGSGLTVGFTDSRKISHSC
	LP24	SGSGTDSRKISHSCTHPFH
	LP25	SGSGISHSCTHPFHHDPPV
β-ribbon (arch1)	LP28	SGSGIGREKFHSRPQHGKE
	LP29	SGSGFHSRPQHGKELPCST
	LP30	SGSGQHGKELPCSTYVQST
	LP31	SGSGLPCSTYVQSTAATTE
	LP32	SGSGYVQSTAATTEEIEVH
Domain B	LP35	SGSGMPPDTPDRTLMSQQS
	LP36	SGSGPDRTLMSQQSGNVKI
	LP37	SGSGMSQQSGNVKITVNGQ
	LP38	SGSGGNVKITVNGQTVRYK
	LP39	SGSGTVNGQTVRYKCNCGG
	LP40	SGSGTVRYKCNCGGSNEGL

Table 3.7, continued: The fifty-nine overlapping peptides used for the peptide-based ELISA cover the CHIKV E2 glycoprotein sequence from amino acids 1 to 362, based on the CHIKV MY/08/065 sequence (accession no. FN295485).

Binding region	Peptide annotation	Sequence[1]
Domain B	LP41	SGSGCNCGGSNEGLTTTDK
	LP42	SGSGSNEGLTTTDKVINNC
	LP43	SGSGTTTDKVINNCKVDQC
	LP44	SGSGVINNCKVDQCHAAVT
β-ribbon (arch 2)	LP47	SGSGNHKKWQYNSPLVPRN
	LP48	SGSGQYNSPLVPRNAELGD
	LP49	SGSGLVPRNAELGDRQGKI
	LP50	SGSGAELGDRQGKIHIPFP
	LP51	SGSGRQGKIHIPFPLANVT
	LP52	SGSGHIPFPLANVTCRVPK
Domain C	LP55	SGSGARNPTVTYGKNQVIM
	LP56	SGSGVTYGKNQVIMLLYPD
	LP57	SGSGNQVIMLLYPDHPTLL
	LP58	SGSGLLYPDHPTLLSYRNM
	LP59	SGSGHPTLLSYRNMGEEPN
	LP60	SGSGSYRNMGEEPNYQEEW
	LP61	SGSGGEEPNYQEEWVMHKK
	LP62	SGSGYQEEWVMHKKEVVLT
	LP63	SGSGVMHKKEVVLTVPTEG
	LP64	SGSGEVVLTVPTEGLEVTW
	LP65	SGSGVPTEGLEVTWGNNEP
	LP66	SGSGLEVTWGNNEPYKYWP
E2 stem	LP69	SGSGQLSTNGTAHGHPHEI
	LP70	SGSGGTAHGHPHEIILYYY
	LP71	SGSGHPHEIILYYYELYPT

[1] The N-terminus of each peptide was biotinylated and followed by SGSG residues as a spacer, and the C-terminus was amidated.

3.5.6 Competitive protein/peptide blocking assay

Soluble recombinant CHIKV proteins (rE1 and rE2 in amounts of 15 μg) were mixed with heat-inactivated immune sera (panels B2 and B3) diluted at 1:200, and incubated for 1 hour at 37°C. CHIKV (MY/08/065) in amounts corresponding to an MOI of 10 was mixed with the pooled serum samples, which were incubated for a further 2 hours at 37°C.

Synthetic peptides with amidated C-termini were obtained from GenScript (LP1, STKDNFNVYKATRPY; LP19v, TITGTMGHFILAR; LP24, TDSRKISHSCTHPFH; LP38, GNVKITVNGQTVRYK; LP47, NHKKWQYNSPLVPRN; LP56, VTYGKNQVIMLLYPD; LP70, GTAHGHPHEIILYYY); 60 μg of each peptide was mixed with immune sera diluted with $1 \times$ DPBS at 1:100 and incubated for 1.5 hours at 37°C. All the synthetic peptides for the blocking assay have a purity grade greater than 95% and are soluble in high-grade water. ICRES1 (sucrose-cushion-purified virus in TE buffer pre-diluted using 2% FBS GMEM) at an amount corresponding to an MOI of 1 was mixed with the pooled serum samples, which were incubated for a further 2 hours at 37°C prior to infection of BHK-21 cells. The plate was replenished with plaque medium, fixed with 4% paraformaldehyde after 15 hours of incubation, and this was followed by ZsGreen fluorescence acquisition. Infectivity corresponded to the fluorescence intensity acquired with a Cellomics HCS reader. The effect on infectivity of antibodies in the presence and absence of blocking peptides was compared.

3.5.7 Antibody-dependent enhancement (ADE) assay

ADE was performed in K562 cells grown in adherent conditions (Konishi *et al.*, 2010). On the same day, the cells were seeded at a density of 2×10^4 cells in a poly-L-lysine pre-coated 96-well CellCarrier-96 optic black plate in 2% FBS RPMI. Heat-inactivated CHIKV immune sera were serially diluted 4-fold (1:50 up to 1:204,800 dilution) or 2-fold (1:50 up to 1:6,400 dilution) in $1 \times$ DPBS. The diluted sera were mixed with rescued CHIKV (ICRES1, CAR, Hybrid CAR or chimeric viruses) pre-diluted with 2% FBS RPMI in equal volume, and the cells were infected at a final MOI of 50. The virus-antibody mixture was incubated for 2 hours at 37°C before being inoculated into K562 cells and further incubated for 2 hours at 37°C. The inocula were decanted and 2% FBS RPMI was added. The plate was fixed with 4% paraformaldehyde after 24, 30 or 48 hours. The plates were only counter-stained with DAPI prior to acquisition of zsGreen fluorescence with a Cellomics HCS reader. The infectivity mediated by virus-antibody complex was expressed as fold-increases of zsGreen expression, which is the mean average fluorescence intensity from a serum sample or virus control relative to the mean average fluorescence intensity from the mock control (cell control).

ADE of active virus production was investigated. After the cells were infected for 24, 30, 48, 72 or 96 hours, the supernatant was harvested and the virus titre was determined by plaque assay on BHK-21 cells. A receptor blocking assay was performed using anti-human CD32 (Biolegend, USA, cat. no. 303202) to define the role of FcγRIIA in mediating ADE. An amount of 2×10^6 K562 cells was incubated with 2 μg of anti-CD32 at 37°C for 1 hour prior to infection with virus-antibody complex. Anti-human CD32 will block the interaction of FcγRIIA with immune complexes.

3.5.8 Complementary neutralising activities of IgM and anti-LP1 IgG antibodies

The human IgG from heat-inactivated sera was precipitated with RIDA RF-Absorbens and 2-fold dilutions prepared from 1:100 to 1:1600. Anti-CHIKV IgM in sera was incubated with virus for an hour, followed by the addition of rabbit anti-LP1 antibody into each pre-diluted serum sample and incubation for another hour at 37°C. Neutralisation assay was performed to verify the percentage of total neutralisation capacity after addition of rabbit anti-LP1 antibody in the presence of anti-CHIKV IgM. Non-neutralising mouse monoclonal antibody F-G6(F6) and rabbit polyclonal antibody anti-E2dA, which both target short linear epitopes (ADAERAGLFV and IKTDDSHDWTKLRY, respectively) in domain A of E2, were included as negative controls. The changes of neutralising capacities were compared either with the addition of neutralising anti-LP1 IgG antibody or non-neutralising antibodies in the presence of anti-CHIKV IgM from human immune sera.

3.6 Computational 3-D structure and epitope analysis

The structural data of CHIKV glycoproteins was obtained from the Protein Data Bank (PDB, ID 3N44 or 3J2W) and viewed with UCSF CHIMERA software (Pettersen *et al.*, 2004). The mapped epitopes of mAbs were highlighted on the structure at surface-exposed and ribbon views. The identified epitopes distributed on CHIKV E2 glycoprotein were compared and aligned with other alphaviruses of the Semliki Forest complex using Clustal W2 (Thompson *et al.*, 2002). The GenBank accession numbers for viral sequences used in this study are: chikungunya (CHIKV/MY/08/065, FN295485; CHIKV/MY/06/37348, FN295483; CHIKV/S27, AF369024; CHIKV/37997, AY726732), O'nyong-nyong (ONNV, ACC97205), Ross River (RRV, AAA47404), Sagimaya (SAGV, AAO33337), Semliki Forest (SFV, CAA27742), Mayaro (MAYV, AAO33335), Middleburg (MIDDV, AA033343), Barmah Forest (BFV, AA033347), and Eastern equine encephalitis (EEEV, AAT96380).

As the LP1 sequence is unresolved in structural data, the structure of the E2 glycoprotein was predicted using the online I-TASSER server (Zhang, 2008; Roy *et al.*, 2010). The electrostatic potential of the E2 structure (amino acid 1-362) was evaluated with PDB2PQR and APBS (Baker *et al.*, 2001; Dolinsky *et al.*, 2004; Dolinsky *et al.*, 2007).

3.7 Statistical analysis

Data are presented as means $\pm$ SD or means $\pm$ standard error of the mean (SEM). Differences between groups and controls were analysed using appropriate statistical tests, as stated in the figure labels (Mann-Whitney U test, Wilcoxon matched-pairs signed rank test, Kruskal-Wallis test, repeated measures ANOVA or two-way ANOVA with Bonferroni multiple comparison test). A *P*-value of <0.05 was considered significant. Statistical analyses were performed with GraphPad Prism 5.

CHAPTER 4: RESULTS

4.1 Production and characterisation of mouse monoclonal antibodies

Recombinant E2 protein
expression and purification

↓

BALB/c mice immunisation
with recombinant E2 protein

↓

Hybridoma production (cell fusion between splenocytes and
myeloma, done by Innobiologics Sdn. Bhd.)

↓

Reactivity screening and
clone selection

↓

Epitope mapping of
monoclonal antibodies

↓

Indirect immunofluorescence assay,
Western blot, ELISA

↓

Computational and epitopes analysis

↓

OUTCOME:
mAb served as an immunological tool
for objectives 2 and 4

Figure 4.1: Flow chart for objective 1, production and characterisation of mouse monoclonal antibodies targeting linear epitopes on chikungunya virus E2 glycoprotein.

4.1.1 Seroreactivity of CHIKV recombinant E2 protein

   Pooled CHIKV human immune sera from ten patients with confirmed CHIKV infection had specific CHIKV-reactive antibodies which recognised the main structural proteins (E1/E2 glycoprotein and capsid) of purified CHIKV virions at 56 kDa and 35 kDa, respectively (Figure 4.2A). The rE2 glycoprotein reacted with the pooled immune sera at 55 kDa (Figure 4.2A). Pooled sera from ten healthy controls exhibited seronegativity as no band was detected by immunoblot (Figure 4.2B).   Mass spectrometry analysis showed the peptides matched to CHIKV E2 protein with significant scores, as shown in Table 4.1.
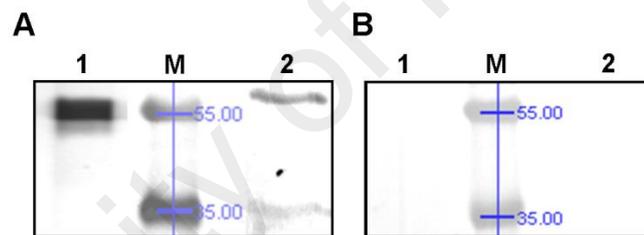
Figure 4.2: Seroreactivity of CHIKV immune sera against recombinant CHIKV E2 protein and sucrose-purified CHIKV. Recombinant E2 (55 kDa) (lane 1) and sucrose-purified CHIKV (with E2/E1 at 55kDa and capsid at 35 kDa) as control (lane 2) were probed with pooled CHIKV immune sera (A) and pooled sera from healthy controls (B) at 1:100 dilution. PageRuler Prestained protein ladder (Thermo Scientific) was used as a marker (M).

Table 4.1: Mass spectrometry analysis of purified CHIKV recombinant E2 protein. Peptides identified in MS and MS/MS were confirmed and matched to chikungunya virus proteins in UniProtKB/Swiss-Prot (accession codes: Q5WQY5 (POLS_CHIKN, GenBank accession no. gi|82050910); Q8JUX5 (POLS_CHIKS, GenBank accession no. gi|341942186) and Q5XXP3 (POLS_CHIKV3, GenBank accession no. gi|82050978)).

Approximate molecular weight (kDa)	Peptide mass fingerprinting (PMF) identified	Protein scores (MS), significant when >53	Protein scores (MS/MS), significant when >25
55	K.WQYNSPLVPR.N K.KWQYNSPLVPR.N K.GETLTVGFTDSR.K R.YMDNHMPADAER.A K.NQVIMLLYPDHPTLLSYR.N	74, 92, 133	68, 84, 109

4.1.2 Generation and production of mAbs specific to CHIKV recombinant E2 protein

An E2-immunised mouse with the highest antibody titre was sacrificed aseptically in 70% alcohol. The enlarged spleen was removed and splenocytes were teased out using a hypodermic needle along with RPMI medium flushing. The extracted splenocytes were washed, counted and fused with myeloma X63.Ag under the action of PEG-6000. Positive hybrid clones which survived under hypoxanthine-aminopterin-thymidine (HAT) medium were expanded and were screened using indirect ELISA with sucrose-purified CHIKV and recombinant E2 protein. The work was carried out by InnoBiologics (Nilai, Malaysia). Clones were selected by automated ClonePixFL and limiting dilution. From a total of 235 parental clones, 37 clones demonstrated reactivity against CHIKV antigen. Eight stable clones were successfully isolated, expanded, and purified with protein G column. Clones A-A9(A1) and BD2(C4) displayed strong affinity against sucrose-purified CHIKV in ELISA, while the rest of the clones demonstrated moderate to weak affinity. The characteristics of the different clones of E2-reactive mAbs are summarised in Table 4.2. Plaque reduction neutralisation test was performed in Vero cells to assess the neutralising activity of purified CHIKV-E2-reactive mAbs at concentrations ranging from 0.001 µg/ml to 100 µg/ml. None of the clones were able to reduce plaque infectivity at concentrations up to 100 µg/ml, suggesting these mAbs did not exhibit any neutralising activity.

Table 4.2: Characteristics of different clones of E2-reactive monoclonal antibodies.

| mAbs | IgG subclass, light chain | Matched epitope | Epitope location[1] | Domain binding site | CHIKV reactivity[2] | | | OD of binding activity[3] |
					Indirect IF	ELISA	WB	
C-B2(C9)	IgG1, κ	LAHCPDCGEGHSCHS	16-30	A	+	+	+	0.42 ± 0.15
F-G6(F6)	IgG2a, λ	ADAERAGLFV	76-85	A	++	+	++	0.54 ± 0.01
O-G12(B1)	IgG1, κ	THPFHHDPPV	126-135	Junction of A and β-ribbon	++	++	+++	1.07 ± 0.09
B-D2(C4)	IgG1, κ	EIEVHMPPDT	166-175	Junction of β-ribbon and B	+++	+++	+++	1.81 ± 0.04
N-B10(B11)	IgG3, κ	EIEVHMPPDT	166-175	Junction of β-ribbon and B	+++	+	+++	0.70 ± 0.07
A-A9(A1)	IgG1, κ	GEEPNYQEEW	301-310	C	++	+++	+++	1.69 ± 0.15
E-G6(A6)	IgG3, λ	VPTEGLEVTW	321-330	C	+++	+	++	0.46 ± 0.05
D-E3(D8)	IgG1, κ	GNNEPYKYWP	331-340	C	++	++	++	1.11 ± 0.11

[1] The first amino acid from E2 is annotated as 1.

[2] CHIKV reactivity was evaluated by indirect immunofluorescence (IF), enzyme-linked immunosorbent assay (ELISA) and western blotting (WB), and rated accordingly: strong (+++), moderate (++) and weak (+).

[3] Mean optical density (OD) ± standard deviation of mAbs binding activity to sucrose-purified CHIKV at 10 µg/ml.

4.1.3 Epitope mapping of CHIKV-E2 reactive mAbs

Epitope mapping of these rE2-reactive mAbs was performed with the PEPSCAN method on 84 biotinylated overlapping synthetic peptides. Each mAb showed similar reactivity/ OD reading to at least 2 overlapping peptides, and the epitope for each mAb was identified as the overlapping 10 amino acid sequence of the 2 peptides (Figure 4.3). In contrast, mAb C-B2(C9) had different reactivity against 2 adjacent peptides; reactivity against peptide 4 (LAHCPDCGEGHSCHS) was higher compared to peptide 5 (DCGEGHSCHSPVALE). The first 5 amino acid residues (LAHCP) in peptide 4 could contribute to the maximum binding capacity of this mAb. Therefore, the sequence of peptide 4 was assigned as the epitope sequence for mAb C-B2(C9). MAbs B-D2(C4) and N-B10(B11) shared the same epitope, although both mAbs were of different IgG subclass (IgG1 and IgG3, respectively). All the matched epitopes of mAbs and their locations are listed in Table 4.2.
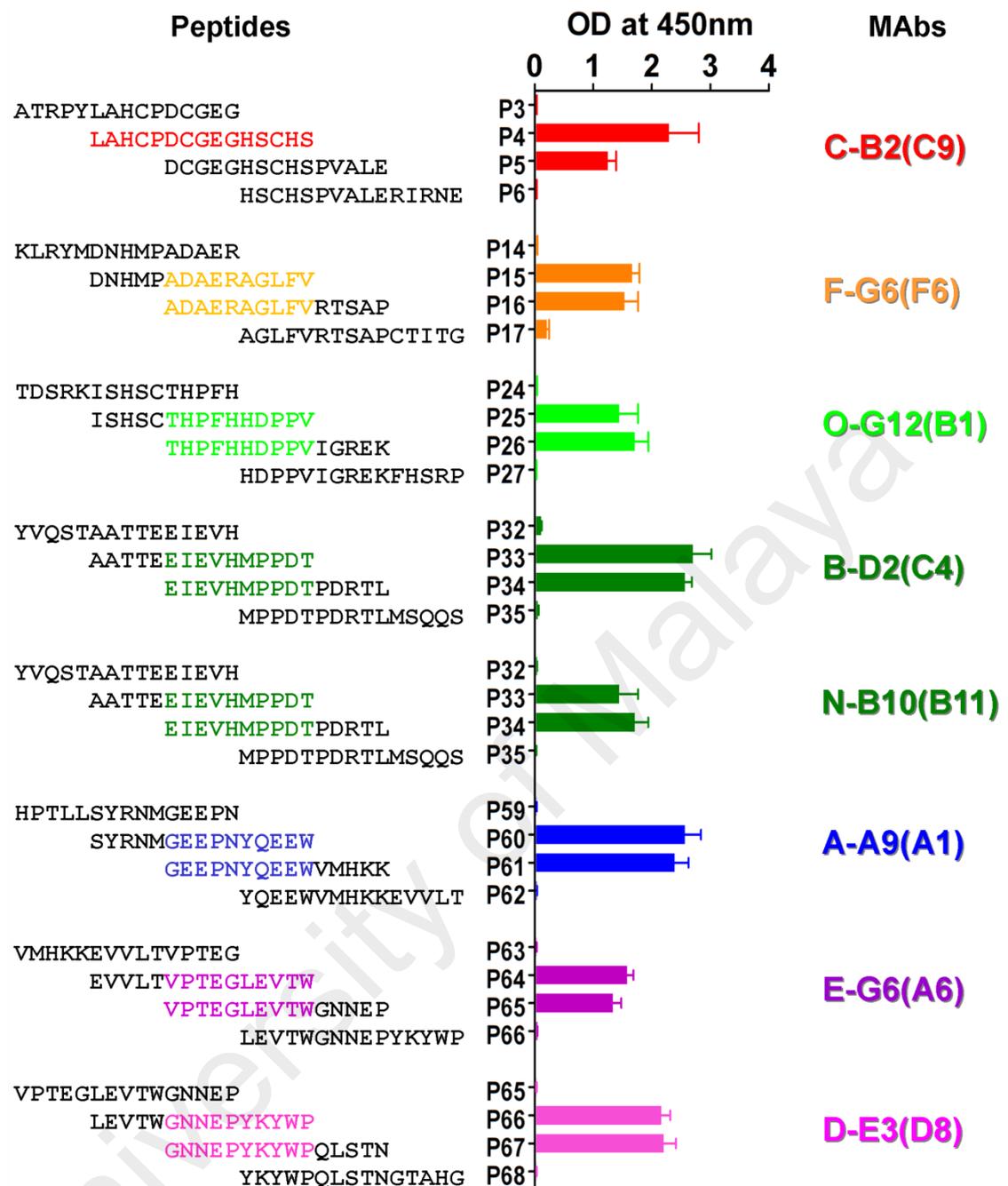
Figure 4.3: Epitope mapping of mAbs by overlapping synthetic peptide-based ELISA, using 1 µg/ml of each mAb. The coloured sequences indicate the respective epitope for each mAb. The assay was performed in triplicate and the error bars indicate the mean and SD.

4.1.4 Binding activity of CHIKV E2-reactive mAbs

Indirect immunofluorescence assay (IF), Western blotting and virus-based ELISA were employed to investigate the binding activity of the mAbs. CHIKV anti-E2 polyclonal antibodies served as positive control in all these assays. For the indirect IF, the use of all the mAbs led to staining of the CHIKV-infected BHK cells with varying intensity. MAbs B-D2(C4), N-B10(B11) and E-G6(A6) stained the infected cells strongly, while mAb C-B2(C9) stained the cells weakly. The mAbs bound to endogenous E2 viral glycoproteins in infected cells, but not to mock-infected cells (Figure 4.4A). Therefore, the mAbs are highly specific to CHIKV E2 antigen but not to other cellular elements.

In Western blotting analysis, the mAbs detected both E2 viral protein from CHIKV and rE2 with varying levels of binding activity (Figure 4.4B). However, when CHIKV antigen was blotted, an additional antigenic fragment at 40 kDa was detected by mAbs A-A9(A1), B-D2(C4) and N-B10(B11), but not the others. A similar observation was noted when polyclonal anti-CHIKV E2 antibody was used as the positive control, and this antigenic fragment could be a proteolytic product of the E2 glycoprotein.

Indirect virus-based ELISA was also performed to examine the reactivity and sensitivity of these mAbs in binding to native virions. All the mAbs bound to the virus in indirect ELISA, suggesting that their epitopes on the E2 glycoprotein could present on the external surface of the virion. Binding of the mAbs to virions exhibited a dose-dependent effect with amounts of mAbs ranging from 10^{-3} to 10^{3} ng (Figure 4.5). MAbs B-D2(C4) and A-A9(A1) showed the strongest reactivity of all the mAbs, and reached binding saturation at 100 ng of mAbs. The reactivity of all the mAbs from the 3 experiments (indirect IF, Western blot and ELISA) are summarised in Table 4.2.
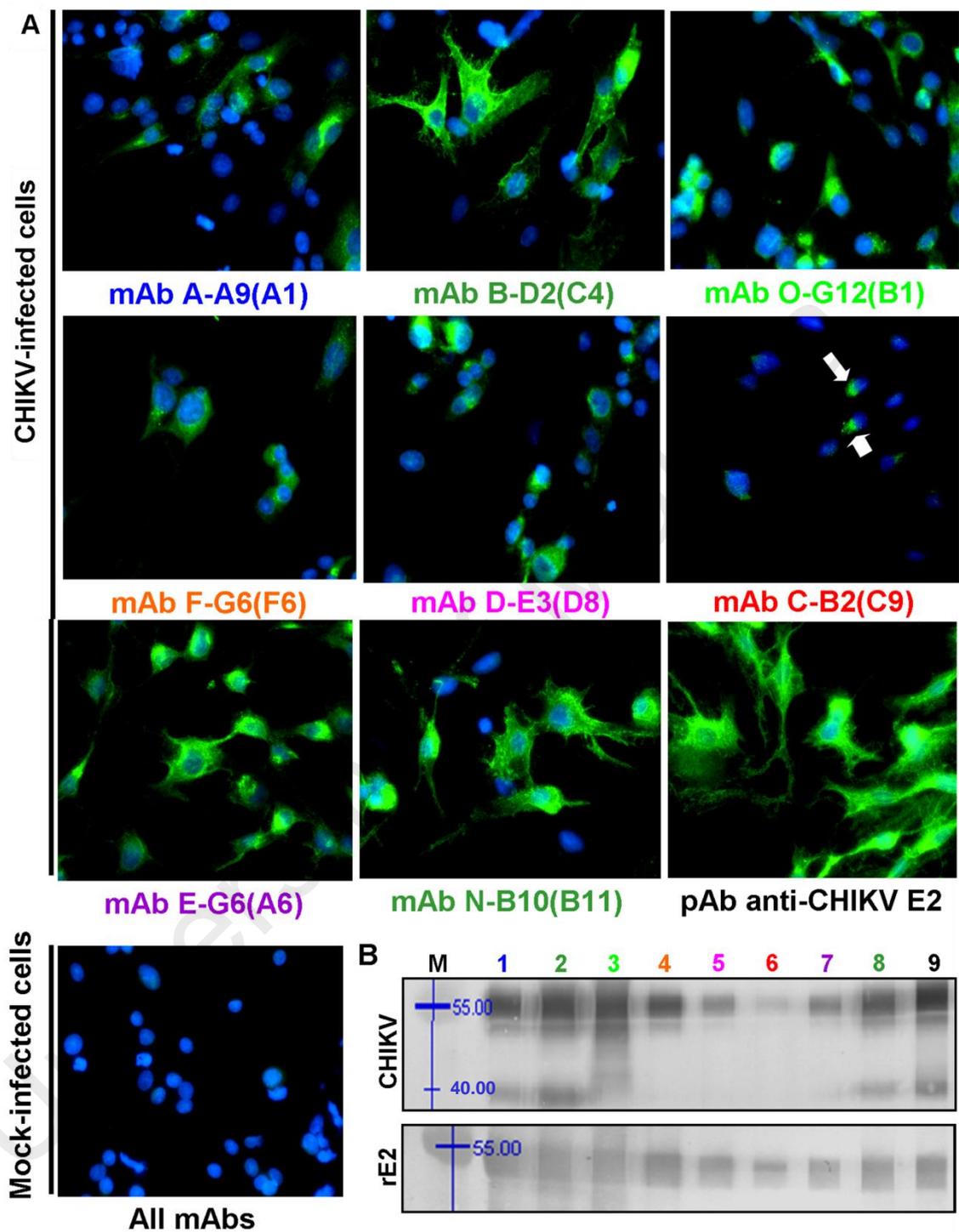
Figure 4.4: The reactivity of all the mAbs from indirect IF, Western blot and ELISA.

Figure 4.4, continued: The reactivity of all the mAbs from indirect IF, Western blot and ELISA. (A) Indirect immunofluorescence assay to investigate the binding of the mAbs in CHIKV-infected BHK-21 cells. Mock-infected BHK-21 cells served as a negative control. The presence of CHIKV antigen was detected as bright green fluorescence. MAb C-B2(C9) stained weakly as indicated by the white arrows. (B) Western blot showing binding of the mAbs to sucrose-purified CHIKV and recombinant E2 protein under denaturing conditions. Lane 1, mAb A-A9(A1); 2, mAb B-D2(C4); 3, mAb O-G12(B1); 4, mAb F-G6(F6); 5, mAb D-E3(D8); 6, mAb C-B2(C9); 7, mAb E-G6(A6), 8, mAb N-B10(B11) and 9, polyclonal anti-CHIKV E2. PageRuler Prestained protein ladder (Thermo Scientific) was used as the marker (M). All mAbs were tested at 5 µg/ml (except mAb N-B10(B11), which was tested at 1 µg/ml for indirect IF due to high background), while polyclonal anti-CHIKV E2 at 1:100 dilution served as a positive control in both experiments. Objective magnification: 20 ×
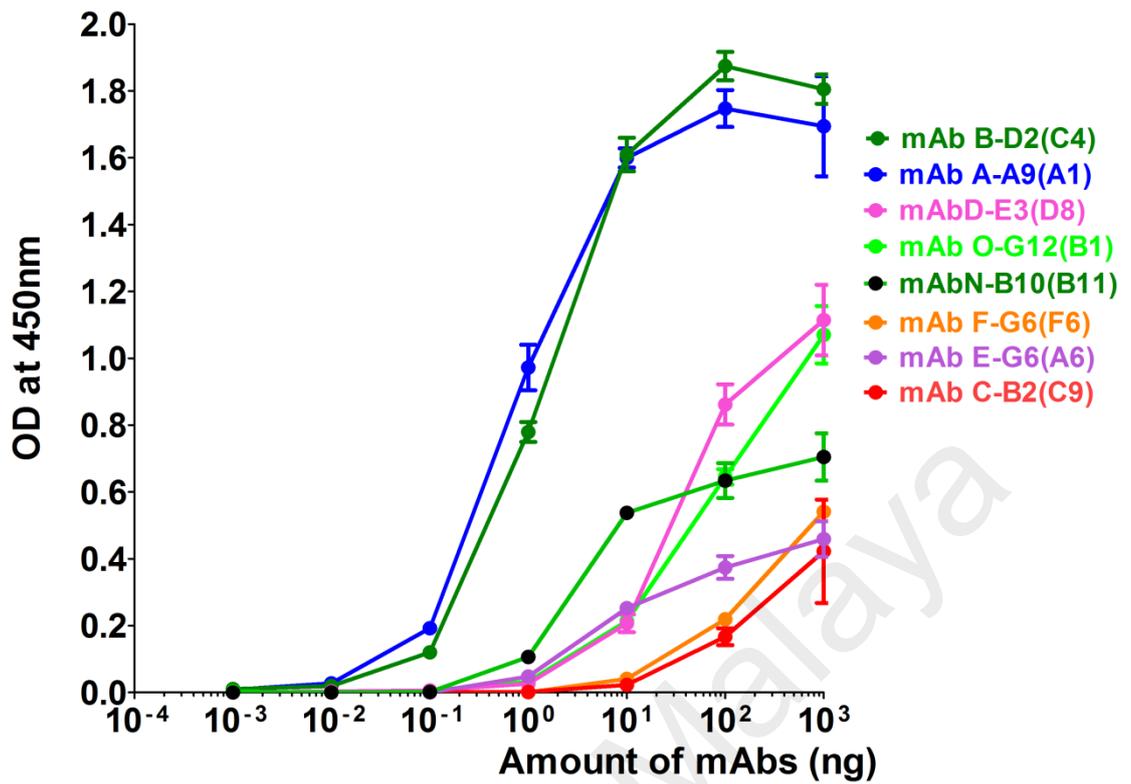
Figure 4.5: Binding activity of different quantities of mAbs against sucrose-purified CHIKV per well (400 ng) using ELISA. The assay was performed in triplicate and the error bars indicate the mean and SD.

4.1.5 Epitope localisation and alignment

Computational modelling was used to map the epitopes of mAbs bound to the native CHIKV particle (Figure 4.6A). Structural visualisation showed that some epitopes are exposed at the surface of the E2 glycoprotein, and some are buried in the E1/E2 heterodimer spikes (Figures 4.6B and 4.6C). Interestingly, the epitopes of the most reactive mAbs such as A-A9(A1), B-D2(C4) and N-B10(B11) are not readily exposed on the surface of the E1/E2 heterodimers.

The epitopes of the mAbs were compared with the sequences of a representative strain from each CHIKV genotype (MY/06/37348, Asian; S27, ECSA prototype; 37997, West African), as well as with other alphaviruses categorised under the Semliki Forest complex. MAbs C-B2(C9), B-D2(C4), E-G6(A6) and D-E3(D8) recognised epitopes which are well conserved within CHIKV in the 4 selected sequences and other published CHIKV sequences (134 sequences in total, data not shown) (Figure 4.7). However, epitopes from mAbs C-B2(C9) and B-D2(C4) are identical with the ONNV sequence. CHIKV epitopes otherwise have differences with the other alphaviruses of this complex.
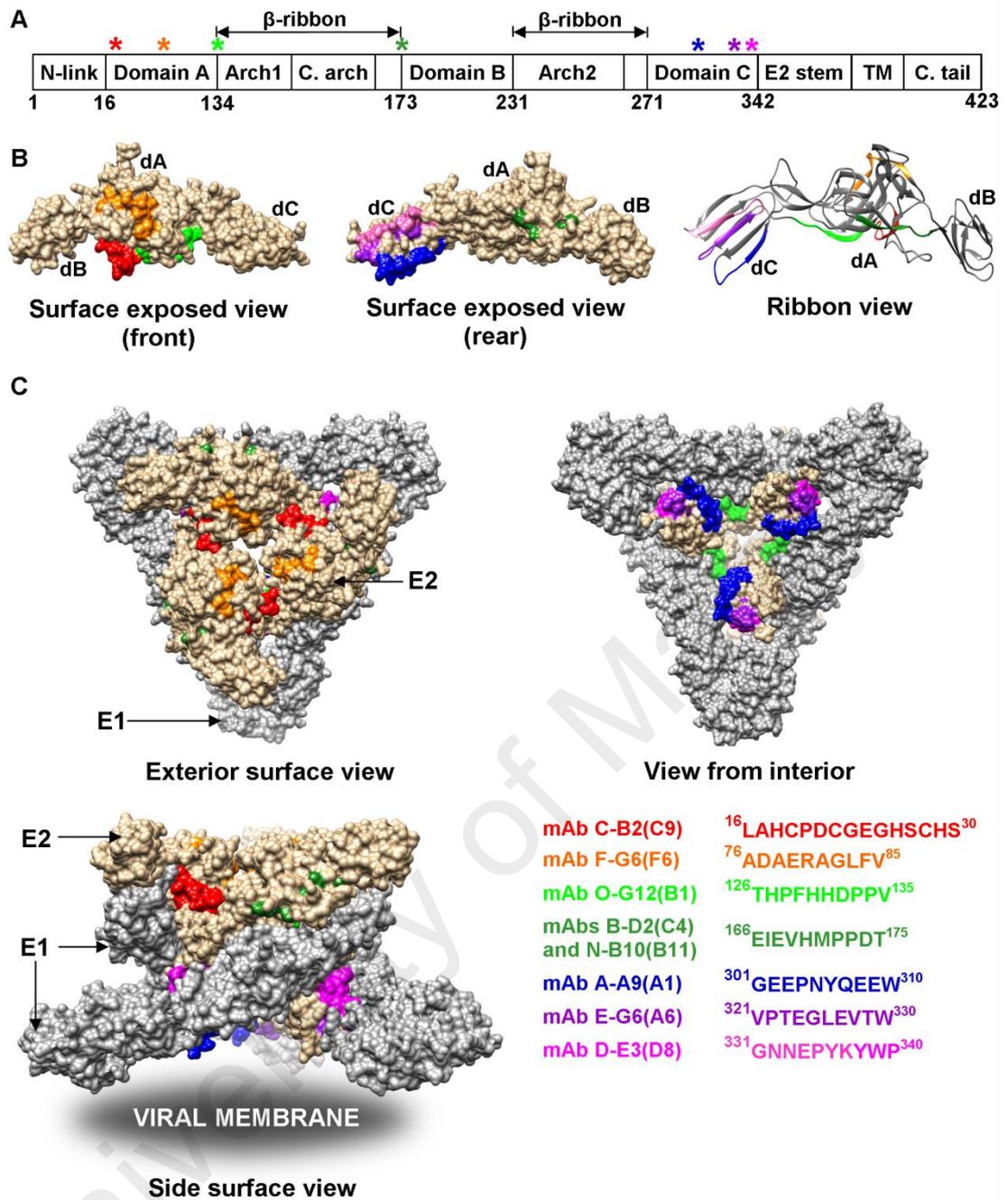
Figure 4.6: Localisation of the CHIKV E2 epitopes of the monoclonal antibodies.

Figure 4.6, continued: Localisation of the CHIKV E2 epitopes of the monoclonal antibodies. (A) Schematic diagram of the E2 protein showing the positions of the domains, with asterisks in different colours corresponding to the mapped epitopes. The numbers refer to the amino acid positions within the CHIKV E2 demarcating the domains. C. arch, central arch; TM, transmembrane; C. tail, cytoplasmic tail. (B) The coloured regions on the structure of the E2 glycoprotein correspond to the listed epitopes. dA, domain A; dB, domain B; dC, domain C. (C) Localisation of epitopes on triplets of E1 (in grey) and E2 (in beige) of the E1-E2 heterodimer complex (based on PDB ID 3J2W).

MAb clone	C-B2(C9)$_{16-30}$	F-G6(F6)$_{76-85}$	O-G12(B1)$_{126-135}$	N-B10(B11)$_{166-175}$
FN295485/CHIKV/08/065	LAHCPDCGEGHSCHS	ADAERAGLFV	THPFHHDPPV	EIEVHMPPDT
FN295483/CHIKV/06/37348	....	....	....	....
AF369024/CHIKV/S27	....	...G......	....	....
AY726732/CHIKV/37997	....	........L.	.......E...	....
AAC97205/ONNV	....	V..D.S....	MH.......L	....
AAA47404/RRV	..Y.A...D.YF.Y.	QESK.DS.R.	KVQYK...LP	..DM.T...I
AAO33337/SAGV	..Y.A...D.QF.Y.	KE.N.DS.Q.	KVQYK...AP	..DM.T...I
CAA27742/SFV	I.Y.A...A......	EN.V.SS.K.	RIQY....QP	..DM......
AAO33335/MAYV	V.Y.A...M......	.E.A.ST.K.	RIAYR.EQRL	..DM.....V
AAO33343/MIDDV	I.Y.S...N.QF.Y.	E.TD.NS.E.	RIAYK.RV..	...M.VAHDV
AAO33347/BFV	IGW.A...LAG..P.	QE...ST.S.	RT..S.K.RF	..TM.V...V
AAT96380/EEEV	I.D..N..HSR-.D.	KSIKIDN.H.	.VAHKVEFRP	YV.M.Q.GLV

MAb clone	A-A9(A1)$_{301-310}$	E-G6(A6)$_{321-330}$ D-E3(D8)$_{331-340}$
FN295485/CHIKV/08/065	GEEPNYQEEW	VPTEGLEVTWGNNEPYKYWP
FN295483/CHIKV/06/37348	....	....
AF369024/CHIKV/S27	....	....
AY726732/CHIKV/37997	.Q....H...	....
AAC97205/ONNV	.RI.D.H...	..A........D......
AAA47404/RRV	.A..HPY...	.TE..I.YQ....P.VRL.A
AAO33337/SAGV	.AD.RPY...	.TED.I.YR....P.VRL.A
CAA27742/SFV	..D.Q.H...	.PVD.M.YH....D.VRL.S
AAO33335/MAYV	.R..VFD.R.	.PV..V.YR...HK.QRL.S
AAO33343/MIDDV	..K.EHHT..	.PE....YT...HA.VRL.A
AAO33347/BFV	.KD.SHSQ..	.GA..V.YV....N.VRL.A
AAT96380/EEEV	.SDA.PTRQ.	.TG....Y....HP.KRV.A

Figure 4.7: Alignment of the mAb epitopes sequences with E2 sequences of CHIKV strains of different genotypes and other alphaviruses from the Semliki Forest complex. The E2 sequence from ECSA genotype CHIKV/08/065 was used as the reference sequence. Amino acid differences between CHIKV and other alphaviruses are highlighted in grey, while differences within CHIKV isolates are highlighted in black.

4.2 Characteristics of cross-neutralising antibody efficacy and epitopes in human immune sera
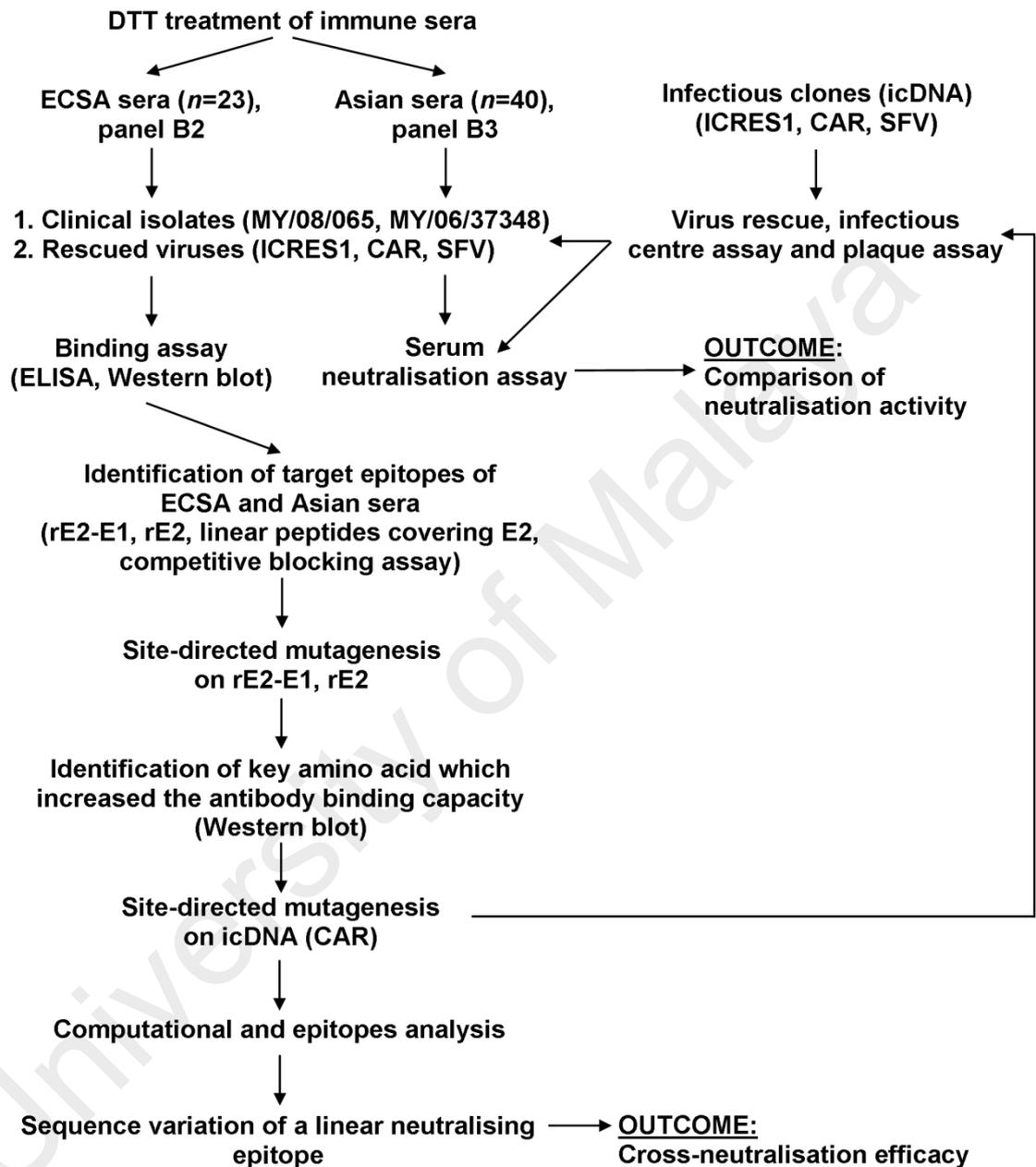
Figure 4.8: Flow chart for objective 2, characteristics of cross-neutralising antibody efficacy and epitopes of antibodies in human immune sera.

4.2.1 Neutralising and binding capacity of ECSA and Asian sera against clinical isolates and rescued viruses

A sensitive neutralisation assay was developed with the use of previously characterised mAb B-D2(C4) to compare the neutralising capacity of the different sera panels against CHIKV isolates MY/08/065 (ECSA) and MY/06/37348 (Asian). For this objective, ECSA serum panel was collected from a nationwide ECSA outbreak during 2008 and 2010 from patients admitted 1 to 6 months previously to UMMC with CHIKV. The Asian serum panel was collected from individuals 1 year after they were affected by the Asian outbreak in 2006 in Bagan Panchor, Perak, before the introduction of ECSA genotype into Malaysia. Both ECSA and Asian serum panels were able to neutralise both viruses (Figures 4.9 and 4.10). The heat-inactivated intact sera (containing both IgM and IgG) and DTT treated sera (containing only IgG) from both panels had similar neutralising capacity against ECSA genotype MY/08/065 (Figure 4.11). ECSA sera demonstrated strong neutralising capacity against ECSA genotype CHIKV compared to Asian genotype CHIKV (Figure 4.12A), with the NT_{50} against MY/08/065 of a median 2.67 (range, 1.40 - 4.61) times greater than the NT_{50} against MY/06/37348 (Figure 4.12B). Unexpectedly, Asian sera demonstrated better neutralising capacity against ECSA genotype CHIKV compared to Asian genotype CHIKV (Figure 4.12A), with the NT_{50} against MY/08/065 of a median 1.44 (range, 0.70 – 3.19) times greater than the NT_{50} against MY/06/37348 (Figure 4.12B). The greater neutralising capacity corresponded to stronger antibody binding to ECSA genotype MY/08/065 compared to Asian genotype MY/06/37348 by quantitative ELISA (Figure 4.12C). To test if this finding is consistent, neutralisation assay was performed using different rescued viruses from icDNA of ECSA and Asian genotypes. Again, both ECSA and Asian sera demonstrated better neutralising capacity against the ECSA genotypeICRES1 CHIKV compared to the Asian genotypeCAR CHIKV (Figure

4.12D).

Immunoblotting showed stronger reactivity of serum with the whole viral antigen (with a band of about 50 kDa consistent with E1 or E2, a known immunodominant antigen in alphaviruses) and recombinant E2 glycoprotein of similar size derived from ECSA, compared to the Asian genotype. Under non-reducing conditions, ECSA sera had stronger antibody binding to the homotypic CHIKV MY/08/065, ICRES1 and recombinant E2 glycoprotein (rE2) (Figures 4.12E and 4.12F). Asian sera bound similarly to both genotypes of viruses (clinical isolates and rescued viruses), and more strongly to rE2 glycoprotein of heterotypic MY/08/065 of ECSA genotype (Figures 4.12E and 4.12F). Under reducing conditions, both sets of sera retained stronger binding to ECSA CHIKV and rE2 of ECSA CHIKV. Both sets of sera had a similar proportion of total antibodies binding to rE2 (median 50%, range 20–63% for ECSA serum; median 50%, range 16–63% for Asian serum) (Figure 4.12G), and these percentages suggest that antibodies also target sites other than E2. Taken together, CHIKV serum from both ECSA and Asian panels show strong neutralising capacity and binding to CHIKV, particularly of the ECSA genotype, and the epitopes may be presented as part of the conformational E1-E2 glycoprotein and/or as linear determinants in the E2 glycoprotein.
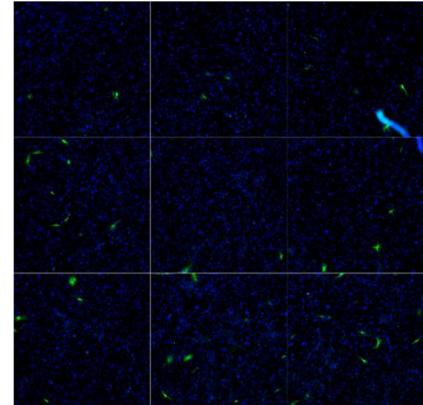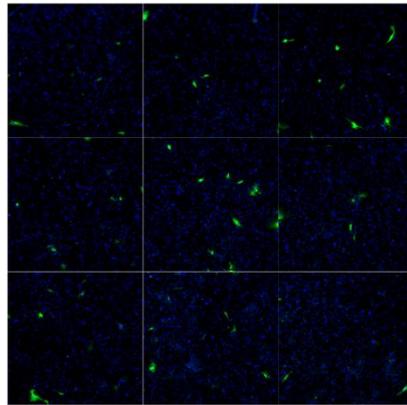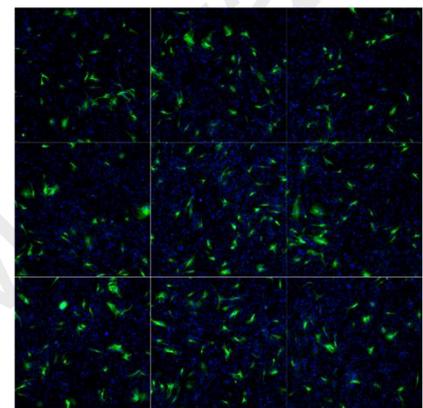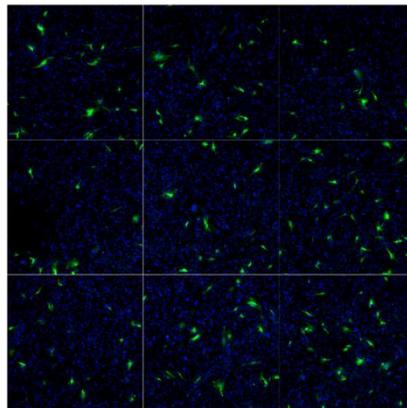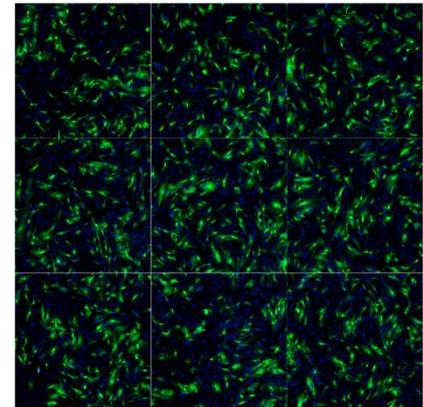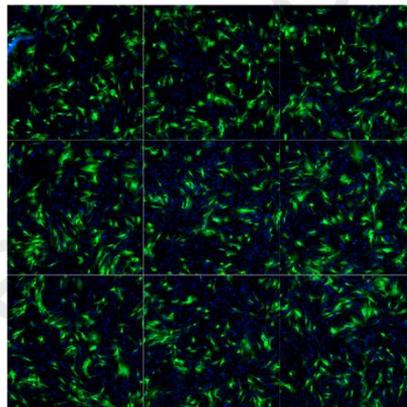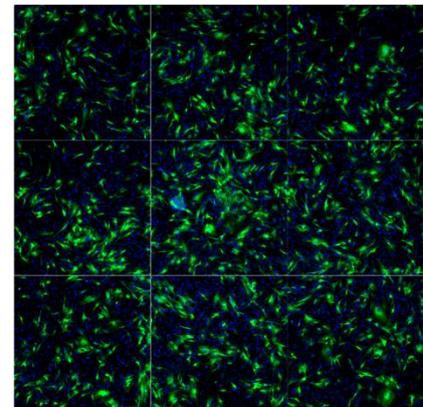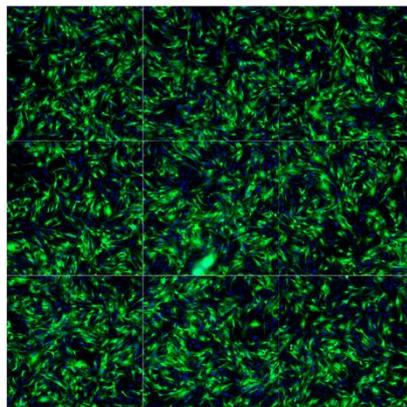
Figure 4.9: Neutralisation of ECSA and Asian CHIKV genotypes using the ECSA sera panel.

Figure 4.9, continued: Neutralisation of ECSA and Asian CHIKV genotypes using the ECSA sera panel. Representative acquired immunofluorescence microscopic images of pooled serum at dilutions of 1:100, 1:400, and 1:1600, and virus control against clinical CHIKV isolates MY/08/065 (ECSA) and MY/06/37348 (Asian). CHIKV was stained using mAb B-D2 (C4) as the primary antibody. Each image contains 9 combined fields within a well (96-well format). Objective magnification: 5 ×.
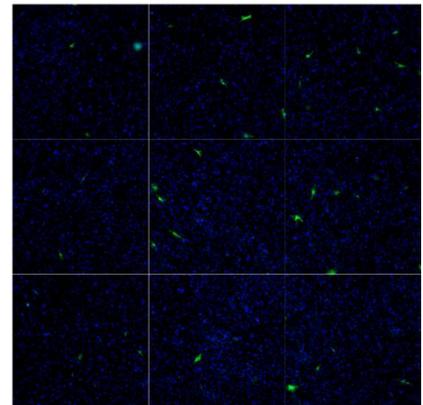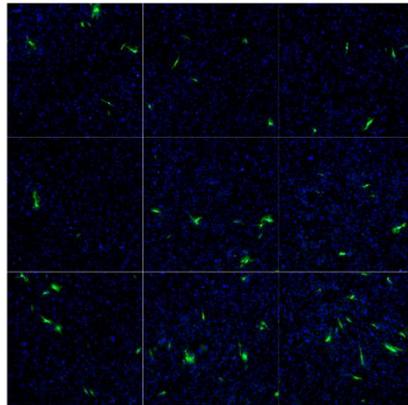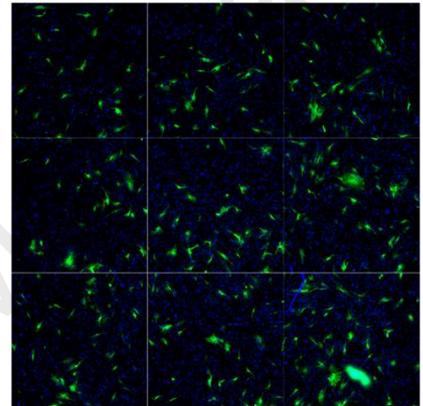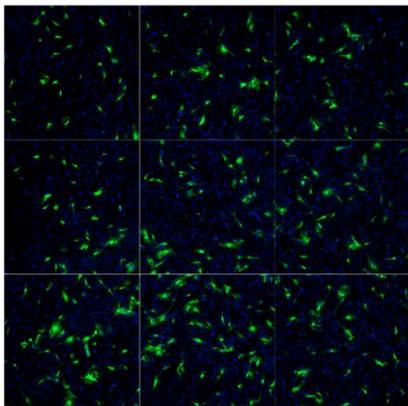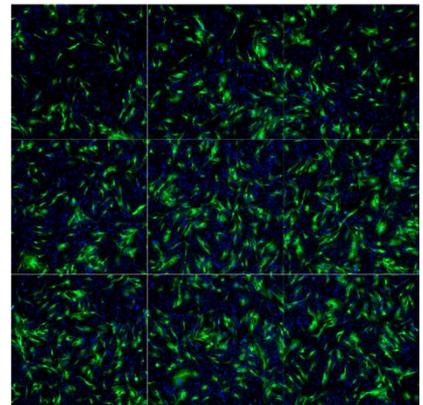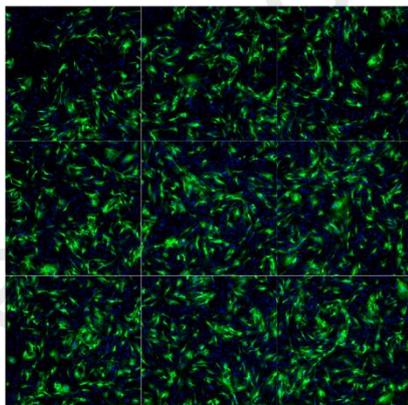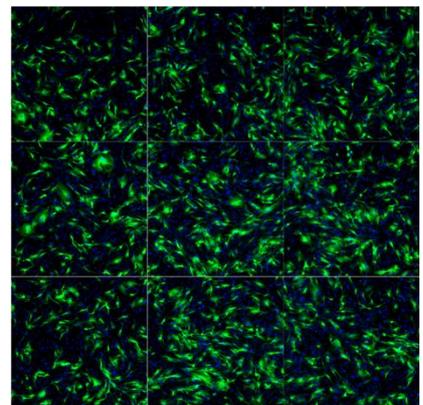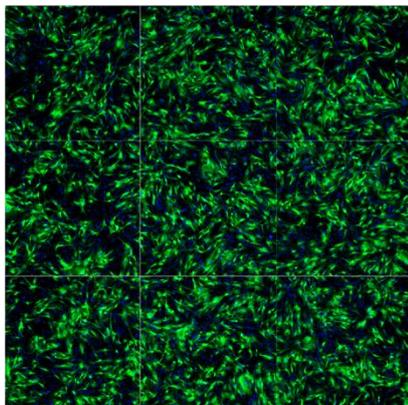
Figure 4.10: Neutralisation of ECSA and Asian CHIKV genotypes using the Asian sera panel.

Figure 4.10, continued: Neutralisation of ECSA and Asian CHIKV genotypes using the Asian sera panel. Representative acquired immunofluorescence microscopic images of pooled serum at dilutions of 1:100, 1:400, and 1:1600, and virus control against clinical CHIKV isolates MY/08/065 (ECSA) and MY/06/37348 (Asian). CHIKV was stained using mAb B-D2 (C4) as the primary antibody. Each image contains 9 combined fields within a well (96-well format). Objective magnification: 5 ×.
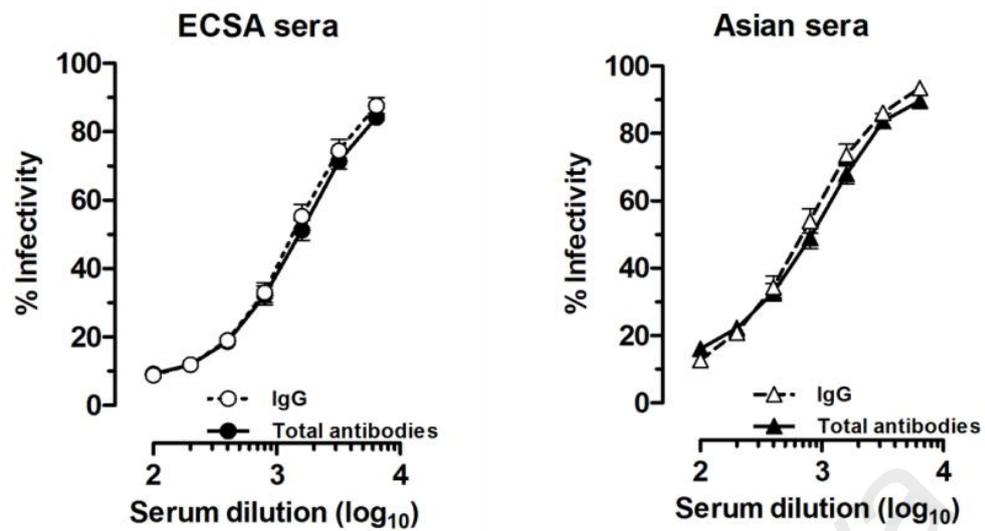
Figure 4.11: Comparison of neutralising capacity of heat-inactivated intact sera and DTT treated sera. DTT treated sera (containing IgG only) had similar neutralising capacity to intact sera, which contains a mixture of IgG and IgM. All sera were assayed up to 1:6400 dilution. The neutralisation data was based on experiments performed against ECSA CHIKV (strain MY/08/065). Data are presented as means ± SEM; $n=23$ for ECSA sera, $n=40$ for Asian sera.
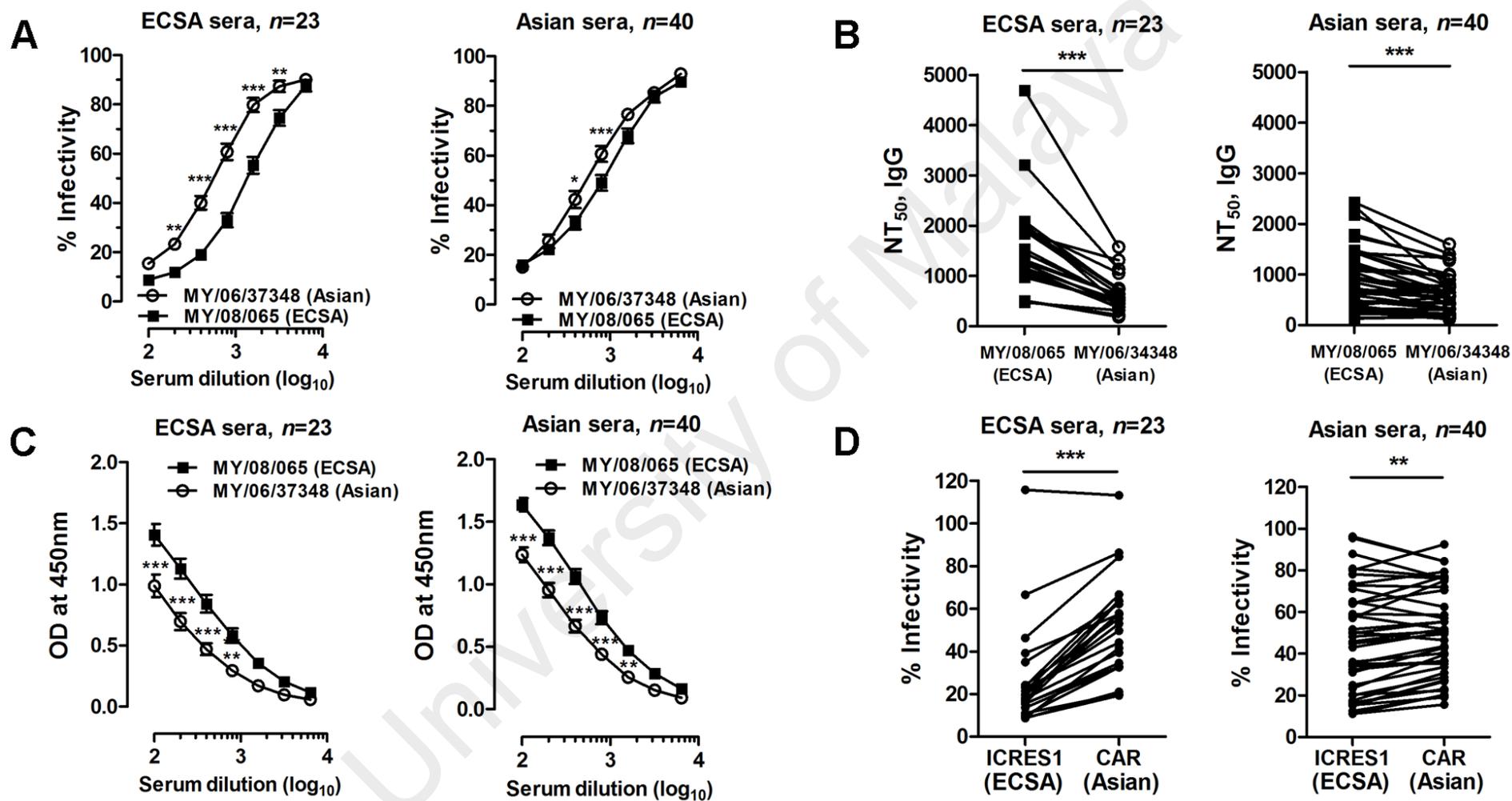
Figure 4.12: Differential neutralisation capacity and antibody binding properties of immune sera against ECSA and Asian CHIKV.

Figure 4.12, continued: Differential neutralisation capacity and antibody binding properties of immune sera against ECSA and Asian CHIKV.

(A) Sera collected from ECSA and Asian CHIKV outbreaks have differential neutralising capacity against MY/08/065 (ECSA) and MY/06/37348 (Asian) isolates of CHIKV. Results are expressed as a percentage of virus control. *P<0.05, **P<0.01, ***P<0.001, two-way ANOVA with Bonferroni multiple comparisons test. Data are presented as means ± SEM from 23 (ECSA) and 40 (Asian) individual serum samples. (B) Neutralisation titres (NT_{50}) of DTT treated sera were determined by non-linear regression fitting. ***P<0.001, Wilcoxon matched-pairs signed rank test. (C) ECSA and Asian sera were cross-screened against both CHIKV isolates (10^5 pfu, treated with 1% Triton X-100) in ELISA at different serum dilutions. Data are presented as mean ± SEM from 23 (ECSA) and 40 (Asian) individual serum samples. **P<0.01, ***P<0.001, two-way ANOVA with Bonferroni multiple comparisons test. (D) Neutralisation was performed against different strains of CHIKV, ICRES1 (ECSA) and CAR (Asian), which were rescued from icDNA CHIKV, at a serum dilution of 1:800. **P<0.01, ***P<0.0001, Wilcoxon matched-pairs signed rank test.
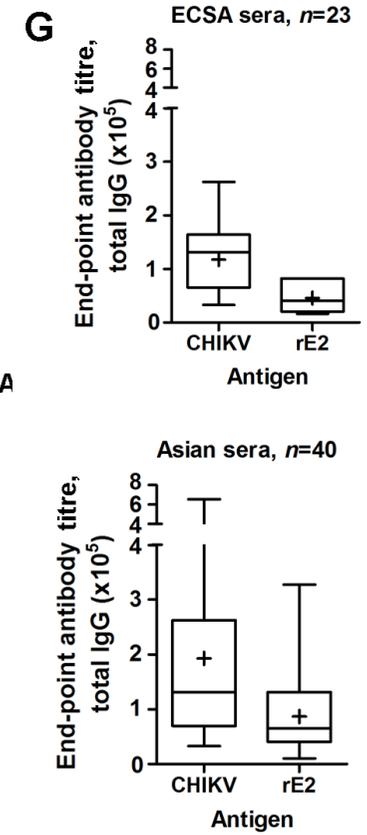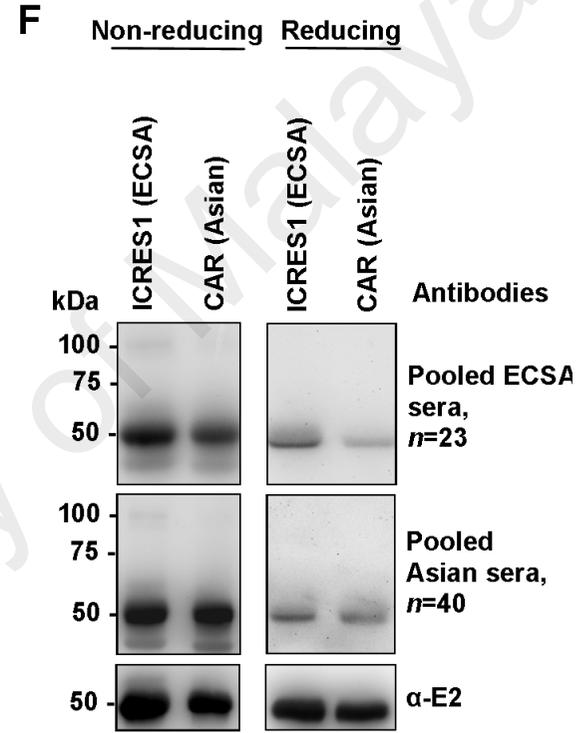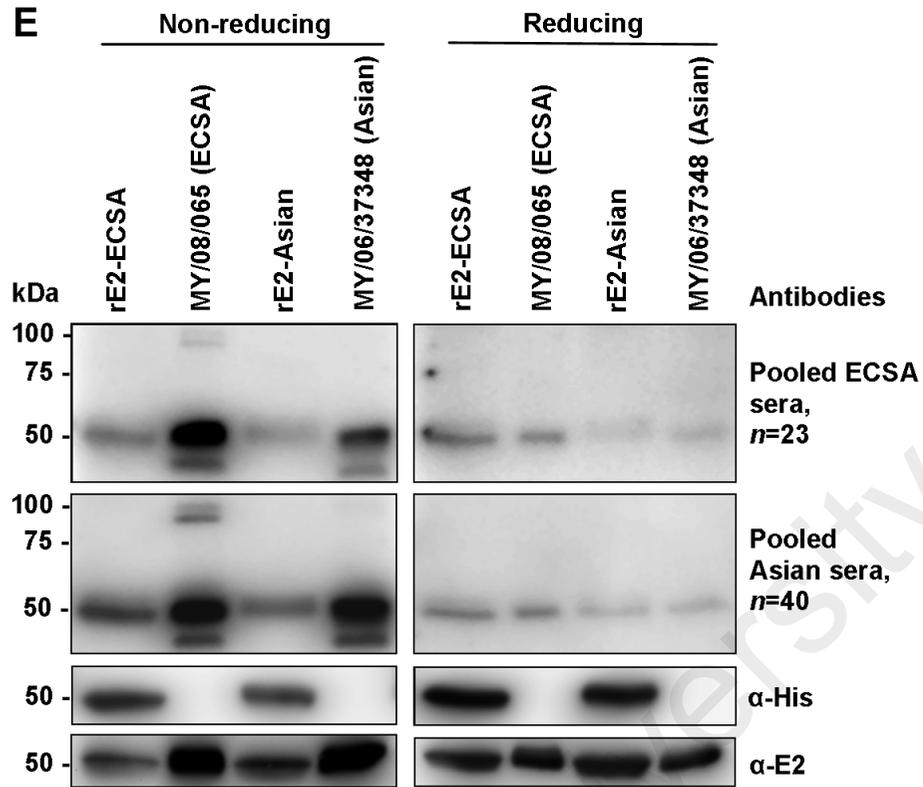
Figure 4.12, continued: Differential neutralisation capacity and antibody binding properties of immune sera against ECSA and Asian CHIKV.

Figure 4.12, continued: Differential neutralisation capacity and antibody binding properties of immune sera against ECSA and Asian CHIKV.
(E) Immunoblotting was performed under non-reducing and reducing conditions against rE2 and CHIKV from ECSA genotype MY/08/065 and Asian genotype MY/06/37348. Mouse anti-His was used as a control and pooled sera were diluted at 1:1000. (F) Immunoblotting was performed under non-reducing and reducing conditions against ICRES1 and CAR. Mouse anti-E2 was used as a control and pooled sera were diluted at 1:1000. (G) Antibody titres of CHIKV immune sera (IgG) were quantified by end-point titre ELISA using whole virus antigen and recombinant E2 (rE2) derived from ECSA genotype MY/08/065. Middle line, median; plus sign, mean; upper and lower boundaries of the box, inter-quartile range; whiskers, range of values.

4.2.2 Neutralising epitope targets in ECSA and Asian sera

To determine if CHIKV immune serum targets E1, recombinant E1 glycoprotein (rE1) was probed in an ELISA with serially diluted pooled sera, and signal was detected at low serum dilutions from 1:100 to 1:800 (Figure 4.13A). A competitive protein blocking assay was performed, and blocking of ECSA and Asian sera with native rE1 alone did not significantly alter the neutralising capacity (Figure 4.13B). However, when the sera were blocked by a mixture of rE1 and rE2, significant increases of infectivity were observed in both panels of sera compared to unblocked sera or sera blocked by either rE1 or rE2 alone. It was hypothesised that antibodies may target conformational epitopes on E1 and E2 glycoproteins together. To test this hypothesis, 2 chimeras were constructed in which the ecto-domain regions of the E2 and E1-E2 glycoproteins were swapped with those of Semliki Forest virus (SFV) (Figure 4.13C). Both sets of sera demonstrated a low degree of cross-neutralisation against SFV, another alphavirus which is a member of the same antigenic complex as CHIKV (Figure 4.14). At 1:100 serum dilution, loss of neutralising effect for both sets of sera was observed when CHIKV E2 was replaced with SFV E2. Furthermore, in ECSA serum, loss of neutralisation activity was much higher against the chimera with E1-E2 from SFV compared to the chimera with SFV E2 alone (Figure 4.13C). This provides further evidence that neutralising antibodies are not solely targeting E2, but are also targeting epitopes spanning E1-E2 glycoproteins, particularly in ECSA serum. Alternatively, E1 may affect the conformation of E2 and alter its epitopes.
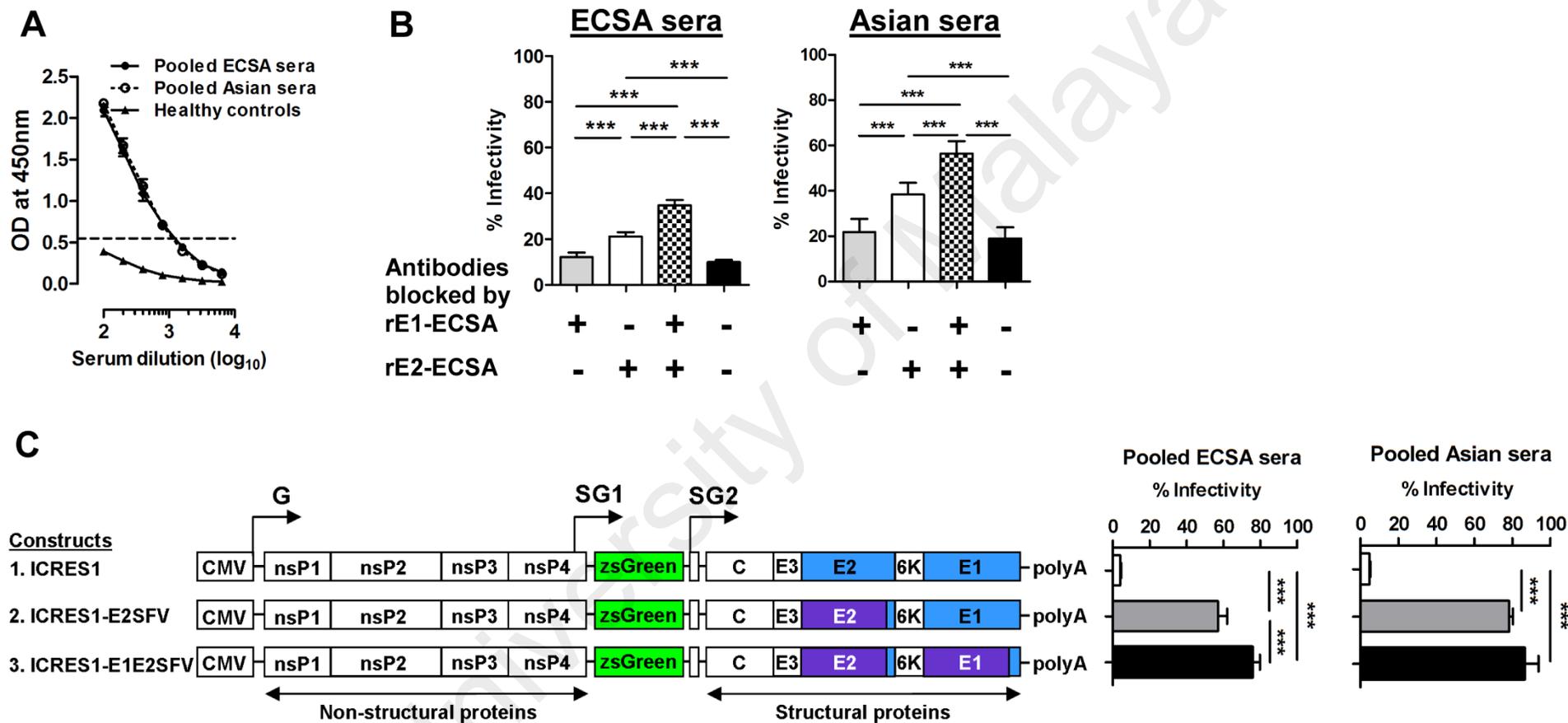
Figure 4.13: Neutralising antibodies of immune sera interact with the epitopes on E2 and E1-E2 glycoproteins.

Figure 4.13, continued: Neutralising antibodies of immune sera interact with the epitopes on E2 and E1-E2 glycoproteins. (A) CHIKV antibody titre against recombinant E1 glycoprotein (100 ng) was determined in ELISA. The ELISA was performed at different serum dilutions using pooled sera. The dotted line represents the cut-off value (mean + 3SD) derived from healthy controls. (B) Competitive blocking assay was performed at 1:200 dilution in triplicate using 7 pools of ECSA and Asian sera, with similar neutralising titres in each pool. Data are expressed as percentages of infectivity of an infection control and are presented as means ± SEM. ***$P < 0.001$, repeated measures ANOVA with Bonferroni multiple comparison test. (C) Schematic diagram showing the construction of chimera viruses with replacement of E2 or E1/E2 from SFV into the CHIKV ICRES1 backbone. Neutralisation was performed against the chimera constructs and the percentage of infectivity was compared to that obtained with ICRES1. Data are represented as means ± SD from 4 independent experiments at a serum dilution of 1:100 (pooled sera). ***$P < 0.01$, Kruskal-Wallis test. G, genomic promoter; SG, subgenomic promoter.
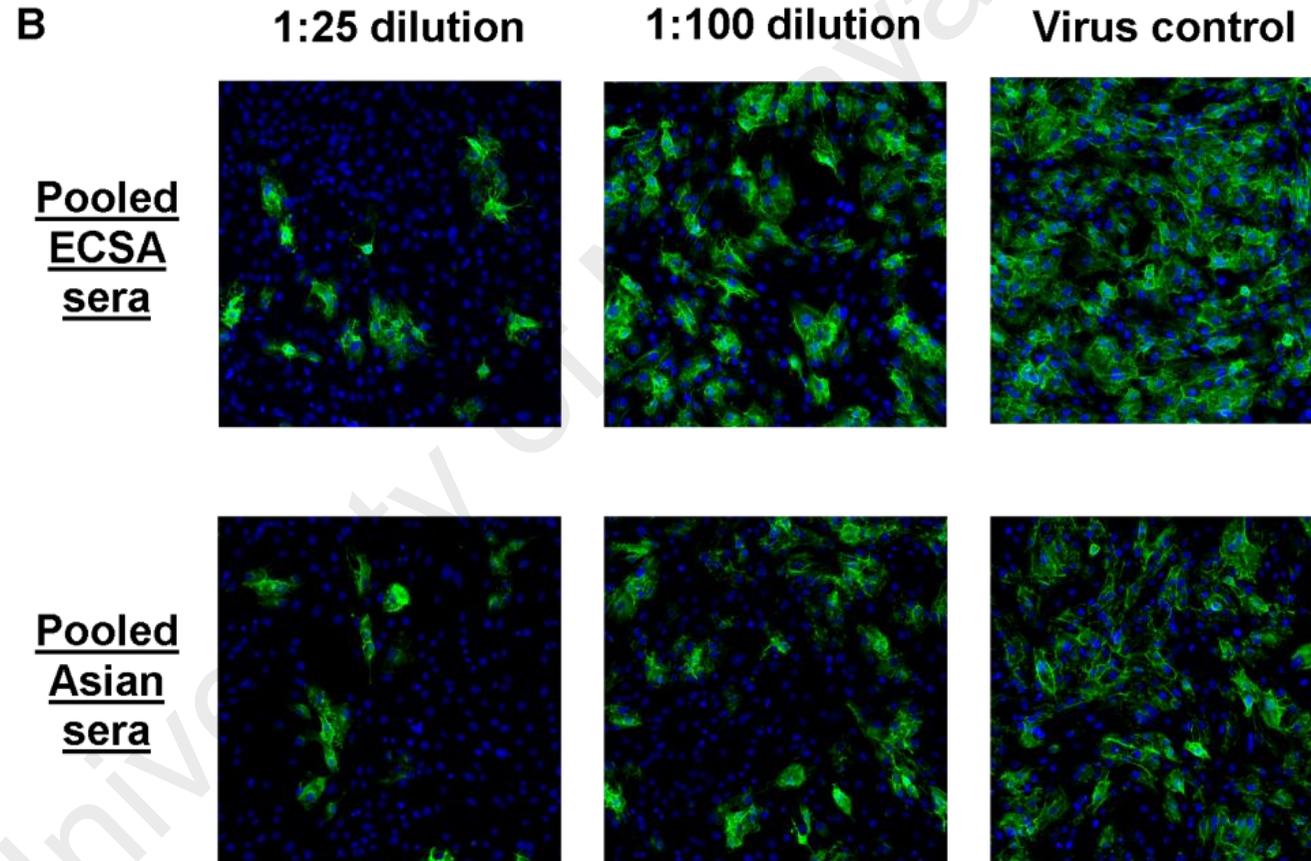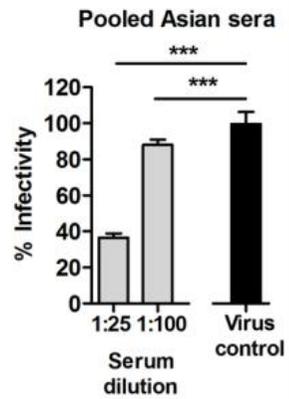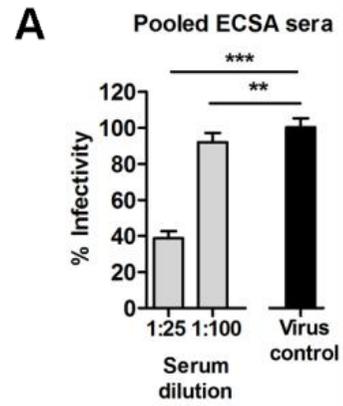
Figure 4.14: Neutralisation of CHIKV immune individuals against Semliki Forest virus (SFV).

Figure 4.14, continued: Neutralisation of CHIKV immune individuals against Semliki Forest virus (SFV). (A) Neutralisation was performed against SFV at 1:25 and 1:100 serum dilutions using pooled sera. Data are expressed as percentages of infectivity over infection control and are presented as means ± SD from 3 independent experiments. **$P<$ 0.01, ***$P<0.001$, Mann-Whitney U test relative to virus control. (B) Representative acquired immunofluorescence microscopic images of pooled serum at dilutions of 1:25 or 1:100 and virus control against SFV rescued from icDNA SFV6. Objective magnification: 10 ×.

4.2.3 Correlation between antibody titre and neutralisation titre

The amount of antibodies present in immune individuals was then correlated with neutralising titre against ECSA genotype MY/08/065. High titres of CHIKV-specific antibodies in Asian sera, particularly anti-E2 antibodies, were correlated with high neutralising titres (Figure 4.15). However, no correlation was noted for ECSA sera. This lack of correlation between anti-E2 antibodies and neutralising antibodies supports the earlier finding that ECSA immune serum targets sites other than E2 alone.

Whole CHIKV antigen (MY/08/065)

rE2
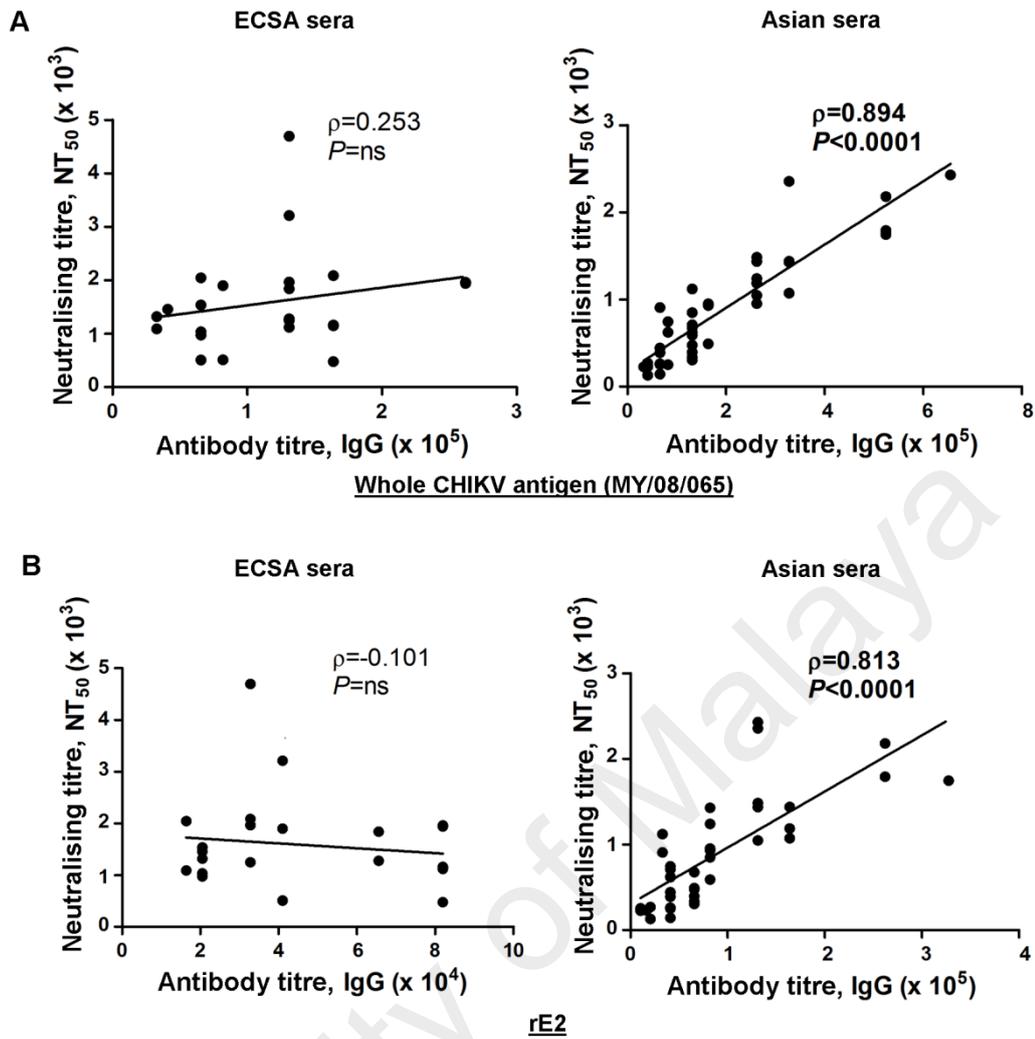
Figure 4.15: High titre of CHIKV-specific antibodies in Asian sera correlated with high neutralising titres. The relationships between NT_{50} and antibody titres against (A) ECSA genotype MY/08/065 and (B) recombinant E2 glycoprotein were assessed. Spearman's rank correlation coefficients (ρ) and *P*-values are shown. *ns,* not significant.

4.2.4 E1-E211K amino acid change in neutralisation activity of ECSA serum

To further determine the importance of conformational epitopes resulting from interactions between E1 and E2, four sets of fusion E1-E2 glycoproteins were constructed. Each hybrid fusion protein contained E1 and E2 sequences from either MY/06/37348 (ECSA) or MY/08/065 (Asian), transiently expressed as secreted native recombinant proteins in insect cells (Figure 4.16A). The antibody binding capacity of ECSA sera against fusion E1-E2 proteins significantly increased when either the E1 or E2 sequence was changed from that of Asian genotype MY/06/37348 to that of ECSA genotype MY/08/065, as shown in immunoblotting (Figure 4.16B) and quantitative ELISA (Figure 4.16C). Asian sera had almost equal antibody binding capacity for the 4 fusion glycoproteins, suggesting that Asian serum was not sensitive to sequence changes in E1-E2 glycoproteins under non-reducing conditions, rather that it was sensitive to sequence changes in E2 glycoprotein under reducing conditions (Figures 4.12E and 4.12F). This data shows that the greater binding and neutralisation of the ECSA isolate MY/08/065 by ECSA sera (Figures 4.12A, 4.12C and 4.12D) is due to critical conformational epitopes on the E1-E2 heterodimer, which are sequence-dependent.

There are 10 amino acids differences in E1 between the strains of ECSA (MY/08/065 and ICRES1) and Asian (MY/06/37348 and CAR) genotypes of CHIKV used in this study (Figure 4.16D). Using the fusion rE2-E1-Asian construct as a template, site-directed mutagenesis was performed independently to replace each amino acid of Asian origin with the corresponding ECSA residue, and the proteins were expressed in insect cells. The antibody binding significantly increased with amino acid changes at A145T, E211K, A226V and M269V, in comparison to hybrid rE2$_{Asian}$-E1$_{ECSA}$ recombinant proteins (Figure 4.16E). Neutralising capacity of serum was increased against a recombinant virus carrying E1-211K (compared to the parental virus clone CAR), and

slightly decreased against a virus with the E1-145T change (Figures 4.16F and 4.16G). The critical 211K amino acid is localised at the surface of the E1-E2 heterodimer (Figure 4.16H).
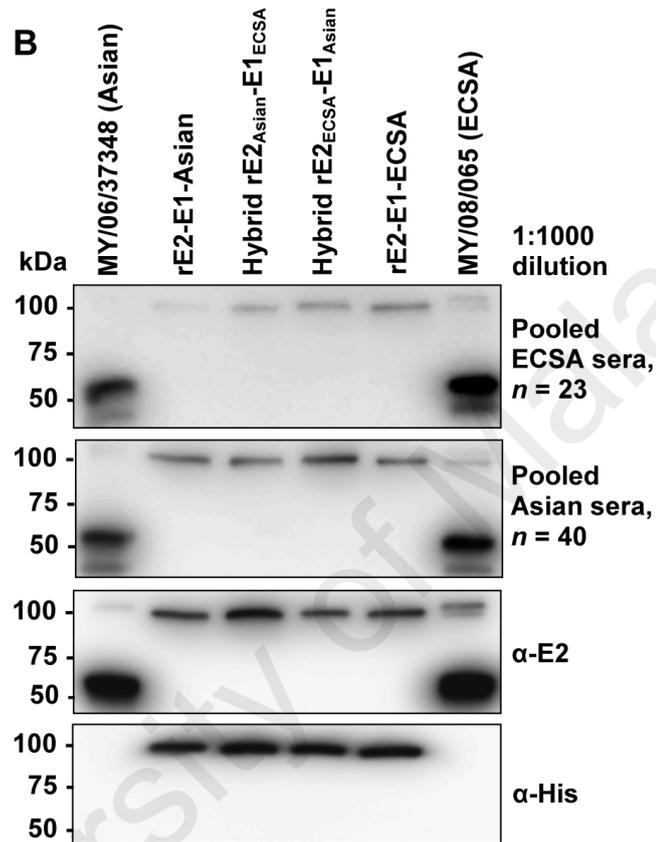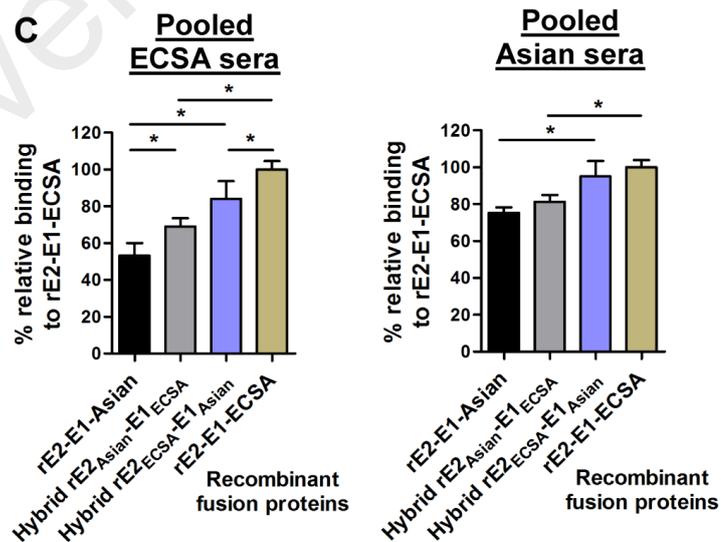
Figure 4.16: Neutralisation activity of ECSA serum is enhanced against CHIKV with the E1-E211K amino acid change.

Figure 4.16, continued: Neutralisation activity of ECSA serum is enhanced against CHIKV with the E1-E211K amino acid change. (A) Schematic diagram showing the generation of fusion recombinant E2 (amino acids 1-362) and recombinant E1 (amino acids 1-412) with a 16 residue linker which has glycine/ serine spacers and octa-histidine sequence. The rE2 and rE1 in each fusion protein are from either MY/08/065 (ECSA) or MY/06/37348 (Asian) virus isolates. (B) Immunoblotting was performed under non-reducing condition. Mouse anti-E2 and mouse anti-His monoclonal antibodies were used as controls. (C) The relative binding capacity of ECSA and Asian sera (1:1000 dilution) with the fusion E2-E1 proteins were determined by ELISA as (OD samples/mean OD samples tested with rE2-E1-ECSA) $\times$ 100. Data are presented as means $\pm$ SD (n= 4). *P<0.05, Mann-Whitney U test.

D

	I	II	FL	II	I	II	I	III		E1 stem
1	36	83	101	13	168		272	293	393	412
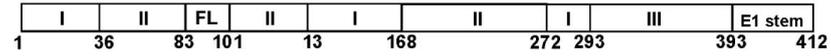
ICRES1 (ECSA)	N	A	T	K	A	V	V	E	P	L
MY/08/065 (ECSA)	N	A	T	K	A	V	V	E	P	L
Amino acid position	72	98	145	211	225	226	269	284	304	397
MY/06/37348 (Asian)	S	T	A	E	S	A	M	D	S	P
CAR (Asian)	S	T	A	E	S	A	M	D	S	L

E

Non-reducing

1:1000 dilution

kDa	S72N	T98A	A145T	E211K	S225A	A226V	M269V	rE2-E1-Asian	Hybrid rE2$_{Asian}$-E1$_{ECSA}$	
100										ECSA sera
100										α-His

F

% Infectivity

CAR, CAR-E1-A145T (*), CAR-E1-E211K (***), CAR-E1-A226V, CAR-E1-M269V

G

% Infectivity (***)

CAR, CAR-E1-E211K

H

E2
E1
K211

Side surface view

Figure 4.16, continued: Neutralisation activity of ECSA serum is enhanced against CHIKV with the E1-E211K amino acid change.

136

Figure 4.16, continued: Neutralisation activity of ECSA serum is enhanced against CHIKV with the E1-E211K amino acid change. (E) Immunoblotting was performed against fusion E2-E1 glycoprotein under non-reducing conditions, with each named amino acid change from the Asian to the ECSA sequence introduced independently. Mouse anti-His antibody was used as a control. (F) Neutralisation was performed against different constructs carrying the indicated mutations in the CAR-2SG-ZsGreen backbone, which were rescued from the corresponding icDNA clones of CHIKV. Data are represented as mean ± SD from 4 independent experiments at a serum dilution of 1:800 (pooled sera). *$P<0.05$, ***$P<0.001$, Mann-Whitney U test, relative to CAR. (G) Neutralisation was performed against the CAR-E1-E211K rescued virus at a serum dilution of 1:800 with 23 individual serum samples. ***$P<0.0001$, Wilcoxon matched-pairs signed rank test. (H) The amino acid position of K211, which affects neutralisation activity, is localised on the structural E1-E2 heterodimer complex (based on PDB 3J2W).

4.2.5 E2-I2T, H5N, G118S and S194G amino acids changes in neutralisation activity of ECSA and Asian sera

To study the linear epitopes in the immunodominant E2 glycoprotein (based on strain MY/08/065, of the ECSA genotype), overlapping synthetic peptides (Table 3.7) covering amino acids 1–362 were mapped by peptide-ELISA using the ECSA and Asian sera (Figures 4.17A, 4.17B and 4.17C). Both ECSA and Asian sera mapped to the same 9 peptides, and the Asian sera mapped to an additional 3 peptides (Table 4.3). There are 15 amino acid differences in E2 (from amino acids 1–362) between the strains of ECSA (MY/08/065, ICRES1) and Asian (MY/06/37348, CAR) genotypes of CHIKV used in this study, of which 4 amino acid differences fall within the identified linear epitopes (Figure 4.17D). Using the rE2-Asian construct as a backbone, site-directed mutagenesis was performed to replace each amino acid of Asian origin with an ECSA residue, and the proteins were expressed in insect cells. The antibody binding of the pooled sera significantly increased with proteins carrying I2T, H5N, G118S, R149K and S194G substitutions in comparison to the original rE2-Asian recombinant protein (Figures 4.17E and 4.17F). Neutralising capacity of serum was increased against recombinant viruses carrying either amino acid E2-2T, 5N, 118S or 194G substitution (compared to the parental virus clone CAR), and decreased against viruses with the E2-R149K amino acid change (Figure 4.17G). Competitive peptide blocking assay indicated that the anti-CHIKV antibodies interact with the LP1, LP24 and LP38 peptides that cover amino acid sites 2, 5, 118 and 194 on E2 (Figure 4.17H). These 4 neutralising linear epitopes are localised on the surface of the E1-E2 heterodimer complex (Figure 4.17I). An additional set of competitive peptide blocking assays was performed with LP19, LP47, LP56 and LP70, peptides showing high reactivity with immune sera relative to LP1. The blocking assay indicated LP19 and LP47 interact with CHIKV neutralising antibodies (Figures 4.18A and 4.18B).
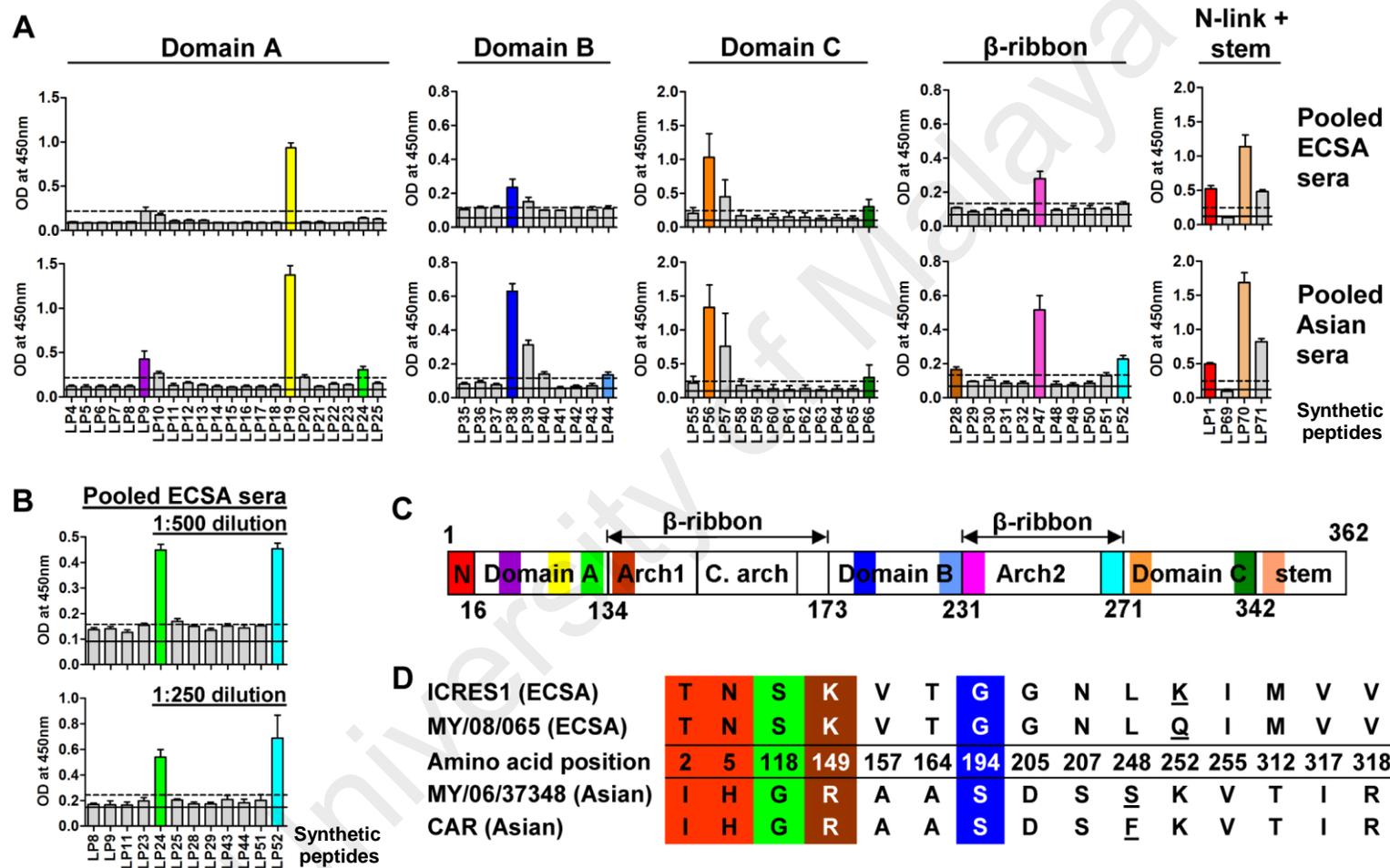
Figure 4.17: Neutralisation activity of ECSA and Asian serum is enhanced against CHIKV with E2-I2T, H5N, G118S and S194G substitutions within linear neutralising epitopes.

Figure 4.17, continued: Neutralisation activity of ECSA and Asian serum is enhanced against CHIKV with E2-I2T, H5N, G118S and S194G substitutions within linear neutralising epitopes. (A) Overlapping synthetic peptides covering the E2 glycoprotein of MY/08/065 and its domains from amino acids 1–362 were screened with CHIKV immune sera at 1:1000 dilution. The black solid line represents the mean OD value of healthy controls and the dotted line represents the cut-off value (mean + 3SD). The average results from 2 independent experiments are presented. In the event of two adjacent positively-mapping peptides, the peptide with the highest OD reading was taken. Key positive mapping peptides are colour-coded. (B) Selected synthetic peptides were re-screened with pooled ECSA sera at lower dilutions of 1:500 and 1:250, in tetraplicate. The black solid line represents the mean OD value of healthy controls and the dotted line represents the cut-off value (mean + 3SD). Key positive mapping peptides are colour-coded. (C) Schematic diagram of the E2 protein showing the positions of the colour-coded mapped epitopes. The numbers refer to the amino acid positions demarcating the E2 domains. N, N-link; C.arch, central arch. (D) Schematic representation of the E2 glycoprotein with the numbers indicating the amino acid positions of the glycoprotein and its domain proteins. The amino acid differences between E2 glycoproteins of ECSA (MY/08/065, ICRES1) and Asian (MY/06/37348, CAR) strains were tabulated and mapped (from amino acids 1–362). Amino acid differences within a genotype are underlined. Amino acid changes which fall within the identified linear epitopes are colour-coded.

Table 4.3: Sequences of identified B cell epitopes on the E2 glycoprotein of MY/08/065.

Domain binding site	B cell epitope sequence [1]	Amino acid positions [2]	Peptide annotation
N-link	STKDNFNVYKATRPY	1-15	LP1
A	ATDGTLKIQVSLQIG	41-55	LP9
A	CTITGTMGHFILARC	91-105	LP19
A	TDSRKISHSCTHPFH	116-130	LP24
β-ribbon (Arch1)	IGREKFHSRPQHGKE	136-150	LP28
B	GNVKITVNGQTVRYK	186-200	LP38
B	VINNCKVDQCHAAVT	216-230	LP44
β-ribbon (Arch 2)	NHKKWQYNSPLVPRN	231-245	LP47
β-ribbon (Arch 2)	HIPFPLANVTCRVPK	256-270	LP52
C	VTYGKNQVIMLLYPD	276-290	LP56
C	LEVTWGNNEPYKYWP	326-340	LP66
Stem	GTAHGHPHEIILYYY	346-360	LP70

[1] Underlined sequences indicate common epitopes recognised by both ECSA and Asian sera.

[2] The first amino acid in E2 is numbered as 1.
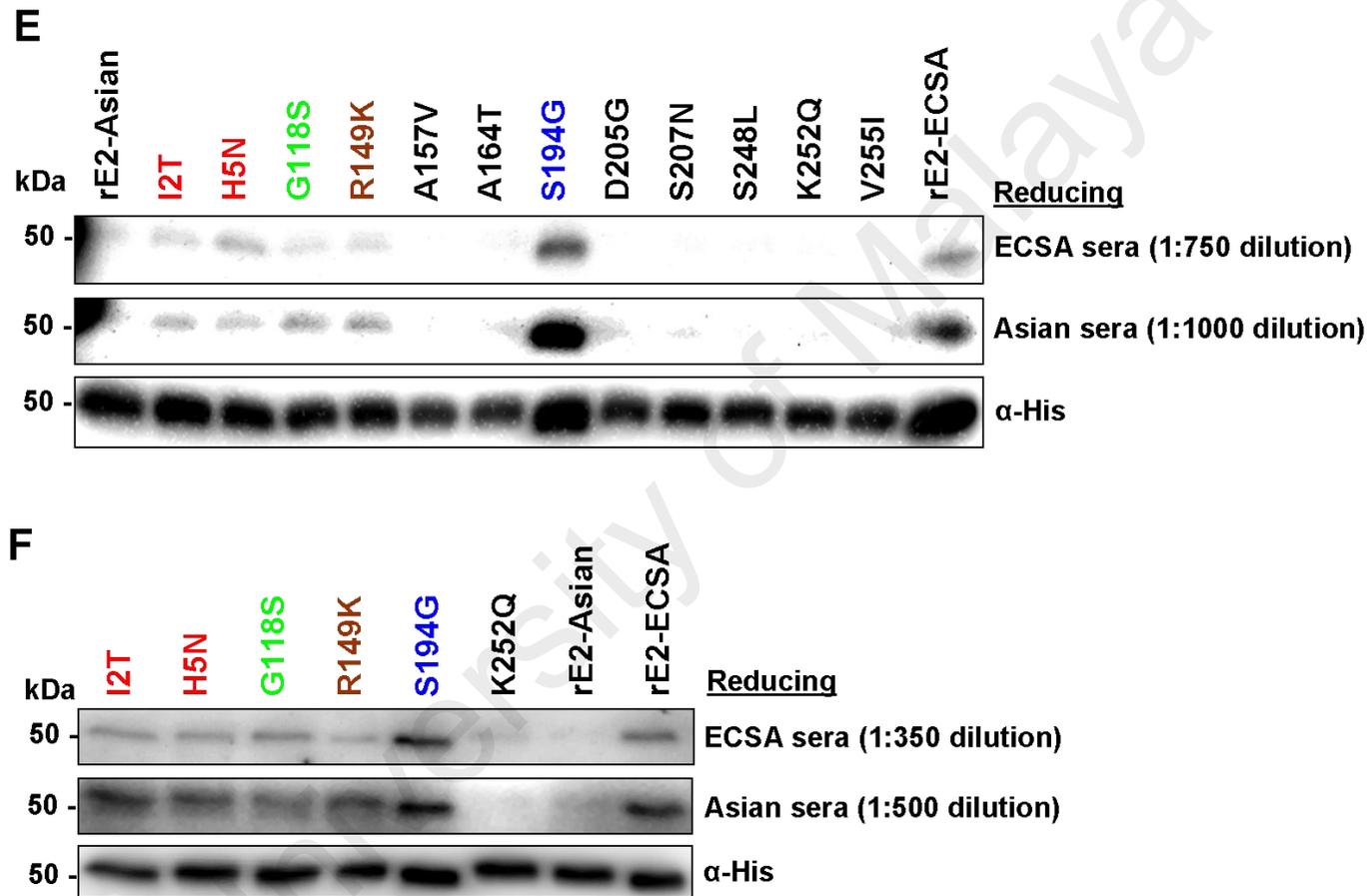
Figure 4.17, continued: Neutralisation activity of ECSA and Asian serum is enhanced against CHIKV with E2-I2T, H5N, G118S and S194G substitutions within linear neutralising epitopes.

Figure 4.17, continued: Neutralisation activity of ECSA and Asian serum is enhanced against CHIKV with E2-I2T, H5N, G118S and S194G substitutions within linear neutralising epitopes. (E and F) Immunoblotting was performed against recombinant E2 glycoproteins under reducing conditions at different serum dilutions, with each named amino acid change from the Asian to the ECSA sequence introduced independently. Mouse anti-His antibody was used as a control. Site-directed mutagenesis was not performed for amino acid positions 312, 317 and 318 as these are predicted not to be exposed on the protein surface.
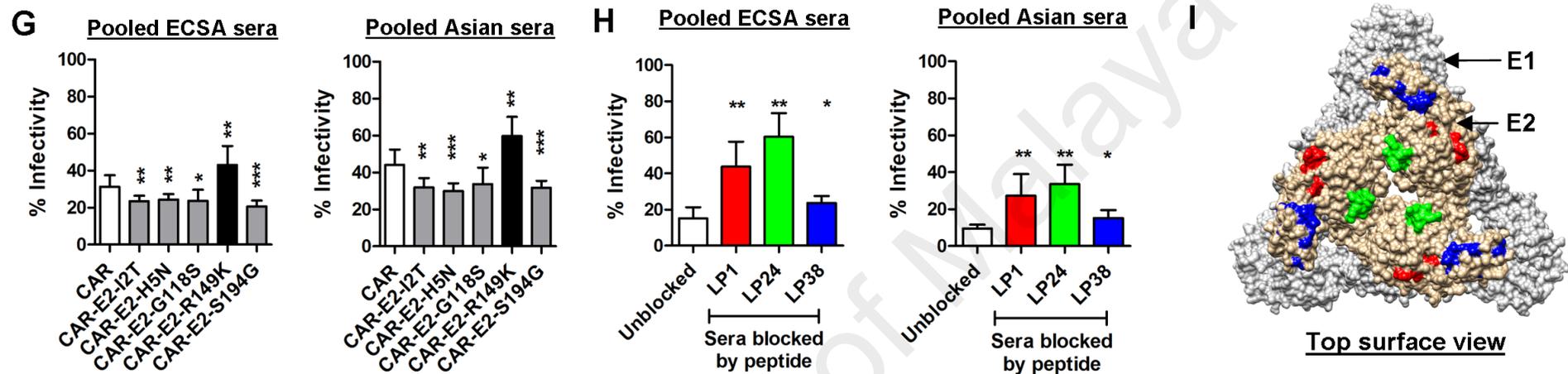
Figure 4.17, continued: Neutralisation activity of ECSA and Asian serum is enhanced against CHIKV with E2-I2T, H5N, G118S and S194G substitutions within linear neutralising epitopes. (G) Neutralisation was performed against different constructs with the CAR-2SG-ZsGreen backbone, which were rescued from the corresponding CHIKV icDNAs. Data are represented as means ± SD from 4 independent experiments at a serum dilution of 1:800 (pooled sera). *$P<0.05$, **$P<0.01$, ***$P<0.001$, Mann-Whitney U test. (H) Competitive peptide blocking assay was performed at 1:100 dilution with either pooled ECSA or Asian sera against ICRES1 at an MOI of 1. Data are expressed as percentages of infectivity of an infection control and are presented as means ± SD from 2 independent experiments. *$P<0.05$, **$P< 0.01$, Mann-Whitney U test, relative to unblocked condition. (I) The colour-coded mapped neutralising epitopes are localised on the structural E1-E2 heterodimer complex (based on PDB 3J2W). The epitope sequence of LP1 is only partially localised as the 3D structure is not fully resolved.
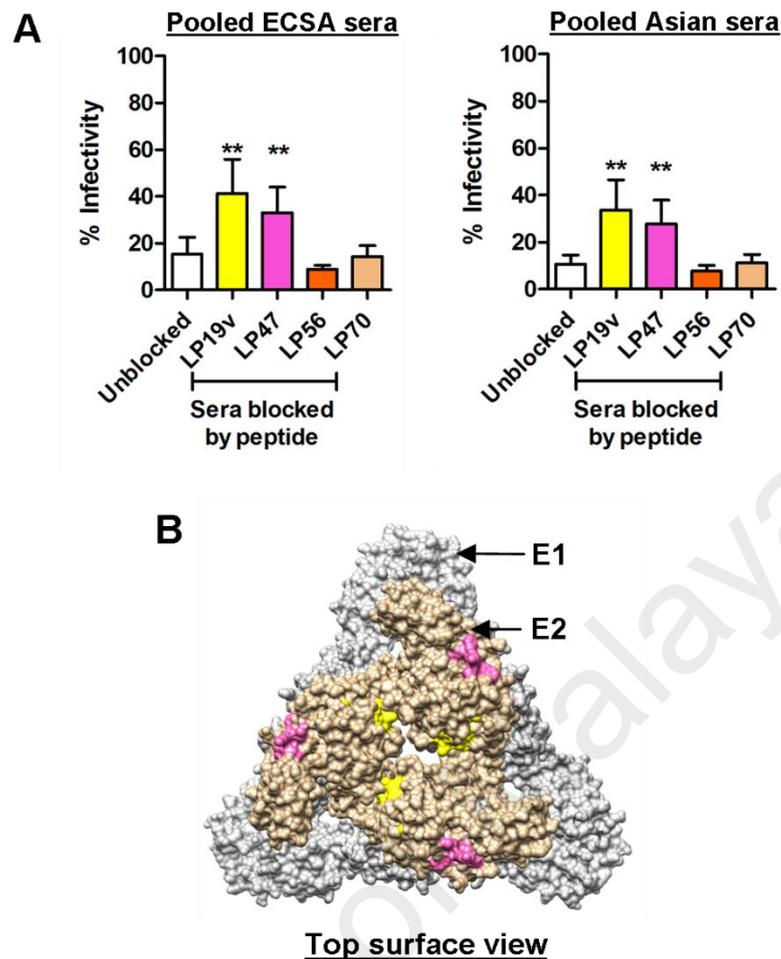
Figure 4.18: Functional characterisation of high linear epitope responders on the E2 glycoprotein. (A) Competitive peptide blocking assay was performed at 1:100 dilution with either pooled ECSA or Asian sera against ICRES1 at an MOI of 1. Sera blocked by LP19v and LP47 resulted in increases in infectivity. LP19v is a soluble peptide without cysteine residues at N- and C-terminuses of LP19. Data are expressed as percentages of infectivity of an infection control, and are presented as means ± SD from 2 independent experiments, run in triplicate. **$P< 0.01$, Mann-Whitney U test, relative to unblocked control. (B) The colour-coded mapped neutralising epitopes (LP19 and LP47) are localised on the structural E1-E2 heterodimer complex (based on PDB 3J2W).

4.2.6 The effect of sequence variation of a neutralising linear epitope in cross-genotype neutralisation

As naturally-acquired infection of the Asian genotype of CHIKV leads to higher cross-neutralising efficacy against ECSA CHIKV, it was hypothesised that an epitope-based vaccine derived from the Asian genotype might provide a substantial level of cross-protection against ECSA CHIKV. The peptide LP1 (STKDNFNVYKATRPY) is similar to E2EP3, a peptide derived from ECSA virus which has been found to be highly immunogenic in eliciting neutralising antibodies in an animal model (Kam *et al.*, 2012b). A variant, LP1A (S<u>I</u>KD<u>H</u>FNVYKATRPY) was generated which was derived from the sequence of the Asian virus. Rabbit polyclonal antibodies were commercially prepared against LP1A and LP1. Peptide-ELISA was performed using human ECSA and Asian serum with LP1A and LP1 as antigens. Human ECSA serum bound to LP1 but not LP1A (Figure 4.19A). Rabbit anti-LP1 antibody showed the lowest binding capacity against CAR (Asian), and demonstrated poor neutralising activity against the CAR virus harbouring the LP1A sequence (infectivity 91 ± 10 %, Figure 4.19B). Anti-LP1 binding capacity and neutralisation efficacy were partially restored with the mutations I2T and H5N. The anti-LP1 antibody had maximum binding capacity and neutralising efficacy against CAR-E2-I2T-H5N (Figure 4.19B), which has the LP1 sequence, a finding in line with the antibody binding of ECSA immune sera against LP1 peptide (Figure 4.19B).

Asian serum could recognise LP1A, although binding was marginally higher to LP1 (Figure 4.17C), which supports the earlier finding that Asian serum has stronger binding against LP1 with I2T and H5N amino acid changes (Figures 4.17E and 4.17F). Unexpectedly, rabbit anti-LP1A did not demonstrate significant neutralising activity against CHIKV with either the LP1A or LP1 sequences (Figure 4.19D). However, a competitive peptide blocking assay indicated that neutralising antibodies from Asian

sera could still recognise and interact with both LP1A and LP1 peptides (Figure 4.19E). The electrostatic potential of the E2 surface was computed based on the CAR ecto-domain region to study the charge distribution of these epitopes which affect binding affinity. The I2T change leads to higher electrostatic potential, which is associated with improved binding capacity and neutralisation efficacy (Figure 4.19F). LP47, another linear neutralising epitope in humans, also failed to induce any functional neutralising antibodies in rabbits in this study. The results implied that the choice of vaccine strain with amino acid variation or differential neutralising epitopes may impact cross-protection against different genotypes.
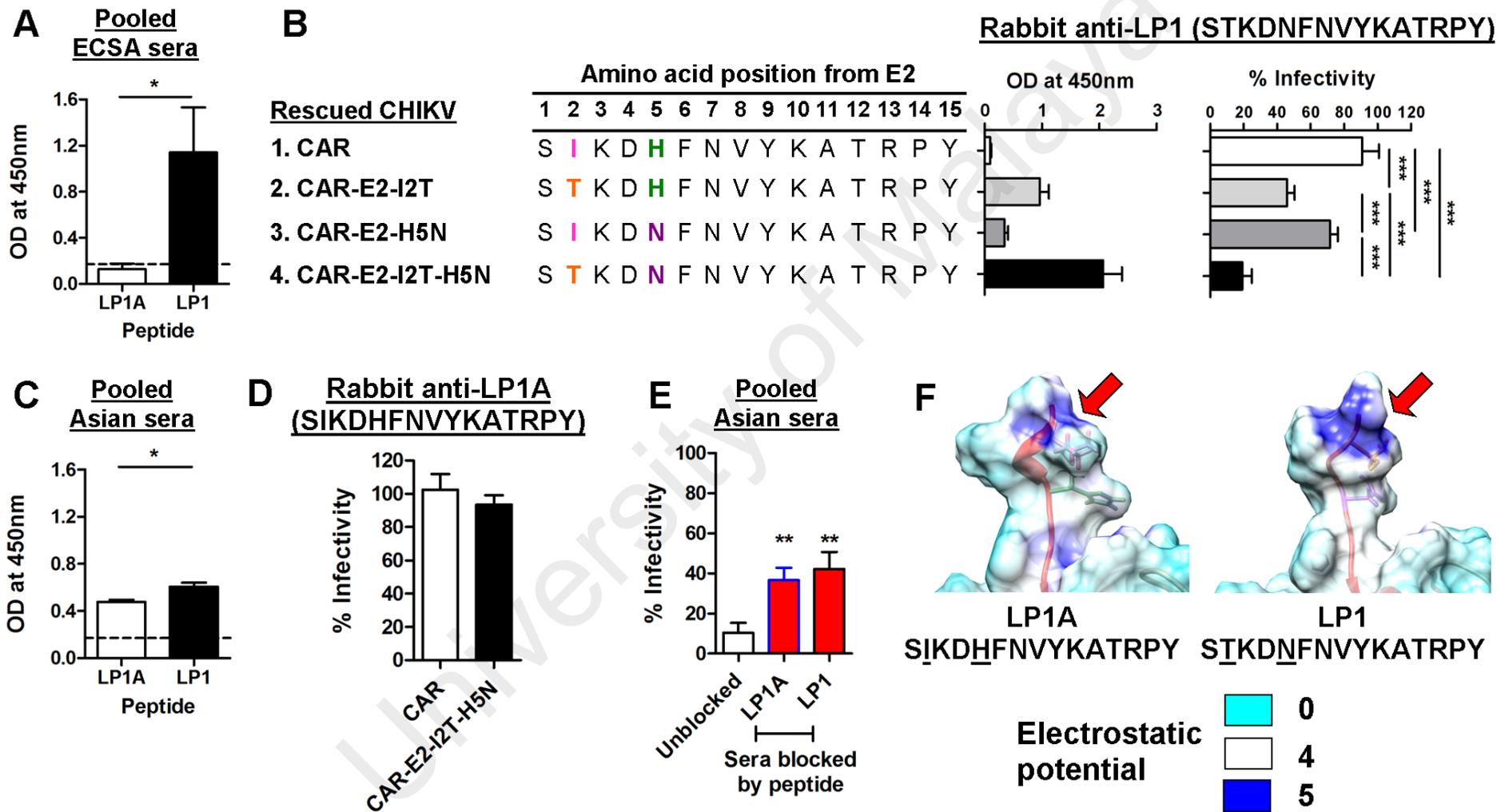
Figure 4.19: Sequence variation of a linear neutralising epitope influences the spectrum of cross-neutralisation across genotypes.

Figure 4.19, continued: Sequence variation of a linear neutralising epitope influences the spectrum of cross-neutralisation across genotypes. (A) Synthetic peptides LP1A and LP1 were screened with ECSA immune sera at 1:500 dilution in tetraplicate. The dotted line represents the cut-off value (mean + 3SD). *P<0.05, Mann-Whitney U test. (B) Recombinant viruses were pre-treated with 1% Triton X-100, then coated at 5×10^5 pfu per well, and a binding assay was performed with 1 µg/ml of antibody. The neutralisation assay was performed with infection at an MOI of 50 against 25 µg/ml of antibody. Data are represented as means ± SD from 3 independent experiments. ***P<0.001, repeated measures ANOVA with the Bonferroni multiple comparisons test. (C) Synthetic peptides LP1A and LP1 were screened with Asian immune sera at 1:500 dilution in tetraplicate. The dotted line represents the cut-off value (mean + 3SD). *P<0.05, Mann-Whitney U test. (D) Neutralisation of anti-LP1A against CAR and CAR-E2-I2T-H5N at an antibody concentration of 25 µg/ml. Data are represented as means ± SD from 3 independent experiments. (E) Competitive peptide blocking assay was performed at 1:100 dilution with either LP1A or LP1 in pooled Asian sera against ICRES1 at an MOI of 1. Data are expressed as percentages of infectivity of an infection control and are presented as means ± SD (n=6). **P< 0.01, Mann-Whitney U test, relative to unblocked condition. (F) Structural images illustrating the changes of surface electrostatic potential due to differences in amino acid positions 2 and 5 within LP1A (Asian) and LP1 (ECSA) sequences (red arrows).

4.3 Evidence for antibody-dependent enhancement (ADE) of chikungunya virus infection
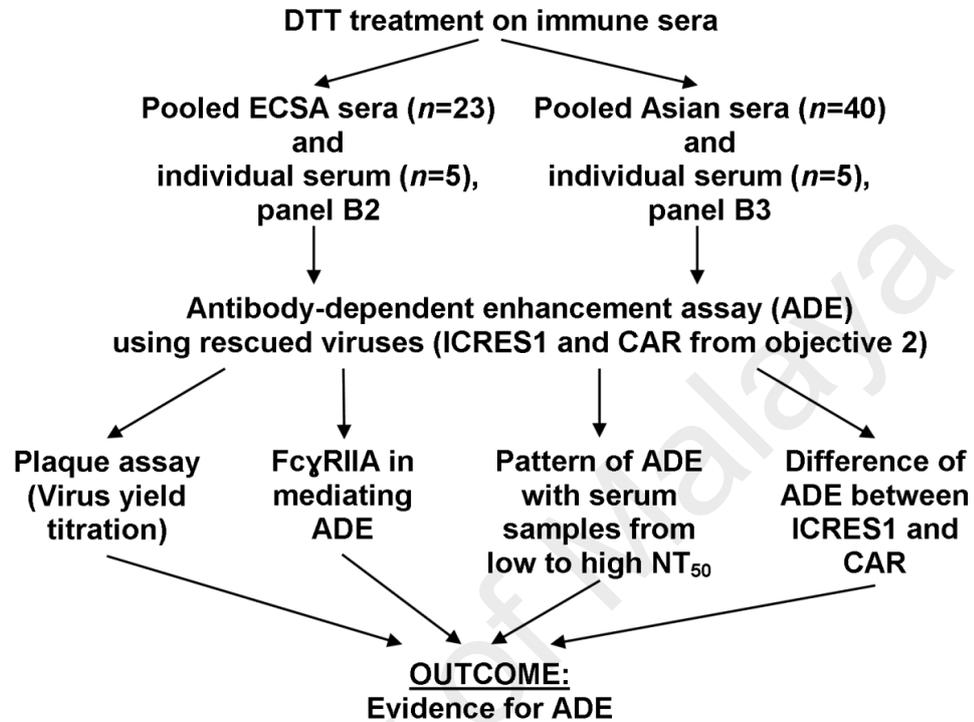
Figure 4.20: Flow chart for objective 3, evidence for antibody-dependent enhancement of chikungunya virus infection.

4.3.1 ADE of CHIKV in K562 cells

In section 4.2, the cross-neutralising antibody efficacy was described, which shows the importance of antibodies in providing protection. However, in certain circumstances, antibodies may mediate pathology, which is known as antibody-dependent enhancement (ADE).

To study CHIKV ADE, two different cell lines bearing the Fc gamma receptor (FcγR) and ECSA rescued virus (ICRES1) with zsGreen reporter were utilised. Using RAW 264.7 murine macrophage cells, ADE was previously demonstrated for RRV (Linn *et al.*, 1996), another alphavirus in the same antigenic complex as CHIKV; while K562 human leukaemia cells have been extensively used to study ADE for DENV. In this study, ADE of CHIKV infection was demonstrated in the presence of CHIKV immune sera from dilutions of 1:50 to 1:3,200 at 24 hours in K562 but not RAW 264.7 cells (Figure 4.21A). Pooled sera from healthy controls did not mediate CHIKV ADE. The evidence for CHIKV ADE was accompanied by supportive and active virus production. Peak enhancement was seen at a serum dilution of 1:800 at 24 hours with virus titre > 10^6 pfu/ml compared to virus control <10^5 pfu/ml (Figure 4.21B). The maximum virus titre was achieved at 24 hours post-ADE, while the maximum relative increase in virus titres relative to the virus control was at 48 hours (Figure 4.21C). Blocking of FcγR, particularly FcγRIIA by anti-CD32, inhibited the interaction of virus-antibody complex with receptors and abrogated CHIKV ADE (Figure 4.21D). K562 was poorly permissible to CHIKV in the absence of immune sera and had a similar infection background relative to the mock control, while in the presence of immune serum, ADE of CHIKV was detected by zsGreen reporter (Figure 4.21E). In short, K562 expressing FcγRIIA supports CHIKV ADE with active virus production.
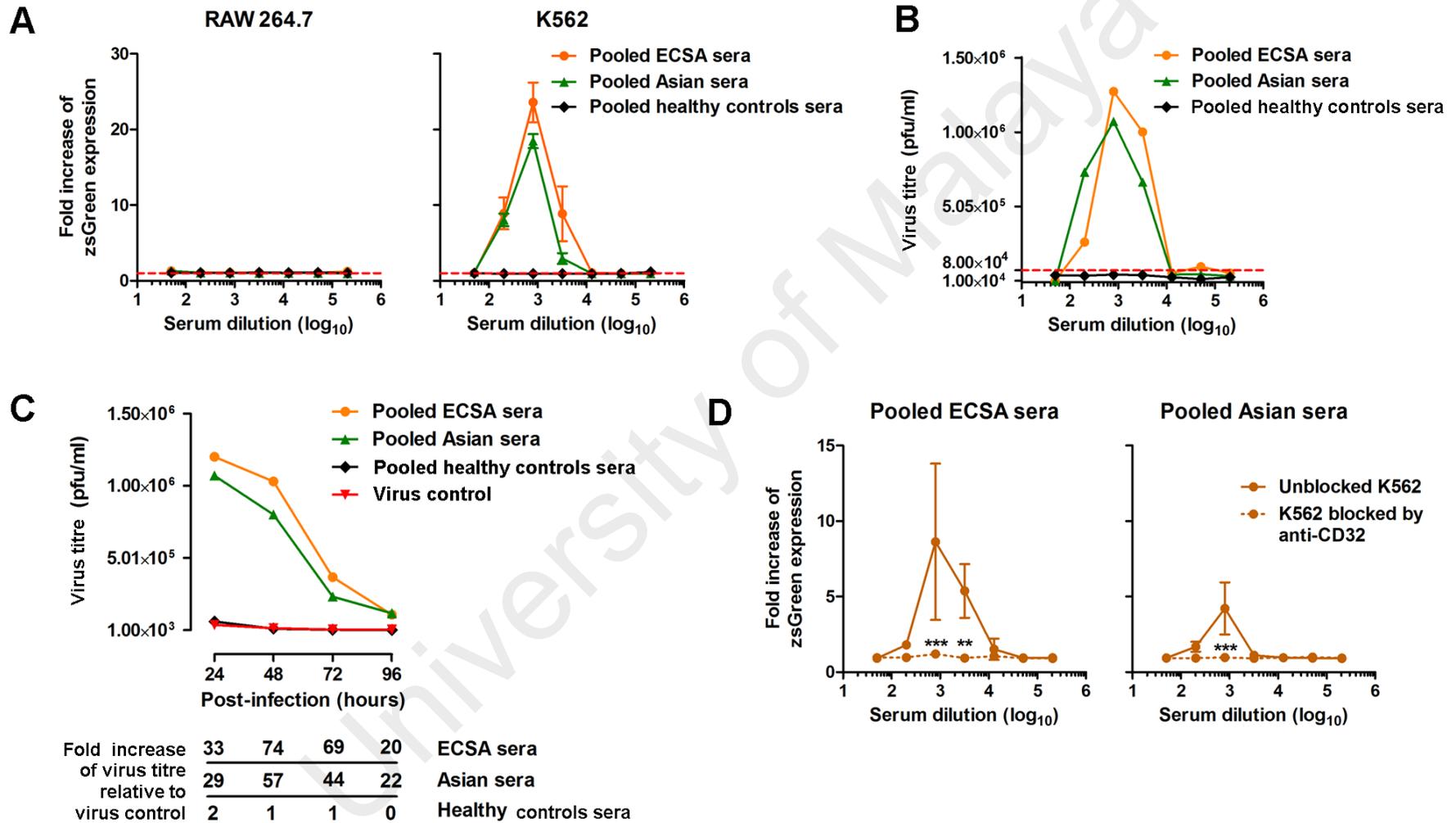
Figure 4.21: K562 expressing FcγRIIA supports ADE with active virus production.

Figure 4.21, continued: K562 expressing FcγRIIA supports ADE with active virus production. (A) ADE of CHIKV infection was seen in K562, but not RAW 264.7 cells. Peak enhancement, as shown as maximum zsGreen expression, was achieved at a serum dilution of 1:800 with CHIKV immune sera. Experiments were performed in triplicate. The red dotted line indicates the fold change of zsGreen expression of the virus control relative to the mock control. (B) ADE of CHIKV in K562 supported active virus production, measured by plaque assay using pools of virus supernatant from triplicate. The red dotted line indicates the basal titre of the virus control (8×10^4 pfu/ml). (C) ADE of CHIKV in K562 achieved the highest virus production at 48 hours relative to the virus control at a serum dilution of 1:800. (D) Blocking of FcγRIIA by anti-CD32 in K562 inhibited ADE. Experiments were performed in triplicate. Fluorescence acquisition was carried out at 48 hours. **P<0.01, ***P<0.001 by two-way ANOVA with Bonferroni multiple comparisons test. Data are presented as means ± SD.

E

Pooled ECSA sera Pooled Asian sera Virus control

1:800 serum dilution

1:3200 serum dilution

Mock control
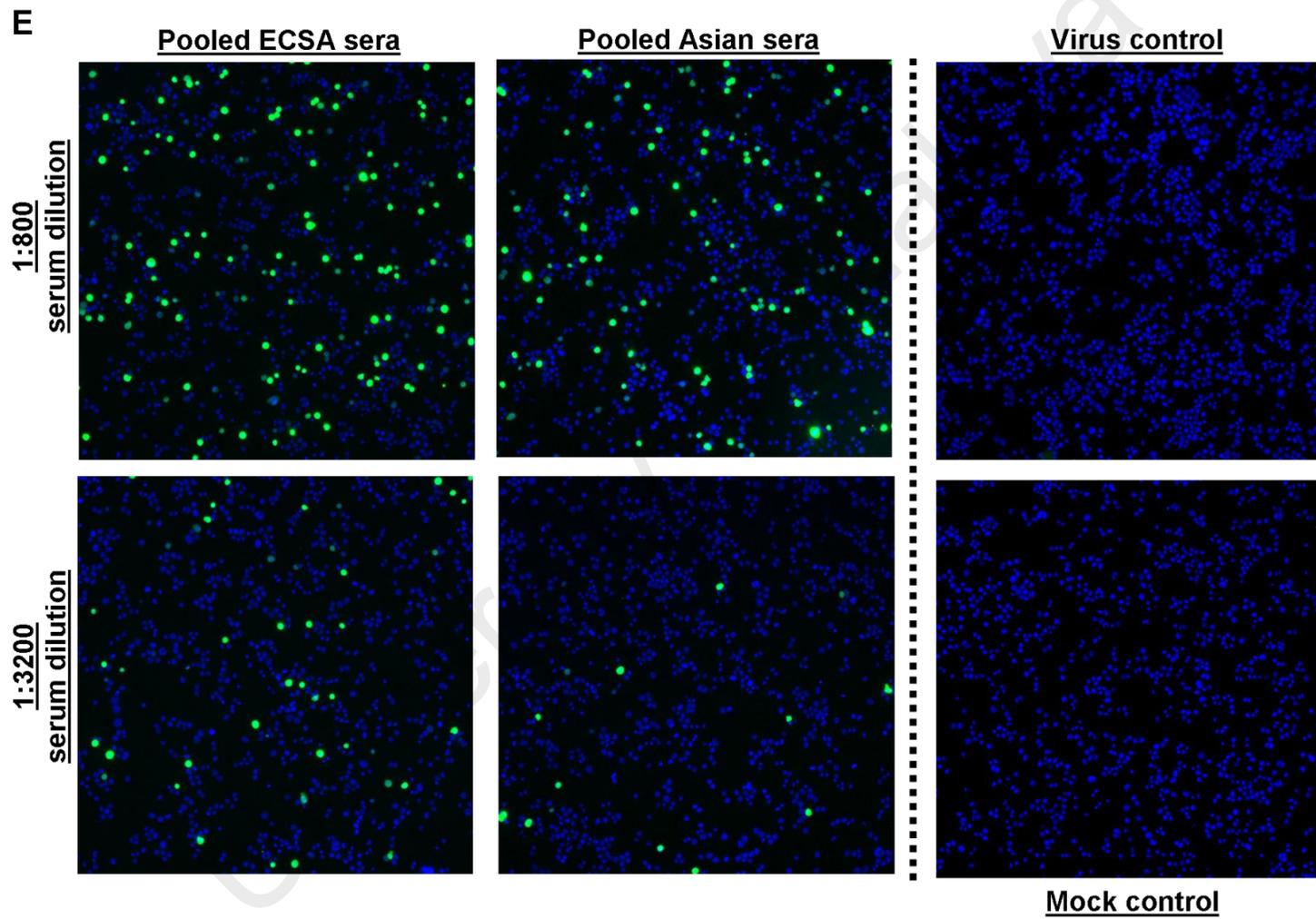
Figure 4.21, continued: K562 expressing FcγRIIA supports ADE with active virus production.

Figure 4.21, continued: K562 expressing FcγRIIA supports ADE with active virus production. (E) Representative immunofluorescent microscopic images of infected K562 which mediated ADE at 1:800 and 1:3200 serum dilutions at 24 hours. Objective magnification: $5 \times$ from 1 field of view. No zsGreen fluorescence was observed with virus and mock controls. All the ADE assays were performed with rescued ICRES1 virus (ECSA).

4.3.2 Pattern of CHIKV ADE using immune sera of variable neutralisation titres

Ten immune serum samples from the ECSA and Asian panels with known neutralising titres ranging from low to high were selected to investigate the pattern of ADE, with 2 samples from healthy individuals as negative controls. The peak enhancements (maximum zsGreen expression) were observed at serum dilutions of 1:200, 1:800 and 1:3200, which also neutralised virus at low levels (Figure 4.22). As the neutralising titres of the serum samples increased, the serum dilutions at which peak enhancement was achieved also increased. Healthy control serum in the absence of anti-CHIKV IgG did not neutralise CHIKV and did not mediate ADE. Overall, CHIKV ADE could be mediated at sub-neutralising levels.
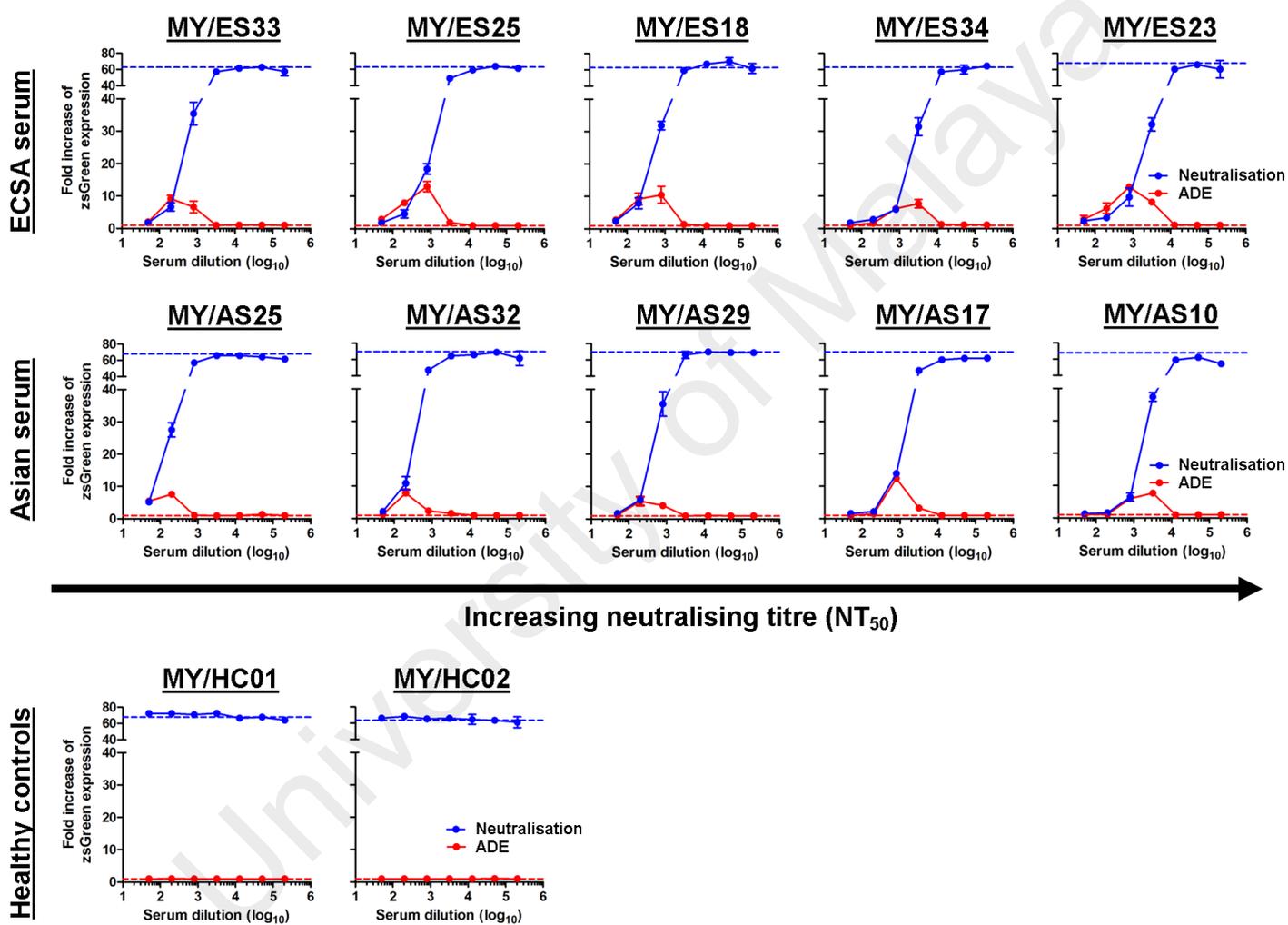
Figure 4.22: CHIKV human immune sera with variable neutralising titres mediate ADE of CHIKV.

Figure 4.22, continued: CHIKV human immune sera with variable neutralising titres mediate ADE of CHIKV. Ten immune serum samples from ECSA and Asian panels were selected to investigate the pattern of ADE. ADE and neutralisation assays were performed with 4-fold serially diluted immune sera from 1:50 to 1:204,800 in K562 and BHK-21 cells. The peak enhancements were achieved at 1:200, 1:800 and 1:3200 serum dilutions at 24 hours. Two healthy controls were included as negative controls without neutralising antibodies, and no ADE was observed. All the ADE and neutralisation assays were performed with rescued ICRES1 virus (ECSA). The red and blue dotted lines indicate the fold changes of zsGreen expression of the virus control relative to the mock control. Experiments were performed in triplicate and data are presented as means ± SD.

4.3.3 ADE of ECSA and Asian rescued viruses

Serum from ECSA and Asian panels were investigated for their ability to enhance infection with ECSA (ICRES1) and Asian (CAR) rescued viruses. Unexpectedly, ADE was shown only for ECSA rescued virus, with significant levels of virus replication detected at 30 hours, and not for Asian rescued virus (Figures 4.23A and 4.23B). Virus titres of Asian CHIKV did not increase at 48 hours at a serum dilution of 1:800 (Figure 4.23C). Taken together, ADE of ECSA rescued virus only was demonstrated in K562 with more than 10-fold increase virus production detected. ADE of Asian rescued virus was not demonstrated which could be due to inability of this virus to infect K562 cells.
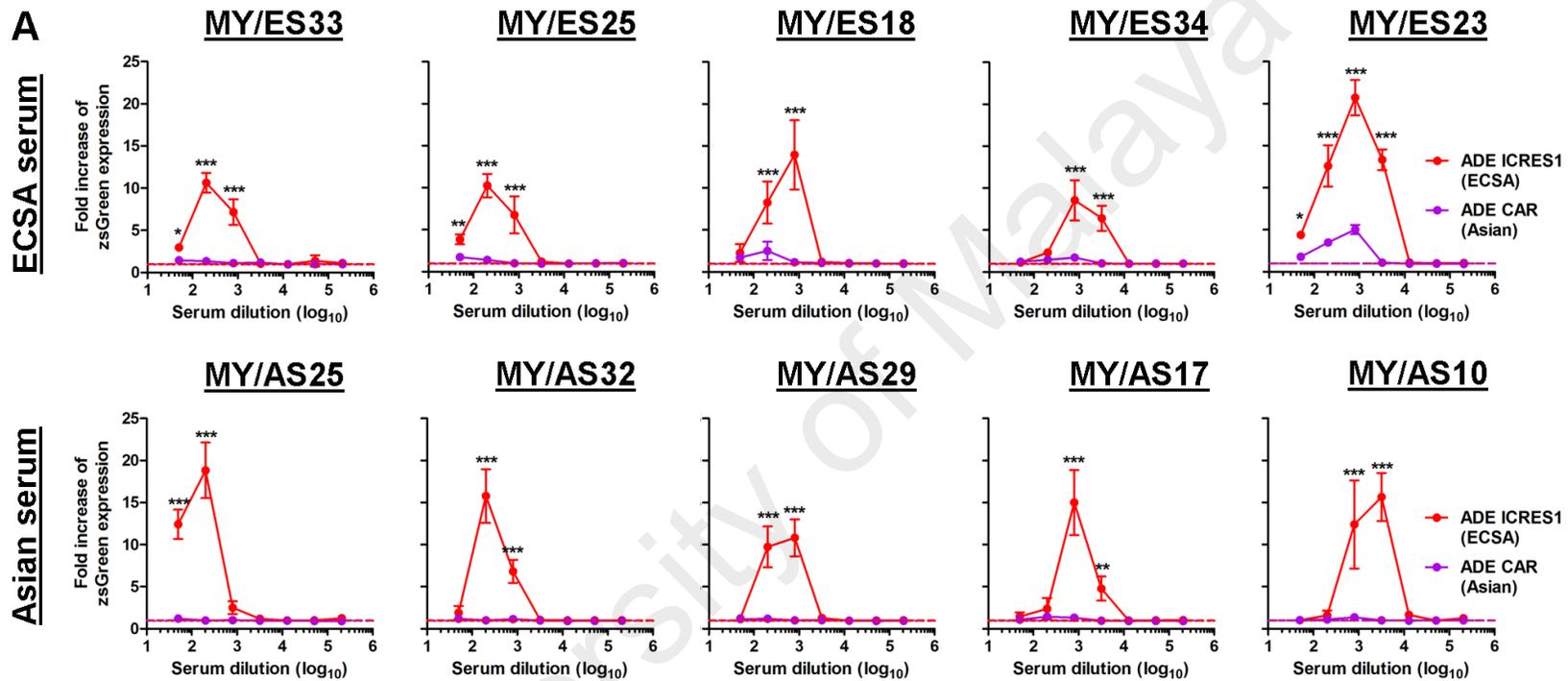
Figure 4.23: Immune serum enhances infection of ECSA rescued virus, but not Asian rescued virus. (A) Ten immune sera from ECSA and Asian panels were used to study ADE of ECSA and Asian rescued viruses. The acquisition of zsGreen was performed at 30 hours. The red and purple dotted lines indicate the fold changes of zsGreen expression of the virus control relative to the mock control. Experiments were performed in triplicate and data are presented as means ± SD. *$P<0.05$, **$P<0.01$, ***$P<0.001$ by two-way ANOVA with Bonferroni multiple comparisons test.
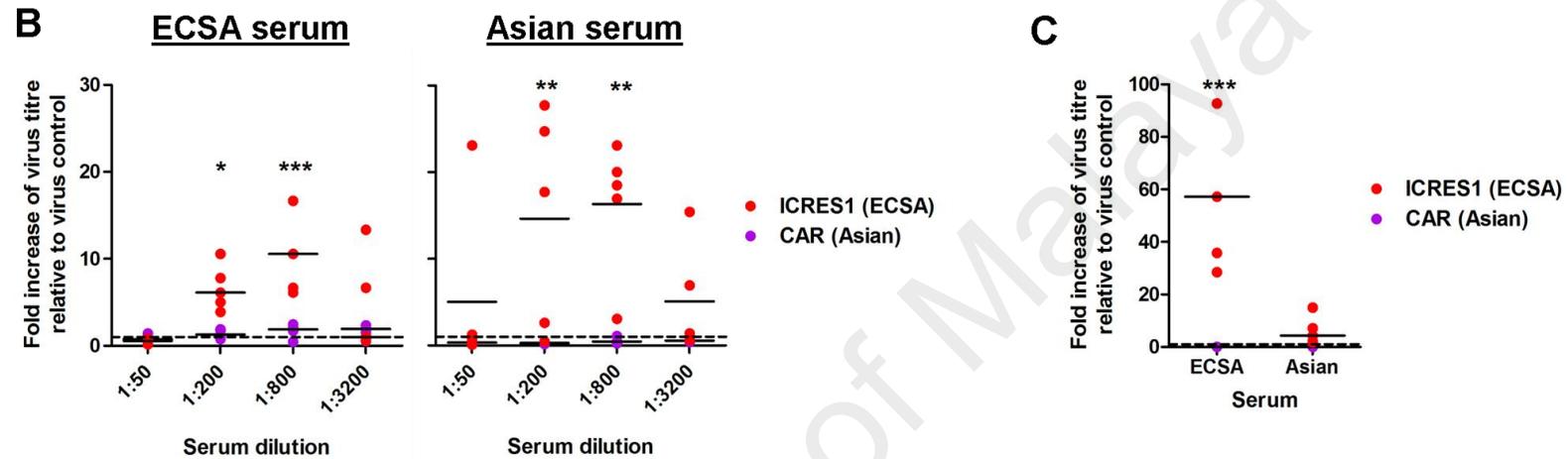
Figure 4.23, continued: Immune serum enhances infection of ECSA rescued virus, but not Asian rescued virus. (B) The virus yield was titrated by plaque assay (pool of virus supernatant from triplicates) at 30 hours post-ADE. The dotted lines indicate the basal level of virus controls. ADE by ECSA and Asian serum led to a more than 10-fold increase of virus titres relative to virus controls at serum dilutions of 1:200 (Asian) and 1:800 (ECSA and Asian), shown by the median black line. (C) The virus yield was titrated at a serum dilution of 1:800 at 48 hours post-ADE. The dotted line indicates the basal level of virus controls. ADE mediated by ECSA serum resulted in a more than 50-fold increase of virus titres relative to virus control, shown by the median black line. *P<0.05, **P<0.01, ***P<0.001 by two-way ANOVA with Bonferroni multiple comparisons test.

4.3.4 CHIKV RNA transfection in K562 and BHK-21 cells

To test the hypothesis that Asian rescued virus (CAR) is unable to infect K562 cells, *in vitro* 5' capped RNA transcripts transcribed from icDNAs and replicons of ECSA and Asian genotypes were electroporated into cells. This allows the study of CHIKV replication and infection in K562 cells to be carried out while bypassing receptor-mediated entry. Transcribed RNA is similar to positive-sense RNA, which is encapsidated in the CHIKV virion, and viral proteins (non-structural and structural proteins from icDNA, but only structural proteins from replicons) are translated in the cell cytoplasm. DNA-launched plasmids are not suitable for electroporation in K562, due to poor transfection efficiency and DNA toxicity (Ponsaerts *et al.*, 2003; Ryser *et al.*, 2006; Melhem *et al.*, 2008). The expression of viral proteins from ECSA and Asian genotypes was examined and compared with the aid of zsGreen reporter. Fluorescent zsGreen expression was observed at 24 and 48 hours after the cells were electroporated with RNA transcribed from icDNA and replicon ICRES1 (Figures 4.24A and 4.24B). However, no zsGreen expression was detected at 24 hours from cells electroporated with RNA transcribed from icDNA and replicon CAR, and low zsGreen expression could only be observed after 48 hours from replicon CAR (Figures 4.24A and 4.24B). In a parallel experiment, RNA was similarly electroporated into BHK-21 cells as a control. High expression of zsGreen in BHK-21 cells confirmed that the RNA transcripts from both ICRES1 and CAR were functional, and the cells displayed signs of CPE upon electroporation of infectious RNA transcribed from icDNA (Figures 4.24A and 4.24B). Electroporated K562 with RNA transcribed from replication-competent icDNA ICRES1, but not from icDNA CAR, led to the production of infectious virus which was detected by plaque assay (Table 4.4). In this pilot study, high expression of zsGreen in K562 could likely reflect active expression of viral proteins in parallel, after transfection of RNA transcribed from ECSA CHIKV-based vectors, but

not of Asian genotype. This supports the finding of ADE of ECSA virus in K562 cells. In contrast, the failure to demonstrate ADE of Asian CHIKV is due to the inability of CAR derived from an infectious clone to infect and replicate in K562 cells.

A

	K562			BHK-21		
	Bright field	zsGreen	Merged	Bright field	zsGreen	Merged
Mock						
icDNA ICRES1						
Replicon ICRES1						
icDNA CAR						
Replicon CAR						
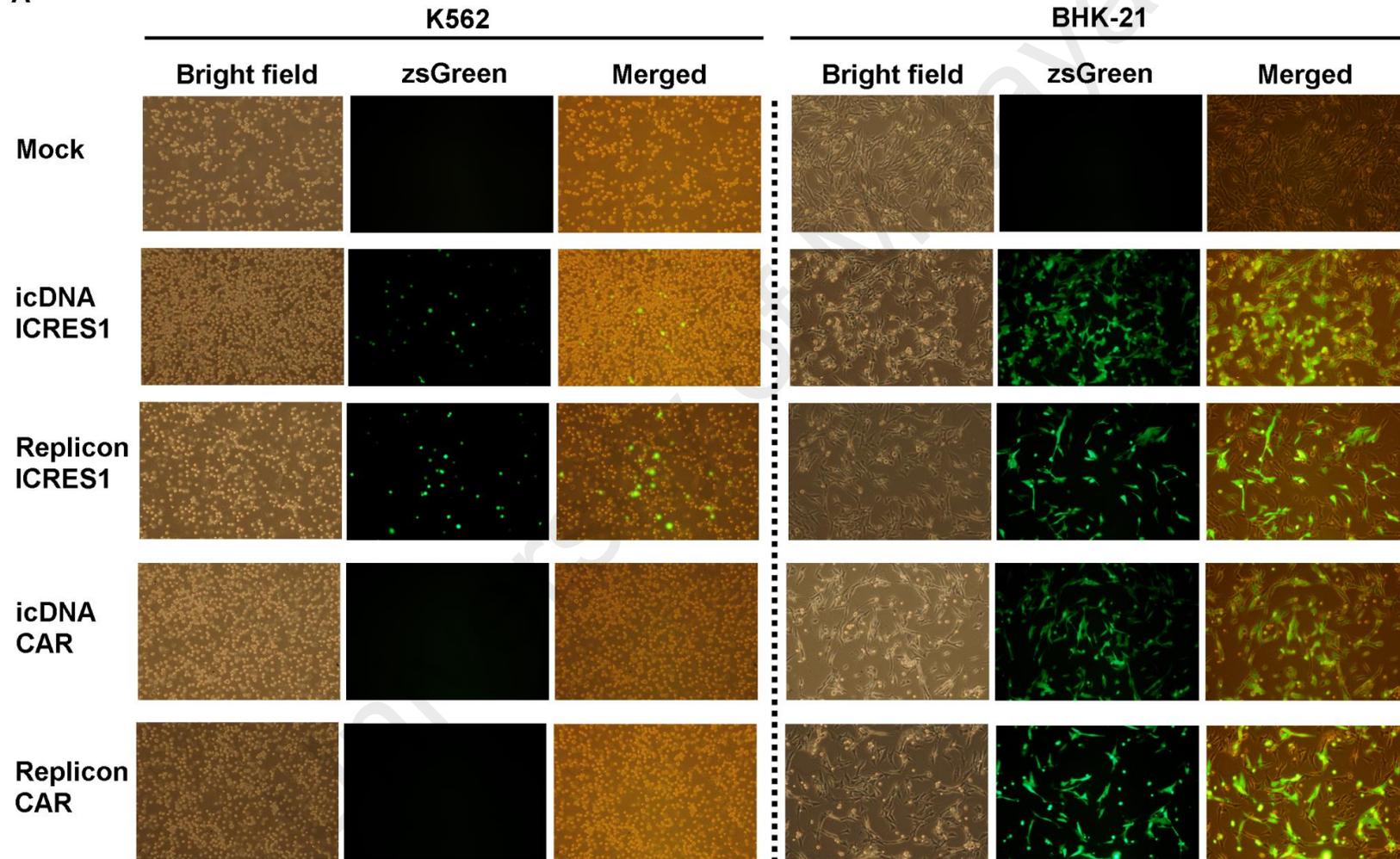
Figure 4.24: CHIKV RNA transfection in K562 and BHK-21 cells.

B
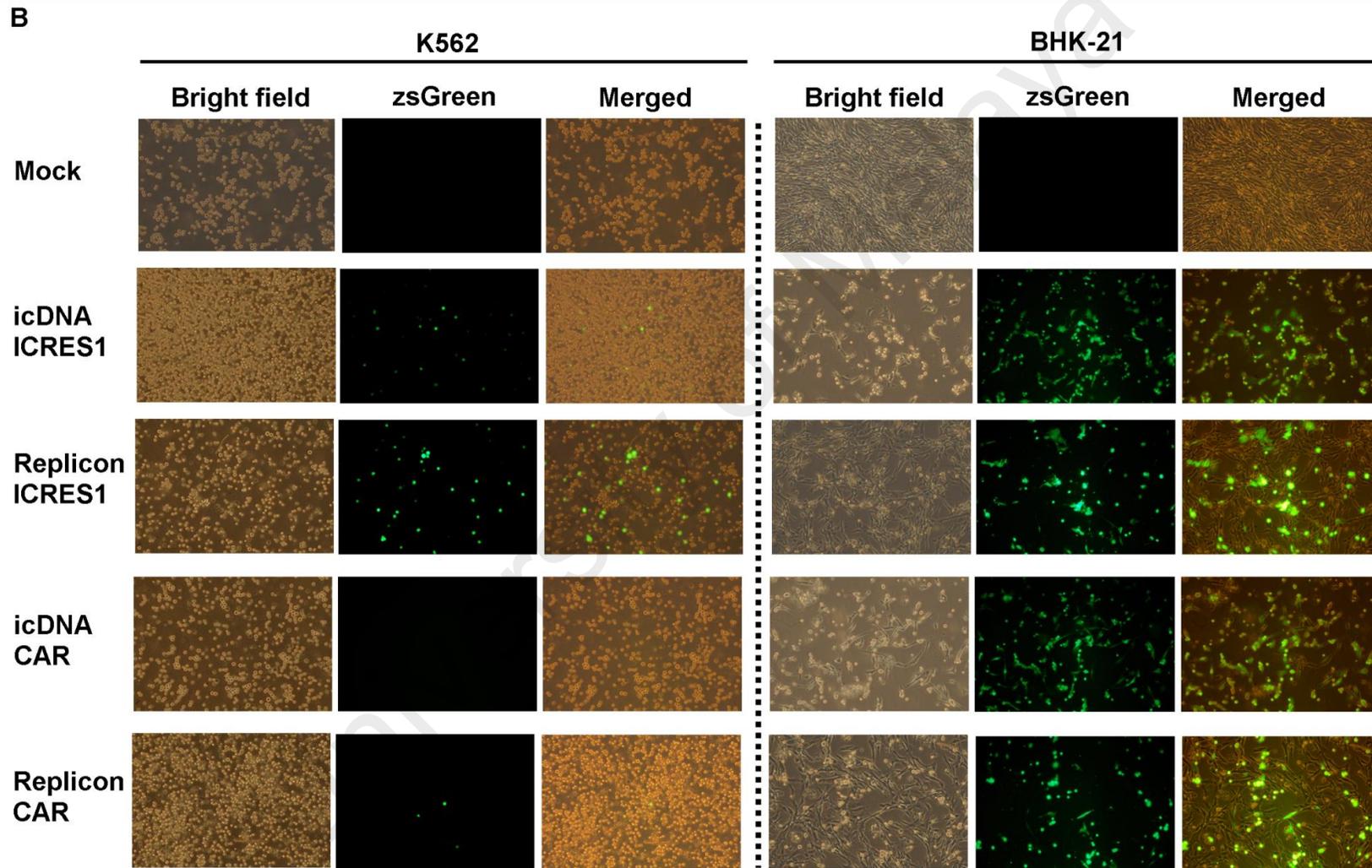
Figure 4.24, continued: CHIKV RNA transfection in K562 and BHK-21 cells.

Figure 4.24, continued: CHIKV RNA transfection in K562 and BHK-21 cells. RNA (transcribed from icDNA and replicons) was electroporated into K562 and zsGreen expression was examined at (A) 24 hours and (B) 48 hours. RNA was also electroporated into BHK-21 cells to verify the integrity of RNA transcripts. Electroporated BHK-21 cells with RNA transcribed from icDNA displayed progressive signs of CPE. The fluorescence was observed under a Nikon Eclipse TE2000-E fluorescence microscope with 20 × objective magnification.

Table 4.4: Virus titration of K562 and BHK-21 culture supernatants after transfection of RNA transcribed from CHIKV-based vectors.

CHIKV-based vector	K562 (pfu/ml)[1,2]		BHK-21 (pfu/ml)[1,3]	
	24 hours	48 hours	24 hours	48 hours
Mock	<3	<3	<3	<3
icDNA ICRES1	5.33×10^5	1.00×10^5	7.33×10^8	5.33×10^8
Replicon ICRES1	<3	<3	<3	<3
icDNA CAR	<3	<3	3.00×10^8	1.13×10^8
Replicon CAR	<3	<3	<3	<3

[1] Detection limit of plaque assay was 3 pfu/ml.

[2] From 10 ml cell culture supernatant, 10 μg RNA transcripts were electroporated into approximately 2.5×10^6 cells.

[3] From 10 ml cell culture supernatant, 5 μg RNA transcripts were electroporated into approximately 5.0×10^6 cells.

4.3.5 Antibodies against CHIKV envelope glycoproteins in enhancing capacity

Since both panels of immune sera have stronger antibody binding capacity against ICRES1 compared to CAR, it was hypothesised that a higher amount of immune complex formation could be internalised during the entry process and the enhancing capacity could be intensified. A "hybrid CAR" was constructed by replacing the structural region (C-E3-E2-6K-E1) of ICRES1 with that of CAR using pCMV-ICRES1-2SG-zsGreen as the main backbone. Using similar conditions to infect K562, the effect of differential antibody binding to ICRES1 and hybrid CAR in enhancing capacity could be compared. Peak enhancement of ICRES1 infection was shown with both ECSA and Asian sera at serum dilutions of 1:800 (Figure 4.25A). Weaker antibody binding to hybrid CAR shifted the peak enhancements to lower serum dilutions at 1:200 (Figure 4.24A). The next investigation was to identify which regions of the envelope glycoproteins are important in mediating the enhancing capacity. Chimeric viruses were constructed in which the ecto-domain regions of E2, E1-E2 and E2 domains (A and B) glycoprotein of CHIKV (ICRES1) were swapped with those of SFV. These viruses were designated ICRES1-E2SFV, ICRES1-E1E2SFV, ICRES1-E2dASFV and ICRES1-E2dBSFV, respectively. With both sera panels, the peak enhancements were shifted dramatically to lower serum dilutions of 1:50 or 1:100, when CHIKV E2 or either of E2 domains A and B were replaced with that of SFV (Figure 4.25B). Total loss of enhancement activity was only observed when both CHIKV E2 and E1 glycoproteins were swapped with those of SFV (Figure 4.25B).

In summary, the modulation of ADE is highly dependent on the antibody specificity against both E1 and E2 envelope glycoproteins, and the amino acid variation of the envelope glycoproteins between ECSA and Asian viruses shifts the enhancement capacities, likely due to differences in epitope-antibody binding.
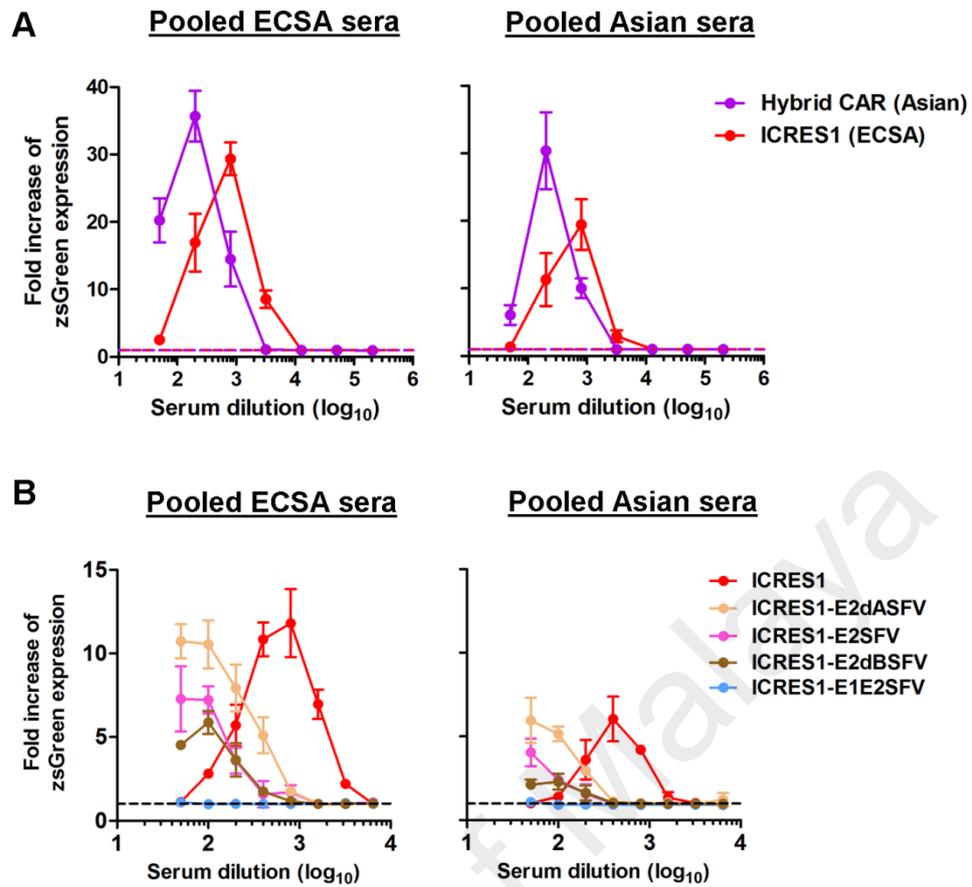
Figure 4.25: Antibodies targeting envelope E1 and E2 glycoproteins influence the enhancing capacity in ADE. (A) Pooled immune sera from ECSA and Asian panels were used to compare differences in enhancement of ICRES1 and hybrid CAR. The acquisition of zsGreen was performed after 24 hours. Experiments were performed in triplicate and data are presented as means ± SD from 2 independent experiments. The red and purple dotted lines indicate the fold change of zsGreen expression of the virus control relative to the mock control. (B) Pooled immune sera from ECSA and Asian panels were used to identify the critical regions in mediating ADE of chimeric viruses. The acquisition of zsGreen was performed after 24 hours. Experiments were performed in triplicate and data are presented as means ± SD. The black dotted line indicates the fold changes of zsGreen expression of the virus control relative to the mock control.

4.4 The neutralising role of IgM during the early phase of chikungunya virus infection
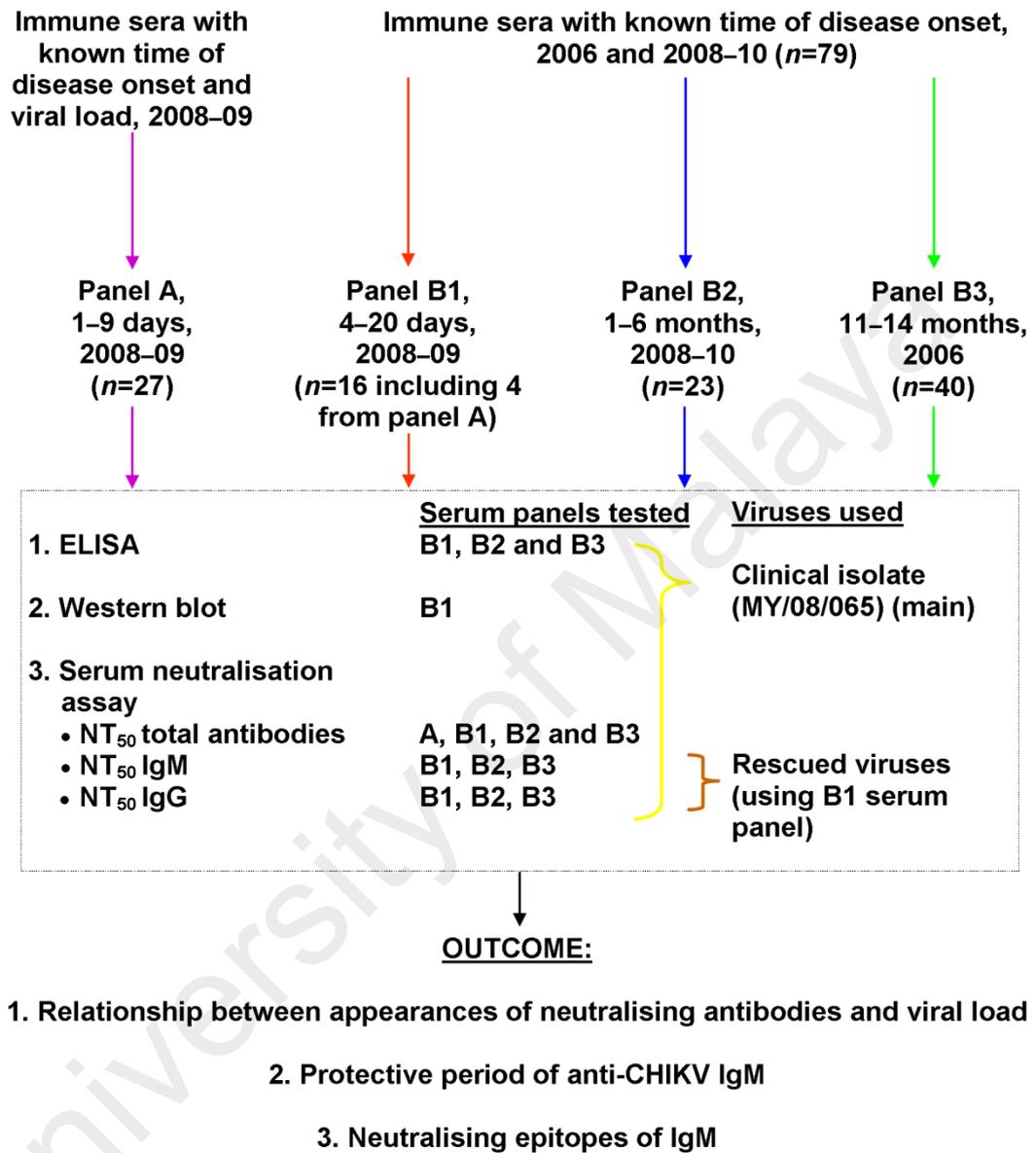
Figure 4.26: Flow chart for objective 4, neutralising role of IgM during the early phase of chikungunya virus infection.

4.4.1 Relationship between neutralising antibodies and viral load

In section 4.2 and 4.3, anti-CHIKV IgG isotype was shown to have protective and pathogenic roles. IgG persists life-long in individuals. In contrast, the IgM isotype is generally present for a shorter period of 1 to 3 months. A study was undertaken to study the exact role of anti-CHIKV IgM during early CHIKV infection.

The IgM and IgG titres were measured after IgG precipitation or IgM inactivation steps to ensure that the neutralising roles of IgM and IgG were examined independently. The efficacies of IgG precipitation and IgM inactivation were 96% and 80%, respectively (Figure 4.27). To understand the relationship between viraemia and the appearance of neutralising antibodies at different days of disease onset, viraemic serum samples with known viral load were examined for the presence of neutralising antibodies. Out of 27 viraemic samples in panel A, 10 samples had neutralising IgM, of which 4 samples had accompanying neutralising IgG (Figure 4.28A). The decrease in viral load corresponded to the rise of neutralising antibodies starting from day 6 after disease onset (Figure 4.28B). Neutralising IgM was detected in all immune sera from day 6 onwards; however, there was variation in neutralising IgG detected within the similar period from days 6 to 9.
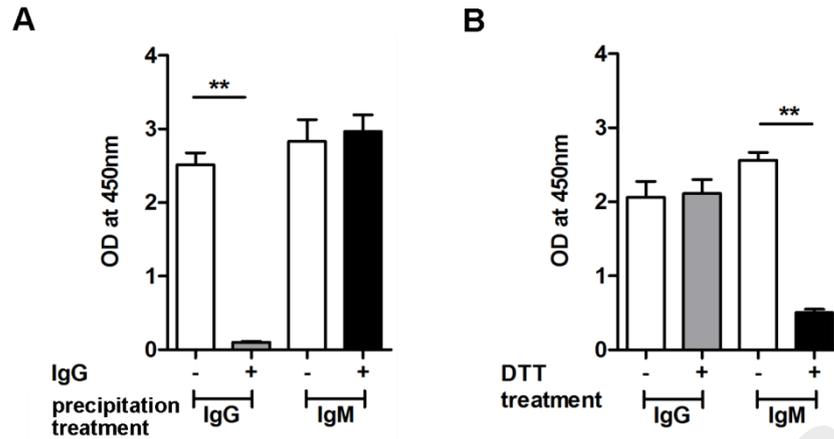
Figure 4.27: Efficacy of IgG precipitation and IgM inactivation in CHIKV immune sera. (A) ELISA to whole virus antigen was carried out to evaluate the efficacy of IgG precipitation from pooled acute sera at 1:100 dilution. The IgM level was maintained after treatment. Data are presented as mean + SD (n=3). **P<0.01, Mann-Whitney U test. (B) ELISA to whole virus antigen was carried out to evaluate the efficacy of IgM inactivation by DTT from pooled acute sera at 1:100 dilution. The IgG level was maintained after treatment. Data are presented as mean + SD (n=3). **P< 0.01, Mann-Whitney U test.
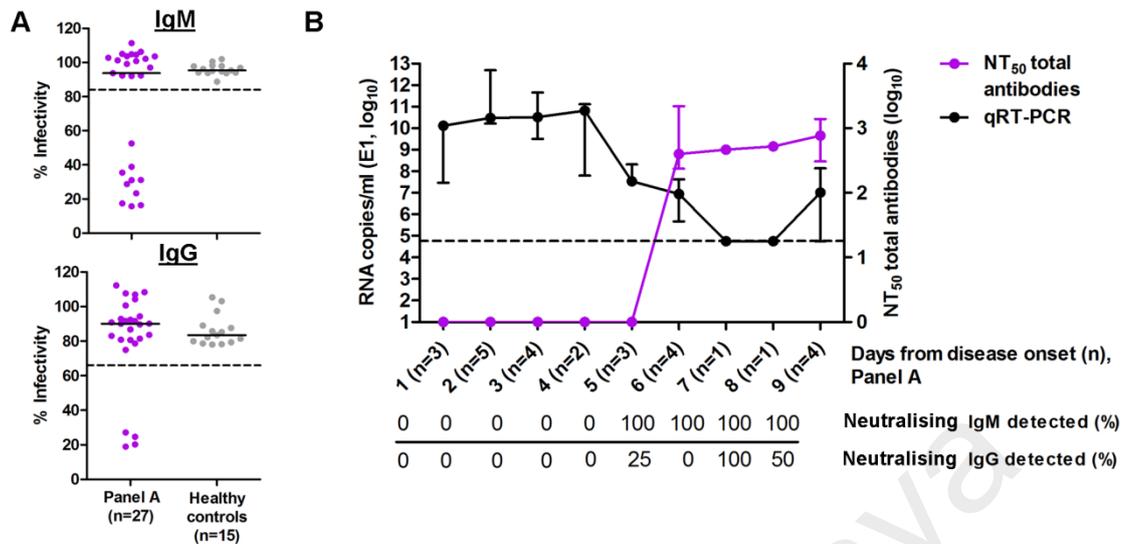
Figure 4.28: The appearance of CHIKV-specific neutralising antibodies is associated with a reduction of viraemia. (A) Neutralisation was performed on sera from panel A which had known viral loads. Experiments were performed in triplicate at 1:100 dilution for detection of neutralising IgM and IgG. The dotted lines represent the cut-off value determined from the mean - SD values from healthy control sera. (B) Neutralisation titres (NT_{50}) of total antibodies and previously-determined viral loads (Chiam *et al.*, 2013) of serum samples (panel A) collected at different times of disease onset were plotted. Each point indicates the median of RNA copies/ml targeting E1 and NT_{50}, with the inter-quartile range. The dashed line indicates the limit of quantification (1 $\log_{10}$ RNA copies/reaction or 4.76 $\log_{10}$ RNA/ml) of the E1 positive-strand PCR assay (Chiam *et al.*, 2013).

4.4.2 Duration of protection of anti-CHIKV IgM

The relative contributions of IgM and IgG towards neutralisation were further characterised using the 79 serum samples from panel B. Anti-CHIKV IgM and IgG seroreactivities were analysed using capture IgM and indirect IgG ELISA. Serum samples were categorised by collection time from disease onset, as either 4–20 days, 1–6 months, or 11–14 months. The anti-CHIKV IgM titres peaked during the early phase of infection (days 4–20) and waned over time (Figure 4.29A); IgM was detectable in most samples at 1–6 months, but was mostly undetectable by 11–14 months. Anti-CHIKV IgG titres rose from days 4 - 20 and were sustained up to 11–14 months (Figure 4.25A). The neutralising titres demonstrated similar patterns as the antibody titres, with the neutralising IgM waning over time while neutralising IgG was sustained up to 11-14 months (Figure 4.29B). IgG generally contributed the most to neutralising activity, but in 4 out of 16 samples from panel B1 (collected at days 4, 8, 9 and 10), the ratio of NT_{50} IgM/NT_{50}IgG was more than 1, indicating a predominant role for neutralising IgM (Figure 4.29C). These samples demonstrated that IgM plays a major neutralising role to inhibit virus infection in the presence of low IgG titres (Figure 4.29D). The inactivation of IgM and precipitation of IgG in immune sera resulted in major loss of neutralising activity (> 75% relative to virus control). Panel B1 was separated into 2 groups of low and high NT_{50} IgG for further analysis, based on Figure 4.29C. For panel B1 samples with low NT_{50} IgG, comprising the 4 samples for which neutralising IgM was predominant, the individual contributions of IgM or IgG towards neutralisation were significantly lower than the total antibodies neutralising capacity (Figure 4.29E). This indicates that the overall neutralising capacity was achieved with the combined presence of IgM and IgG acting in a complementary manner. As for panel B1 samples with high NT_{50} IgG, the total neutralising capacity was similar to neutralising capacity of IgG alone, with no significant additional effect of IgM (Figure 4.29E). Both low and high

NT$_{50}$ IgG groups had similar IgG titres against the whole virus antigen; however, the high NT$_{50}$ IgG group had higher IgG titre against rE2 (Figure 4.29F). These results suggest that anti-CHIKV IgM provides a short period of protection during the early phase of infection for a duration of up to 10 days (varying between patients), before the mounting of an effective and strong anti-CHIKV IgG response targeting E2 glycoprotein.
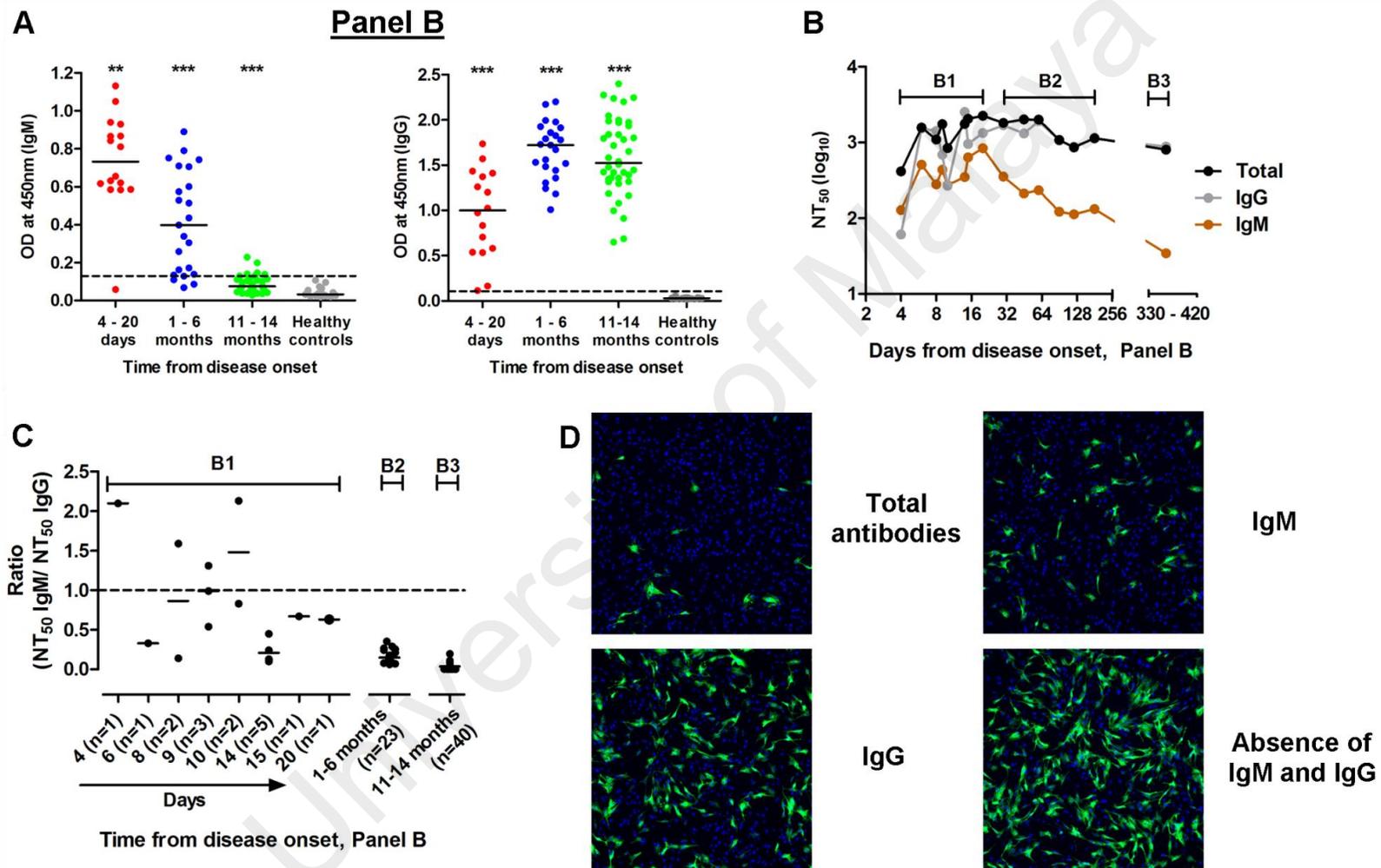
Figure 4.29: Anti-CHIKV IgM provides a short period of protection up to day 10 during early phase of infection.

Figure 4.29, continued: Anti-CHIKV IgM provides a short period of protection up to day 10 during early phase of infection. (A) Anti-CHIKV IgM and IgG were measured by ELISA. Samples were categorised by time from disease onset. **P<0.01, ***P<0.001, Mann-Whitney U test compared to healthy controls. The dotted lines represent the cut-off value. One sample in panel B1 had an optical density reading below the cut-off value, but the NT_{50} IgM was 74. (B) The neutralising titres of IgM, IgG and total antibodies were plotted by days from disease onset. Each point indicates the median of NT_{50}. (C) Ratios of NT_{50} IgM over NT_{50} IgG were calculated and plotted for serum samples by times from disease onset (panel B). A ratio of 1 indicates equal strength of NT_{50} IgM over NT_{50} IgG. (D) Representative immunofluorescent microscopic images of CHIKV-infected cells incubated with 1:100 diluted serum under different treatment conditions. Objective magnification: $5 \times$ from 1 field of view. The serum sample used was from panel B1, collected 4 days post-onset of illness, with IgM and IgG titres of 20,480 and 4096, respectively.
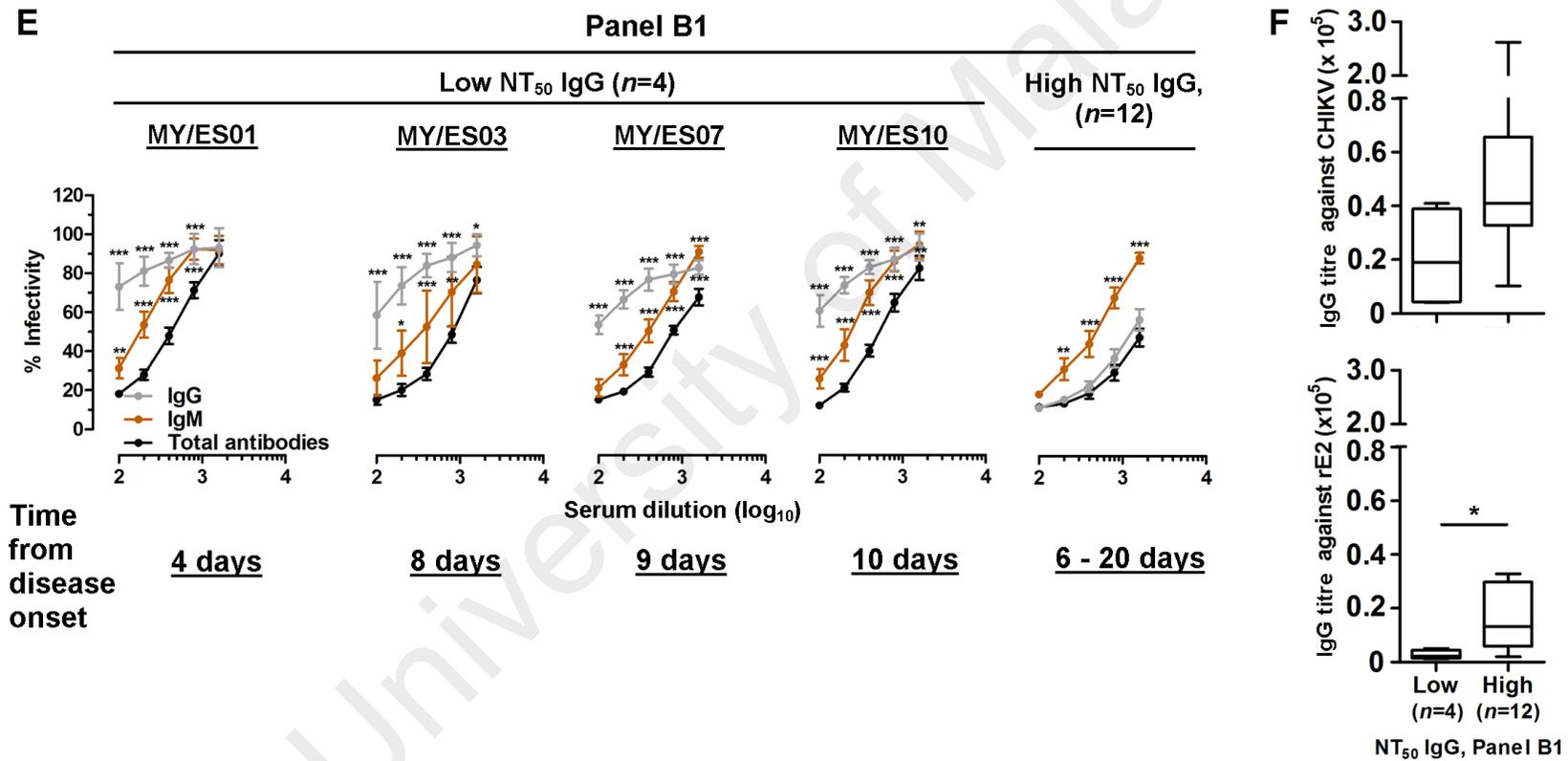
Figure 4.29, continued: Anti-CHIKV IgM provides a short period of protection up to day 10 during early phase of infection.

Figure 4.29, continued: Anti-CHIKV IgM provides a short period of protection up to day 10 during early phase of infection. (E) Panel B1 was divided into groups with either high or low NT_{50} IgG. Neutralisation of virus infectivity due to IgM or IgG was compared to neutralising capacity due to total antibodies. Results are expressed as percentage of virus control. *$P<0.05$, **$P<0.01$, ***$P<0.001$ by two-way ANOVA with Bonferroni multiple comparisons test. Data are presented as mean $\pm$ SD for individual samples with low NT_{50} IgG and mean $\pm$ SEM for 12 samples with high NT_{50} IgG. (F) Panel B1 was divided into groups with either high or low NT_{50} IgG ratio. IgG titres against CHIKV and rE2 were measured. *$P<0.05$, Mann-Whitney U test. The lines in the middle of the boxes indicate medians; the upper and lower boundaries of the box indicate inter-quartile ranges; and the whiskers indicate ranges of values.

4.4.3 Neutralising epitopes of anti-CHIKV IgM

To further understand the neutralization characteristics of IgM, the target epitopes of IgM were investigated using the serum samples from panel B1 against the individual structural envelope glycoproteins. Immunoblotting analysis was performed under non-reducing conditions. At 1:100 serum dilution, anti-CHIKV IgM poorly recognised rE1 and rE2 glycoproteins with undefined bands, but strongly bound to the whole virus antigen, with a band of 50 kDa consistent with E1 or E2 (Figure 4.30A). At higher dilution (1:400), the recognition was only retained against the whole virus antigen, but not against rE1 or rE2 glycoprotein. The poor reactivity of IgM on western blot against rE1 and rE2 was supported by capture ELISA (Figure 4.30B), which showed that more samples had below detectable IgM responses to either rE1 (6 samples) or rE2 (5 samples) compared to whole virus antigen (1 sample; Figure 4.29A). Notably, 4 serum samples with below detectable IgM responses against rE1 or rE2 were from the same patient, yet had detectable IgM against the whole virus. This indicates that IgM can recognise epitopes on rE1 or rE2, but possibly binds to a combination of both glycoproteins. To determine the importance of epitopes resulting from interactions between E1 and E2 glycoproteins, immunoblotting was performed using a fusion E1-E2 glycoprotein as an antigen (rE2-E1-ECSA). At 1:400 serum dilution, IgM recognised this fusion protein under non-reducing conditions (Figure 4.30C) in contrast to individual rE1 or rE2 proteins (Figure 4.30A). The recognition by IgM was diminished when the fusion protein was subjected to reduction by DTT, which would lead to loss of conformation. To further verify the target epitopes of IgM, a neutralisation assay was performed with previously constructed chimeras which had the ecto-domain regions of the CHIKV E2 and E1-E2 glycoproteins swapped with those of SFV. Another parallel experiment was carried out to compare the target epitopes of early IgG. Loss of neutralisation was expected as E1 and E2 are known to contain the main neutralising

epitopes for CHIKV. Loss of neutralisation by both IgM and IgG was observed against the chimera with SFV E2 (Figure 4.30D). Using the chimera with SFV E1-E2 resulted in a significant additional loss of neutralisation activity of IgM, but not of IgG (Figure 4.30D). This shows that neutralising IgM targets epitopes on both E2 and E1-E2 glycoproteins, while the early neutralising IgG mainly targets epitopes on E2. Taken together with the results from antibody binding studies and neutralisation, IgM preferably recognised epitopes spanning E1-E2 glycoproteins, rather than epitopes on individual E1 or E2 glycoprotein.
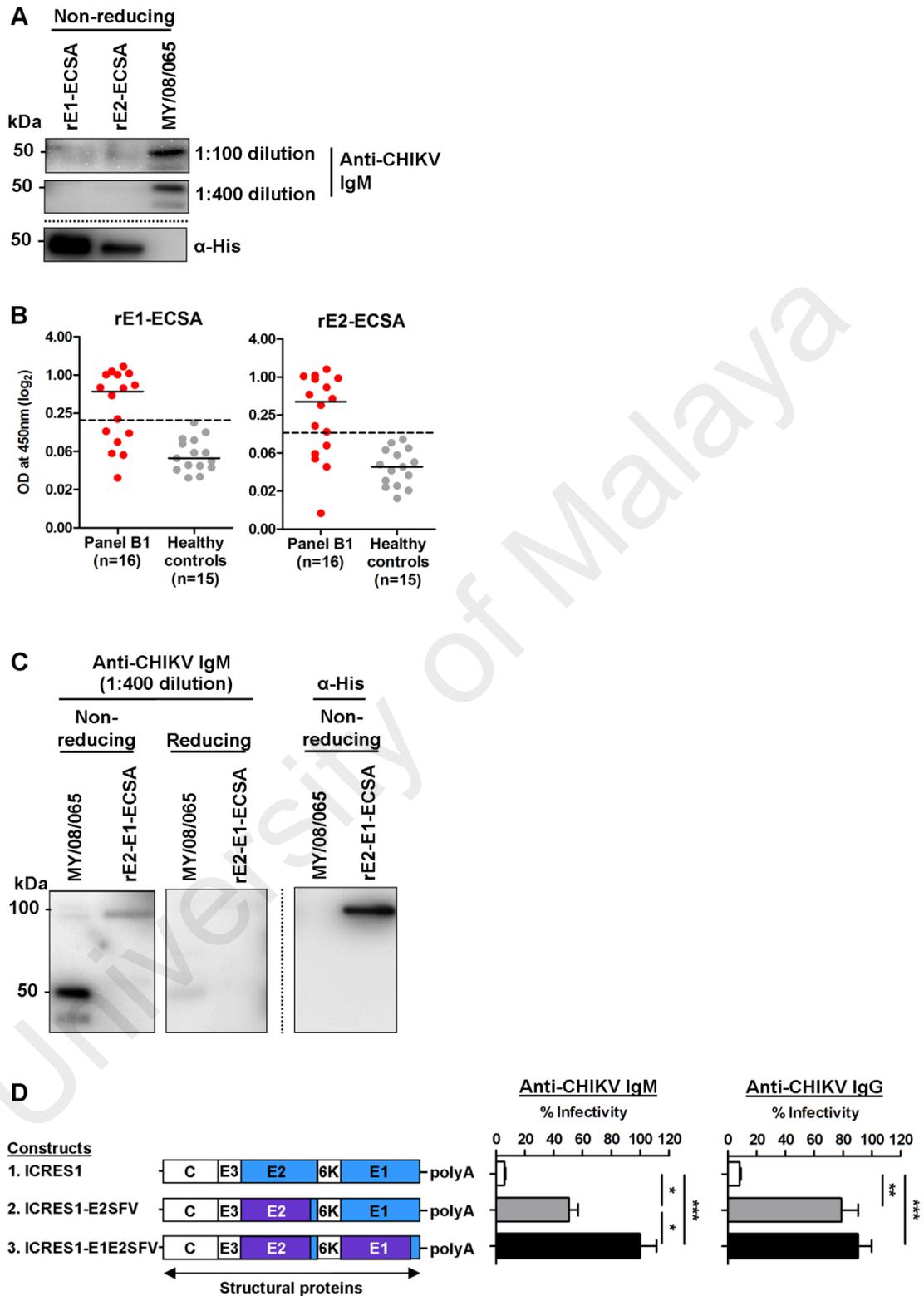
Figure 4.30: Anti-CHIKV IgM preferably targets epitopes on E1-E2 glycoproteins.

Figure 4.30, continued: Anti-CHIKV IgM preferably targets epitopes on E1-E2 glycoproteins. (A) IgM immunoblotting was performed using approximately 1 µg of recombinant protein (E1 and E2) and 2.5 µg of whole virus antigen under non-reducing conditions at serum dilutions of 1:100 and 1:400. Mouse anti-His (α-His) was used as a control. (B) IgM reactivity against rE1 and rE2 in capture ELISA was investigated. The experiments were performed at 1:100 serum dilution. The dotted lines represent the cut-off value. (C) Immunoblotting was performed under non-reducing and reducing conditions at a serum dilution of 1:400 against fusion recombinant E2 and recombinant E1 glycoprotein (rE2-E1-ECSA). (D) Schematic diagram showing the chimeras used in neutralisation with comparison of infectivity using pooled serum samples from panel B1. Data are presented as means ± SD from 4 independent experiments at a serum dilution of 1:100. *$P<0.05$, **$P<0.01$, ***$P<0.001$, Kruskal-Wallis test.

CHAPTER 5: DISCUSSION

5.1 Production and characterisation of mouse monoclonal antibodies

In this first objective of the study, a panel of E2-reactive mAbs was generated and characterised for subsequent use in immunological assays. The epitopes recognised by the mAbs were identified by peptide-based ELISA, and the epitopes positions were located. The E2 glycoprotein was chosen as it is the major viral protein involved in host-cell receptor interaction by alphaviruses (Smith *et al.*, 1995), and the major target of the immune response against CHIKV (Kam *et al.*, 2012b). The E2-reactive antibodies in CHIKV human immune sera could strongly recognise rE2 protein in Western blotting. The E2 glycoprotein belongs to the immunoglobulin superfamily, and consists of 3 structural domains: domain A (spanning residues 16-133), domain B (spanning residues 173-230), and domain C (spanning residues 271-341) (Voss *et al.*, 2010). Bréhin *et al.* (2008) described the first production of mouse monoclonal antibodies against CHIKV. Several mAbs that target domain A and domain B have been described, which block fusion and inhibit the interaction with the cellular receptor (Pal *et al.*, 2013; Sun *et al.*, 2013); however, the mAbs in this study that target linear epitopes distributed on these regions are non-neutralising. Some of the mAbs in this study targeted linear epitopes which were also found to be B-cell linear epitopes shared between human and mice (Lum *et al.*, 2013), such as [301]GEEPNYQEEW[310], [321]VPTEGLEVTW[330], and [331]GNNEPYKYWP[340] distributed in domain C. It is most likely that the major neutralising sites on the CHIKV E2 glycoprotein are present in a conformational-dependent manner instead of as linear epitopes. A single linear peptide "E2EP3", derived from the N-terminus of E2 glycoprotein, has been shown to induce neutralising antibodies in humans, and antibodies which provide protective immunity in a mouse model (Kam *et al.*, 2012b).

Binding activities of the mAbs were assessed by various immunological assays. Indirect ELISA and immunofluorescence assay demonstrated that mAbs A-A9(A1) and B-D2(C4) have superior binding to native virus particles compared to the other mAbs. Poor antibody affinity could be an explanation for the weak reactivity of mAbs C-B2(C9) and F-G6(F6) despite their epitopes being exposed at the surface tips of the spikes, while mAb A-A9(A1) could possess a strong affinity for its epitope, despite its unexposed location within the E1-E2 heterodimers. A further possibility is that the number of accessible epitope sites could be different in native virus particles compared to endogenous newly synthesised viral proteins, due to alteration of structural proteins under different conditions. The ELISA results support this possibility, as none of the mAbs except A-A9(A1) and B-D2(C4) had achieved binding saturation at 10^3 ng of CHIKV (the highest amount used), suggesting that the binding sites had not been fully occupied. It will be of interest for future study to compare the reactivity of these mAbs against infected cells without detergent (Triton X-100) treatment in order to determine if these antibodies can recognise linear E2 epitopes at the plasma membrane.

The homologous sequences of each epitope were compared with other representative alphaviruses in the Semliki Forest complex, including different genotypes of CHIKV, in an attempt to predict cross-reactivity. There were a number of differences between CHIKV and the other alphaviruses, except for the epitopes [16]LAHCPDCGEGHSCHS[30] and [166]EIEVHMPPDT[175], which were identical in CHIKV and ONNV. CHIKV and ONNV have been previously described as sharing one-way cross-reactivity, with anti-CHIKV antibodies reacting against both CHIKV and ONNV (Powers *et al.*, 2000). This likely cross-reactivity would limit potential diagnostic applications of the three mAbs involved (C-B2(C9), B-D2(C4) and N-B10(B11)) in countries where both viruses circulate. At present, ONNV has only been described in East, West and Central Africa (Powers *et al.*, 2000; Posey *et al.*, 2005). The homologous sequences were also

compared within representatives of different genotypes of CHIKV. Epitopes derived from the West African genotype have at least 1 amino acid difference from the other genotypes, while epitopes [321]VPTEGLEVTW[330] (mAb E-G6(A6)) and [331]GNNEPYKYWP[340] (mAb D-E3(D8)) are well conserved within CHIKV, and differ from ONNV (1 amino acid difference) and the other alphaviruses. These mAbs E-G6(A6) and D-E3(D8) could be useful in diagnosis or surveillance, to differentiate CHIKV from other alphaviruses and arboviruses which share similar clinical manifestations (Sam *et al.*, 2011), although the mAb specificities should be confirmed.

5.2 Characteristics of cross-neutralising antibody efficacy and epitopes of antibodies in human immune sera

CHIKV has become a major public health concern worldwide and causes considerable socio-economic burden. Protective adaptive immunity is mainly provided by specific antibodies, particularly those directed against epitopes on the E2 and E1 glycoproteins (Voss *et al.*, 2010; Mallilankaraman *et al.*, 2011). Understanding cross-immunity resulting from infections with different genotypes is particularly important and timely. Many Asian countries now have both endemic Asian and epidemic ECSA strains circulating, and the recent widespread outbreaks in the Americas are due to the Asian genotype rather than the previously epidemic ECSA strains, indicating that viruses from both genotypes are capable of global spread.

In this second objective of the study, the differences in cross-genotypic neutralisation efficacy of immune sera against ECSA and Asian genotypes of CHIKV were demonstrated and the key amino acids which alter the neutralisation potential were identified. Both ECSA and Asian serum had greater neutralising capacity against ECSA genotype (MY/08/065 and ICRES1) than Asian genotype (MY/06/37348 and CAR), indicating that neutralising antibodies regardless of initial infecting genotype

preferentially recognised the epitopes presented by the ECSA genotype. The presence of cross-genotype neutralisation was clearly shown lasting up to 14 months post-infection. The clinical significance of the differential cross-protective capacity of ECSA and Asian sera remains unclear, as all the immune sera had more than the minimum neutralising titre (≥10) which appears to correlate with immune protection from symptomatic CHIKV infection in humans (Yoon *et al.*, 2015). This high degree of cross-neutralisation likely contributed to the geographic restriction of CHIKV of different genotypes seen historically, which limited, for example, the spread of ECSA viruses in Asia, at least until CHIKV underwent mutations that facilitated sequential adaptation to the *Aedes albopictus* vector (Vazeille *et al.*, 2007; Tsetsarkin *et al.*, 2011; Tsetsarkin and Weaver, 2011).

Apart from the stronger antigenicity of epitopes of the ECSA genotype, neutralising capacity was also affected by the target and the amount of neutralising antibodies. Both ECSA and Asian sera contain high levels of neutralising antibodies to numerous linear epitopes on the E2 glycoprotein as well as conformational epitopes on the E1-E2 heterodimer complex. This supports recent findings that most of the reported CHIKV neutralising monoclonal antibodies target conformational epitopes on the exposed, topmost outer surfaces of the E2/E1 spike, particularly in domain A and domain B (Fong *et al.*, 2014; Fox *et al.*, 2015; Long *et al.*, 2015; Smith *et al.*, 2015). The findings also suggest that subunit vaccine candidates derived from E1 or E2 glycoproteins alone (Khan *et al.*, 2012; Kumar *et al.*, 2012; Weber *et al.*, 2015) may be insufficient to provide full protection against all genotypes, and that virus-like particle vaccines which present epitopes on E2/E1 in their native configuration may preferentially induce the most highly protective immune response (Akahata *et al.*, 2010; Brandler *et al.*, 2013; Metz *et al.*, 2013; Chang *et al.*, 2014).

The loss of neutralisation activity against chimeric CHIKV is in line with the finding that total IgG and anti-rE2 antibody titres correlate with the neutralising titre of Asian serum, suggesting that most of the neutralising epitopes are on the E2 glycoprotein. The lack of correlation between anti-rE2 antibodies and neutralising antibodies seen in ECSA serum could be due to the greater importance of conformational epitopes at E1-E2 sites, but it could be differences in potency/quality of the circulating antibodies due to the different timings of collection between the Asian and ECSA serum panels. Correlation between serum neutralisation titres and antibody binding titres has been reported in other viral infections such as dengue and influenza (Puschnik *et al.*, 2013; Pedersen *et al.*, 2014), and is important for developing serological assays which are accurate correlates of protective immunity following infection or vaccination. Therefore, E2, while appropriate for serological assays to diagnose acute or past infection (Cho *et al.*, 2008), may not be a suitable candidate for assays to measure protective immunity due to all CHIKV genotypes. Such assays are necessary for vaccine development.

Amino acid changes in key epitope regions, such as naturally occurring mutations or antigenic variation between different genotypes could affect surface charge distribution and electrostatic interactions between epitopes and antibodies, affect binding affinity and ultimately alter neutralising capacity (Kam *et al.*, 2012a). The E211K mutation in domain II of the E1 glycoprotein is a significant change of a negatively-charged to positively-charged amino acid, and this appears to enhance antibody binding and neutralisation efficacy. During the recent Indian outbreak of ECSA CHIKV, the key amino acid change E1-K211E was shown to be under positive selection pressure (Sumathy and Ella, 2012), which may confer a selective advantage for virus dissemination and escape from the action of neutralisation in humans. In addition, E211 is highly conserved in strains of the Asian genotype. Peptide-specific rabbit polyclonal antibody prepared against a short linear epitope (GDIQSRTPESKDVY, position 201-

214) including 211K did not show neutralisation activity, suggesting that the neutralising activity of immune sera targeting this amino acid is highly conformation-dependent (Appendix C). As for the E2 glycoprotein, I2T, H5N, G118S and S194G changes increased antibody binding and neutralisation efficacy. All these amino acid changes are positioned within linear epitopes, which interacted with neutralising antibodies. This was supported by a previous report of well-characterised human neutralising monoclonal antibodies targeting epitopes that cluster around the LP24 and LP38 peptide regions in our study (Smith *et al.*, 2015). Notably, the linear epitope LP1 in this study is similar to E2EP3, a well-characterised key neutralising linear epitope which has been suggested as a serology marker (Kam *et al.*, 2012b; Kam *et al.*, 2015), and LP1 demonstrated cross-reactivity with ECSA and Asian serum in this study. However, there was no effect of K252Q on antibody binding capacity in this Malaysian cohort, although this was reported recently (Kam *et al.*, 2012a), and this could be due to different immune responses in different populations. Other linear epitopes (LP19, LP47, LP56 and LP70) were identified in this study which had higher binding than LP1, and as all demonstrated binding to both ECSA and Asian sera, they may be potential candidates for diagnostic serological assays. Furthermore, antibodies against LP19 and LP47 demonstrated neutralising characteristics which warrant further investigation as vaccine candidates.

It was interesting that the Asian serum had greater neutralising capacity against the heterologous ECSA isolates. The previously reported human CHIKV monoclonal antibodies 5F10 and 8B10 had a broad neutralisation activity against isolates of the ECSA and West African genotypes, but were also less potent against an Asian isolate from Indonesia (Warter *et al.*, 2011). Monkeys inoculated with a virus-like particle vaccine derived from the West African strain 37997 also developed better neutralising activity to a heterologous ECSA strain LR2006 OPY-1 than to 37997, possibly due to

better presentation of conserved epitopes by LR2006 OPY-1 (Akahata *et al.*, 2010). ECSA and Asian CHIKV genotypes could have induced different immune mediator profiles; as shown in mice, infection with a Caribbean (Asian) strain was associated with a weaker pro-inflammatory Th1 and natural killer cell response and higher IgG1:IgG2c ratio compared to an ECSA CHIKV strain, resulting in less severe joint pathology (Gardner *et al.*, 2010; Teo *et al.*, 2015). Different CHIKV viruses may also trigger differential regulation of key innate immune responses such as TLR3 (Priya *et al.*, 2013), which plays an important role in shaping subsequent neutralising capacity (Her *et al.*, 2015). Further studies are needed to understand how differentially-induced immune mediators modulate the properties of circulating serum antibodies.

Two amino acids in LP1 (2T, 5N) of the ECSA virus are critical for binding and neutralisation activity, and this further highlights the fact that sequence variation could impact vaccine development. The rabbit polyclonal antibody targeting the linear neutralising epitope LP1 from the ECSA virus showed reduced cross-neutralisation against the Asian genotype, and unexpectedly, rabbit anti-LP1A from 4 different rabbits poorly neutralised the homotypic CAR Asian virus. The linear neutralising epitope LP1A from the Asian virus was not recognised by the ECSA sera. However, clearly there are preexisting antibodies against LP1 and LP1A in the Asian sera. LP47, another linear neutralising epitope in humans, which has a sequence that is conserved in both genotypes, did not induce any functional neutralising antibodies in rabbits despite a similar immunisation approach. Future studies will be required to address these apparent underlying differences of neutralising antibody production from either natural infection or immunisation. Nevertheless, the findings indicate that the choice of virus strain for vaccines could impact the spectrum and efficacy of protection across genotypes. For antibody therapy of CHIKV, monoclonal antibodies should retain high potency against a broad diversity of CHIKV isolates (Smith *et al.*, 2015).

5.3 Evidence for antibody-dependent enhancement (ADE) of chikungunya virus infection

Following viral infection, the host immune response leads to production of different types of antibody (e.g. neutralising, non-neutralising, enhancing, non-enhancing, poly-reactive) which can influence anti-viral efficacy (Takada and Kawaoka, 2003; Warter *et al.*, 2012). Rational vaccine design aims to induce antibody production equivalent to naturally-acquired infection; at least a minimum level of protective antibodies needs to be achieved, which is highly dependent on candidate vaccine strains and immunisation parameters. However, little is known about the potential impact of non-protective antibodies which may be present in naturally-acquired or vaccinated individuals.

In the third objective of this study, CHIKV infectivity of poorly permissible K562 cells could be enhanced in the presence of immune sera, demonstrating the phenomenon of antibody-dependent enhancement (ADE). The surface expression of FcγRIIA facilitates the entry of virus-antibody complex (which can be blocked by anti-CD32 antibodies) and subsequent virus production. Humans express nine different FcγR and their binding affinities to immune complexes are dependent on the IgG subclasses. Each receptor carries an immunoreceptor tyrosine-based activation motif (ITAM) or immunoreceptor tyrosine-based inhibitory motif (ITIM) or neither (Bruhns, 2012; Gillis *et al.*, 2014; Chan *et al.*, 2015; Taylor *et al.*, 2015). Surface FcγRIIA carrying ITAM is widely expressed on immune cells, especially monocytes and macrophages, which are the natural targets of CHIKV (Sourisseau *et al.*, 2007a; Her *et al.*, 2010; Hoarau *et al.*, 2010; Labadie *et al.*, 2010; Teng *et al.*, 2012). This study provides evidence for ADE in the presence of sub-neutralising antibody titres, suggesting that a primary CHIKV infection or suboptimal vaccination with a poor antibody response might potentially allow more immune cells to take up virus-antibody complexes and become infected, rather than neutralising the virus. ADE could potentially aggravate pathologic

conditions such as joint swelling and inflammation, or induce a higher viral load in infected individuals which may enhance transmission to mosquitoes. Three cases of possible secondary infection (re-infection) with CHIKV have been reported in humans, although the clinical manifestations did not appear to be unusually severe (Kosasih *et al.*, 2013). Detailed study of the immune responses in these patients was not reported, although one was stated to have unusually rapid acute IgM and IgG responses. A recent CHIKV vaccine study showed that post-vaccine virus challenge of immune mice with suboptimal antibody titres led to foot swelling, suggesting the enhancement of inflammation (Hallengärd *et al.*, 2014a; Hallengärd *et al.*, 2014b).

CHIKV neutralisation and ADE were demonstrated to be dependent on serum dilution. Neutralisation is dose-dependent in a sigmoidal pattern, while ADE is detected at sub-neutralising concentrations of the neutralising antibody in a Gaussian pattern. The nature of the neutralising antibodies is not known. The peak ADE shifted from low to high serum dilutions in parallel with increasing neutralising titres of the samples, which likely reflects the dominant effect of enhancing antibodies over the neutralising antibodies when diluted to a critical level. However, the likelihood of *in vivo* neutralisation or protection cannot be accurately determined based on *in vitro* neutralisation which utilises non-FcγR-bearing cells. Such *in vitro* assays are necessary to detect the balance of enhancing and neutralising antibodies and to assess the minimum protective antibody level required using FcγR-expressing cells for vaccine development (Moi *et al.*, 2010a; Moi *et al.*, 2010b; Sirivichayakul *et al.*, 2014; Byers *et al.*, 2015). In flaviruses such as dengue and West Nile viruses, other arboviruses which also demonstrate ADE, the action of neutralisation and ADE are affected by epitope specificity, epitope accessibility, numbers of antibodies engaged on a virion, antibody subclasses, complement component and virus conformational changes (Pierson *et al.*, 2007; Rodrigo *et al.*, 2009; reviewed in Dowd and Pierson, 2011). Furthermore, ADE

could exploit alternative pathways, such as deployment of cell-intrinsic mechanisms to enhance virus infection by increasing synthesis of the anti-inflammatory cytokine IL-10, suppresion of the activity of antiviral type 1 IFN and hampering TLR signalling (Lidbury and Mahalingam, 2000; Mahalingam and Lidbury, 2002; Suhrbier and La Linn, 2003; Chareonsirisuthigul *et al.*, 2007; Modhiran *et al.*, 2010; Ubol *et al.*, 2010; Tsai *et al.*, 2014).

To minimise the genetic variation due to laboratory adaptation for the ADE assays, low passage recombinant rescued viruses were used in this study, instead of clinical isolates which would be heterogenous populations (Chaichana *et al.*, 2014; Wikan *et al.*, 2015). The ADE of rescued ECSA virus (ICRES1) was clearly shown in K562 and electroporation with infectious RNA into K562 led to active virus production. However, ADE of rescued Asian virus (CAR) was unable to be shown as it did not readily infect K562 cells. Several possible reasons could account for the inability of CAR/Asian virus to replicate in K562 cells, such as the presence of an opal codon in the non-structural region, deletion of amino acids in nsP3, and differences in 3' UTR. The presence of an opal stop codon at the junction of nsP3 and nsP4 (preceding the nsP4) determines if either polyprotein P123 or P1234 is synthesised, which will be cleaved into non-structural proteins (Strauss and Strauss, 1994; Solignat *et al.*, 2009). The translation terminates at the stop codon and results in P123, while read-through of the stop codon occurs with 5 to 20% efficiency and results in P1234 (de Groot *et al.*, 1990; Shirako and Strauss, 1994; Firth *et al.*, 2011). The lack of nsP4 due to premature termination of translation could impair the formation of the replication complex and stall the whole replication process (Kim *et al.*, 2004), while the mechanism of read-through could be cell type-dependent (Li and Rice, 1989). The locus of this stop codon exists in SINV, VEEV, RRV and some isolates of CHIKV, especially in the Asian genotype (Strauss *et al.*, 1988; Firth *et al.*, 2011; Sam *et al.*, 2012; Tan *et al.*, 2015; Stapleford *et al.*, 2016);

while some isolates of SFV, CHIKV and ONNV carry an arginine codon (Lanciotti *et al.*, 1998; Tuittila and Hinkkanen, 2003; Myles *et al.*, 2006; Schuffenecker *et al.*, 2006). Recent CHIKV isolates of the Asian genotype have nsP3 deletions starting at position 376 or 379 and ranging from 4 - 7 amino acids (Sam *et al.*, 2012; Leparc-Goffart *et al.*, 2014; Teo *et al.*, 2015; Chen *et al.*, 2016), which are not present in ECSA isolates. The effect of these nsP3 deletions remains unclear. As for the 3'UTR, the Americas lineage of the Asian genotype has a distinct and longer sequence compared to ECSA and West African genotypes (Chen *et al.*, 2013). However, the variant of 3' UTR is unlikely to affect virus replication in mammalian cells (Stapleford *et al.*, 2016). Therefore, further work is required to identify the key factors resulting in impaired replication of Asian virus. Alternatively, other cell types could be included to study ADE which can support both ECSA and Asian virus replication.

An important observation from this study was that antibodies targeting envelope glycoproteins influence the enhancing capacity in ADE. For vaccine and therapeutic antibody development, the potential of ADE occurrence should be kept to a minimum or avoided. Immune enhancement of disease could be prevented by manipulation of epitopes of the antigen that induce enhancing antibodies or removal of carbohydrate/sugar chains from the heavy chains of antibodies (Goncalvez *et al.*, 2007; Balsitis *et al.*, 2010; Hughes *et al.*, 2012). There were similar levels of ADE of infection using human serum against CHIKV backbone (pCMV-ICRES1-2SG-zsGreen) viruses with either ECSA or Asian (CAR) envelope glycoproteins, suggesting both viruses share common epitopes that mediate ADE. However, differential antibody binding to the glycoproteins of ECSA or Asian virus influenced the peak enhancements at different serum dilutions. Therefore, further study is required to fully understand and mitigate the underlying risk of ADE following natural infection or vaccination.

5.4 The neutralising role of IgM during early chikungunya virus infection

Neutralising antibodies in viral immunity provide protection by clearing viraemia. Other effector functions of IgM and IgG are complement activation, opsonisation of antigens for phagocytosis and antibody-dependent cell-mediated cytotoxicity (Racine and Winslow, 2009; Nimmerjahn and Ravetch, 2010). Neutralising IgG in CHIKV infection, which persists for a long period, has been studied (Lum *et al.*, 2013; Teo *et al.*, 2013; Poo *et al.*, 2014b), particularly from the viewpoint of eliciting protective immunity by vaccination. However, the role of anti-viral IgM, which appears earlier and is normally present for only up to 3 months, is less well understood.

In this fourth objective of the study, the roles of anti-CHIKV IgM and IgG from immune sera were defined independently, and both antibody isotypes were shown to have neutralising characteristics similar to previously reported humanised/mouse monoclonal antibodies (Fric *et al.*, 2013; Pal *et al.*, 2013; Selvarajah *et al.*, 2013; Fox *et al.*, 2015; Lam *et al.*, 2015; Smith *et al.*, 2015). In the cohort of acutely infected patients, anti-CHIKV IgM can be detected as early as day 4 (panel B1), and is present in most cases by day 6 (panel A), suggesting that the high valency of IgM could be important in reducing viraemia before the production of robust neutralising IgG with high affinity. A rapid decrease of viral load at day 5 was seen in the absence of detectable neutralising antibodies. While it is likely at this point that there were low levels of antibodies below the limit of detection of the neutralisation assay, there may also be a role for the innate immune response, involving the activation of cytokines and NK cells prior to full development of the adaptive immune response (Ng *et al.*, 2009; Hoarau *et al.*, 2010; Petitdemange *et al.*, 2011; Simarmata *et al.*, 2016). It was noted that in rare cases (*n*=7), IgM can persist in serum at 11−14 months, similar to reports in La Réunion and Indonesia (Grivard *et al.*, 2007; Borgherini *et al.*, 2008; Kosasih *et al.*, 2013). Studies have described an association of unusually persistent IgM with chronic arthralgia,

destructive arthritis and neurological complications (Malvy *et al.*, 2009; Kashyap *et al.*, 2010; Chusri *et al.*, 2011; Gauri *et al.*, 2012), but the pathophysiological significance of this is unknown. In this study, IgM demonstrated neutralising activity similar to IgG, albeit with a weaker neutralising effect compared to IgG at higher serum dilutions. It will be useful to further evaluate anti-CHIKV IgM as a prognostic marker and to investigate the functional role of IgM from patients with persistent joint disease (Mohd Zim *et al.*, 2013).

Between days 4–10, there was individual variation in the relative contributions of IgM and IgG to overall neutralisation capacity, with a few patients displaying predominant IgM and most others having predominant IgG. After day 10 from disease onset, IgM contributes minimally to overall neutralising activity, as neutralising IgG plays the dominant role. During this period of seroconversion (day 4 to 10), strong overall neutralising capacity can be achieved with synergistic binding action of IgM and IgG against circulating infectious virus. In previous studies, the early IgG response, but not IgM, was shown to target a well-characterised linear epitope E2EP3 on the N-terminus of E2 glycoprotein (Kam *et al.*, 2012b; Kam *et al.*, 2015), similar to linear epitope LP1 in the earlier finding of this study (objective 2). A supplementary experiment was carried out to demonstrate the complementary activities of neutralising IgM and specific anti-LP1 IgG, which reiterates the importance of IgM acting in synergy with specific early IgG (Appendix D). The complementary effect of rabbit anti-LP1 IgG was dose-dependent, while the addition of non-neutralising IgG to IgM did not have any effect on overall neutralising capacity (Appendix D). The timing of appearance and the amount of IgM and IgG may have significant impact on clinical outcome, and it has been shown that early neutralising IgG3 response has been associated with faster viral clearance and reduced risk of persistent arthralgia (Kam *et al.*, 2012c). For the clinical use of therapeutic antibodies, optimal epitope selection and

relative proportions of IgM and IgG should be carefully determined to ensure the optimum synergistic effect instead of competitive binding.

There has been no prior study of IgM epitopes for CHIKV. Current commercial diagnostic serology kits to detect IgM in acute samples showed poor sensitivity when using recombinant E1 glycoprotein alone as the antigen, showing the importance of epitopes on E2 (Rianthavorn *et al.*, 2010; Yap *et al.*, 2010; Blacksell *et al.*, 2011; Sam *et al.*, 2011; Kosasih *et al.*, 2012; Prat *et al.*, 2014; Sam *et al.*, 2015b; Burdino *et al.*, 2016). This was supported by the present study, in which the IgM recognises conformation-dependent and reduction-sensitive epitopes on the E1-E2 fusion glycoprotein. This finding provides the basis for development of an optimised native antigen for reliable IgM detection (Erasmus *et al.*, 2015; Johnson *et al.*, 2016; Prince *et al.*, 2016).

5.5 Limitations of the current study

To understand the nature of genotype-specific antibody immunity, this study utilised serum collected during past outbreaks of Asian and ECSA CHIKV. However, detailed comparative study could not be carried out due to different timings of collection of the samples and different cohorts (rural versus urban) for the ECSA and Asian serum panels. Furthermore, the serum samples were mainly single samples from patients. To fully study the immune response, including the effects of innate immunity in shaping adaptive immunity, a well-designed cohort with longitudinal collection of samples over time will be needed.

CHAPTER 6: CONCLUSION

This study characterised the human antibody responses to CHIKV. An in-house developed monoclonal antibody clone B-D2(C4) was chosen as an immunological tool for serum neutralisation and capture IgM ELISA assays. Serum samples from humans infected with CHIKV of either ECSA or Asian genotypes showed differences in neutralisation and binding capacities against representative clinical isolates and viruses rescued from infectious clones, with both ECSA and Asian sera found to be more effective in binding and neutralising ECSA virus. The key amino acids/epitopes (E1-E211K, E2-I2T, E2-H5N, E2-G118S and E2-S194G) within the E1-E2 surface glycoprotein were identified to impact the efficacy of antiserum in cross-neutralising ECSA and Asian genotypes of CHIKV. The choice of vaccine strain may impact cross-protection against different genotypes, as sequence variation of a known linear neutralising epitope influenced the spectrum of cross-neutralisation across genotypes. Individuals with naturally-acquired CHIKV (both ECSA and Asian genotypes) have enhancing antibodies in the sera that could promote ADE. The ADE capacity was greatly dependent on antibodies targeting the envelope glycoproteins. During the early phase of infection, CHIKV-infected individuals developed neutralising anti-IgM and anti-IgG which mediate the clearing of viraemia. Neutralising IgM is particularly important up to day 10 of infection, when it acts in a complementary manner with the early IgG, after which a robust neutralising IgG response consistently predominates. Anti-CHIKV IgM preferably recognises epitopes on the CHIKV surface E1-E2 glycoproteins rather than E1 or E2 individually. These findings have implications for the continued outbreaks of co-circulating CHIKV genotypes, choice of vaccine strain, effective design of vaccines, therapeutic antibodies and diagnostic serological assays.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Research articles and book chapter related to thesis:

1. Chua CL, Chan YF, Sam IC. Characterisation of mouse monoclonal antibodies targeting linear epitopes on Chikungunya virus E2 glycoprotein. **J Virol Methods** 2014; 195: 126-33.

2. Chua CL, Sam IC, Chan YF. Expression and purification of E2 glycoprotein from insect cells (Sf9) for use in serology. **Methods Mol Biol** 2016, 1426: 51-61.

3. Chua CL, Sam IC, Merits A, Chan YF. Antigenic variation of East/Central/South African and Asian chikungunya virus genotypes in neutralization by immune sera. **PLoS Neg Trop Dis** 2016, 10: e0004960.

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1. Sam IC, Chua CL, Chan YF. Chikungunya virus diagnosis in the developing world: a pressing need. **Expert Rev Anti Infect Ther** 2011; 9: 1089-91.

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