AUTOIMMUNITY IN THE PATHOGENESIS OF SEVERE DENGUE

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ABSTRACT

Dengue, an arboviral disease is transmitted between humans by the bite of infected female *Aedes* mosquitoes. Annually, about 50 million cases of dengue virus (DENV) infections were reported with approximately 500,000 cases of severe dengue and about 5% fatality rate. Understanding the mechanism leading to dengue or severe dengue is crucial, considering the fact that it is not completely comprehended. Autoimmunity has known to be involved in the pathogenesis of dengue. Here, we investigated the association of dengue and autoimmunity by comparing the genetic variant distribution among severe, non-severe dengue and systemic lupus erythematosus (SLE) patients using Ion Torrent sequencing method. Four significant variants were identified; novel variant at chr3:49679737 of BSN gene was associated with susceptibility to severe dengue, while polymorphisms rs76018112 of ABCF1 gene, rs1557370 of Mx1 gene and rs945635 of FCRL3 were associated with protective effects against severe dengue. Theoretical analysis on the effects of novel variant (chr3:49679737) suggests the importance of calcium release in the prevention of severe manifestation, whereas rs76018112, rs1557370 and rs945635 demonstrated that effective early antiviral responses and faster virus clearance associated with protective effects against severe dengue manifestation. SLE patients showed high distribution of all variants related to protection against severe dengue, suggesting that autoimmune disease patients might possess protective factors against acute dengue. We then analysed the protective factor of autoimmunity against dengue by determining the capability of SLE-sera to neutralize DENV using foci reduction neutralization test (FRNT). A total of 82 dengue serology negative sera of SLE patients were collected and FRNT against DENV was performed. Results revealed that 69%, 61% and 52% of the SLE patients' sera showed FRNT50 neutralization against DENV1, DENV2 and DENV3, respectively. SLE-sera significantly neutralized DENV

in comparison to healthy donors, though tested negative for dengue IgG/IgM antibodies, signifying that SLE patient might have an efficient antiviral response against DENV, perhaps via other antibody isotypes or autoreactive antibodies. We further investigated the association of dengue and autoimmunity by observing the role of high mobility group box 1 (HMGB1), a DNA-binding protein commonly linked to the progression of autoimmune diseases, in the pathogenesis of dengue. HMGB1 resides in the nucleus, but translocated out to the cytoplasm and extracellular milieu during DENV infection. HMGB1-knockdown cells showed higher DENV replication and lower interferon-stimulated genes (ISGs) production than wild-type cells, proposing the novel role of HMGB1 in the antiviral response against DENV, through the regulation of innate immune response. Resveratrol (RESV) treatment inhibits the translocation of HMGB1 via Sirt1, and the accumulation of nuclear HMGB1 consequently increased production of ISGs and reduced DENV replication, verifying the role of nuclear HMGB1 in regulating ISGs in the antiviral response against DENV. Nuclear HMGB1 was found to bind to the promoter region of ISG and positively regulated the expression of ISGs. Our findings highlighted the importance of efficient early antiviral responses and protective effects of autoimmunity against severe dengue progression. We also introduce the novel role of nuclear HMGB1 in the antiviral response and RESV as an antiviral drug against DENV.

Keywords: autoimmunity, dengue, antibody, HMGB1, resveratrol

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ABSTRAK

Denggi adalah sejenis penyakit arbovirus yang menjangkiti manusia melalui gigitan nyamuk Aedes betina yang dijangkiti virus denggi (DENV). Setiap tahun, kira-kira 50 juta kes jangkitan DENV dilaporkan, dimana sekitar 500,000 daripadanya adalah kes denggi kritikal dan 25,000 adalah kes kematian. Pemahaman berkenaan proses terjadinya denggi kritikal adalah masih samar, maka kajian untuk merungkai proses mendasari wabak tersebut adalah penting. Tindak balas autoimun telah diketahui terlibat dalam patogenesis wabak denggi. Oleh itu, kaitan antara denggi dan tindak balas autoimun disiasat didalam kajian ini dengan membandingkan taburan genetik polimorfisme di kalangan pesakit demam denggi kritikal, denggi tidak kritikal dan sistemik lupus erythematosus (SLE), menggunakan kaedah penjujukan Ion Torrent. Empat genetik polimorfisme telah dikenalpasti; polimorfisme unik pada chr3: 49679737 gen BSN cenderung kepada denggi kritikal manakala polimorfisme rs76018112 gen ABCF1, rs1557370 gen Mx1 dan rs945635 gen FCRL3 dikaitkan dengan perlindungan terhadap denggi kritikal. Analisis teori mengenai kesan polimorfisme unik pada chr3: 49679737 menunjukkan kepentingan kalsium dalam mencegah manifestasi denggi kritikal, manakala rs76018112, rs1557370 dan rs945635 menunjukkan bahawa tindak balas antivirus yang awal dan efisien, sehingga mampu untuk menyingkirkan virus dengan pantas adalah berkaitan dengan kesan perlindungan terhadap perkembangan denggi kritikal. Pesakit SLE menunjukkan taburan yang tinggi bagi setiap polimorfisme cenderung kepada perlindungan terhadap denggi kritikal, menunjukkan kemungkinan bahawa pesakit autoimun mempunyai faktor perlindungan terhadap denggi akut. Analisis mengenai faktor perlindungan tindak balas autoimun diteruskan dengan menentukan keupayaan serum pesakit SLE untuk meneutralkan DENV menggunakan ujian peneutralan pengurangan virus (FRNT). Sebanyak 82 serum pesakit SLE dengan serologi

negatif denggi dikumpulkan dan FRNT terhadap DENV dijalankan. Hasil kajian menunjukkan 69%, 61% dan 52% daripada sera pesakit SLE menunjukkan pengurangan FRNT50 terhadap DENV1, DENV2 dan DENV3, masing-masing. Serum pesakit SLE mampu meneutralkan DENV berbanding penderma sihat, walau diuji negatif bagi antibodi IgG/IgM denggi, menandakan pesakit SLE mungkin mempunyai tindak balas antivirus efektif terhadap DENV melalui antibodi jenis lain atau antibodi autoreaktif. Kaitan antara denggi dan tindak balas autoimun diteruskan dengan menyiasat peranan high mobility group box 1 (HMGB1), sejenis protein pengikat DNA yang berhubung kait dengan penyakit autoimun dan patogenesis denggi. HMGB1 terletak di dalam nukleus tetapi boleh berpindah ke sitoplasma dan luar sel semasa jangkitan denggi. Sel tanpa HMGB1 menunjukkan replikasi DENV yang lebih tinggi dan pengeluaran 'interferon-stimulated genes' (ISGs) yang lebih rendah daripada sel asal. Keputusan ini mengusulkan peranan HMGB1 dalam tindak balas antivirus terhadap DENV melalui pengawalan imun semulajadi (innate). Resveratrol (RESV) menghalang pemindahan HMGB1 dan mendorong pengumpulan HMGB1 di dalam nukleus, dan seterusnya menyebabkan peningkatan pengeluaran ISGs dan pengurangan replikasi DENV. Ini mengesahkan peranan HMGB1 di dalam nukleus dalam mengawal selia ISGs dalam tindak balas antivirus terhadap DENV. HMGB1 di dalam nukleus mengikat pada promoter ISG dan menyebabkan peningkatan dalam penghasilan ISG. Penemuan dalam kajian ini menekankan kepentingan tindak balas antivirus yang efisien dan faktor tindak balas autoimun dalam mempengaruhi perkembangan denggi kritikal. Kami juga memperkenalkan peranan unik HMGB1 nuklear dalam tindak balas antivirus dan RESV sebagai ejen antivirus DENV.

Kata Kunci: autoimun, denggi, antibodi, HMGB1, resveratrol

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°C	degree celsius
%	percent
ABCF1	ATP-binding cassette sub-family F member 1
ACR	American College Rheumatology
ADE	antibody-dependent enhancement
ADP	adenosine diphosphate
Ae	*Aedes*
ANOVA	analysis of variance
APC	antigen presenting cells
ATCC	American Type Culture Collection
bNAbs	broadly neutralizing antibody
BSN	bassoon
C	capsid
CA16	Coxsackie A16
CD	cluster of differentiation
CLEC5	C-type lectin domain family 5
CMC	carboxymethylcellulose
CO_2	carbon dioxide
DAB	3’ di-amino-benzidine
DENV	dengue virus
DENV1	dengue virus type 1
DENV2	dengue virus type 2
DENV3	dengue virus type 3
DENV4	dengue virus type 4
DF	dengue fever
DHF	dengue hemorrhagic fever
DMEM	Dulbecco’s modified eagle medium
DNTP	deoxyribonucleotide triphosphate
DSS	dengue shock syndrome
E	envelope
EDTA	ethylene-diamine-tetra-acetic acid
EGR1	early growth response protein 1
ER	endoplasmic reticulum
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular-signal-regulated kinases
EV71	Enterovirus 71

FBS	fetal bovine serum
Fc	fragment crystallizable
FCRL3	Fc receptor-like protein 3
FFU	foci forming unit
FRNT	foci reduction neutralization test
FRNT50	50% foci reduction neutralization test
g	relative centrifugal force
hCF	human cytotoxic factor
HI	hemagglutination inhibition
HIV	human immunodeficiency virus
HMGB1	high mobility group box 1
HRP	horseradish peroxidase
IFN	interferon
Ig	immunoglobulin
JAK	Janus kinase
JEV	Japanese Encephalitis virus
JNK	c-Jun N-terminal kinase
kb	kilobase
kDa	kiloDalton
LCMV	lymphocytic choriomeningitis virus
M	molar
mg	miligram
ml	mililiter
mM	milimolar
mmHg	milimeter of mercury
MOI	multiplicity of infection
Mx1	MX dynamin like GTPase 1
n	sample size
NS	nonstructural
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
OD	optical density
ORF	open reading frame
p.i.	post infection
PBS	phosphate buffered saline
PCR	polymerase chain reaction
pH	power of hydrogen
PI3K/Akt	Phosphoinositide 3-kinase/protein kinase B
qRT-PCR	quantitative real-time PCR

RNA	ribonucleic acid
RT-PCR	reverse transcription PCR
shRNA	short hairpin RNA
Sirt1	sirtuin
SLE	systemic lupus erythematosus
SLEDAI	SLE disease activity index
STAT	signal transducer and activator of transcription
TBEV	tick-borne encephalitis virus
TEMED	tetramethylethylenediamine
TM	trademark
UMMC	University Malaya Medical Centre
µg	microgram
µl	microliter
µm	micrometer
w/v	weight per volume
WHO	World Health Organization
WNV	West Nile Virus

CHAPTER 1: INTRODUCTION

1.1. Dengue pathogenesis and autoimmunity

The pathology of dengue disease includes both virus and host immune response factors (Wan *et al.*, 2013). Dengue virus (DENV) comprises of four antigenically distinct serotypes, DENV1, DENV2, DENV3 and DENV4. Based on the phylogenetic study of the envelope protein, each serotype was further characterized into separate genotype (Chen *et al.*, 2011). Each serotype of DENV showed different capacity in inducing severe dengue (Guzman *et al.*, 2010a; Yung *et al.*, 2015). The susceptibility of certain cells also influences the infectivity of the virus. Dendritic cells (Wu *et al.*, 2000) being the first target by DENV and also macrophages (Kou *et al.*, 2008), liver, kidney, spleen, bone marrow, lung, thymus and brain (Martina *et al.*, 2009), suggesting a wide-distribution and diverse receptors of DENV. Thus, the affinity of DENV towards these receptors affected the virulence and the infectivity of the virus.

In addition to viral factors, host immune responses also play roles in the pathogenesis of dengue, particularly severe dengue. The onset of plasma leakage and the upsurge of vascular permeability were commonly observed during the defervescence stage (Chuansumrit *et al.*, 2014), a condition after the removal of DENV by the host immune response. Activation of 'cytokine storm' during dengue infection is initiated by multiple cytokines activation cascade including tumour necrosis factor alpha (TNF-α), vascular endothelial growth factor-A (VEGF-A), IL-6, IL-10, IL-35, CCL3, GM-CSF and human cytotoxic factor (hCF) (Chaturvedi *et al.*, 2000; Srikiatkhachorn *et al.*, 2017), which then induces multiple cytokine cascades that leads to the severe dengue development by several mechanisms, including: i) direct damages against endothelial cells by the cytokines (Chousterman *et al.*, 2017; Pang *et al.*, 2007); (ii) further activation of pro-inflammatory genes and antibodies (Chaturvedi *et al.*, 2000); and iii) production of

autoantibodies against host cells/tissues due to the molecular mimicry between the virus and host cells (Chuang *et al.*, 2014).

Autoimmunity shows correlation with the development of severe dengue (Martina *et al.*, 2009; Wan *et al.*, 2013), where the elements such as autoantibodies production and vigorous immune responses cause injuries to own cells and tissues, hence the development of plasma leakage and vascular permeability. On the other hand, autoimmunity also confers some protection against virus infection, mainly reported in human immunodeficiency virus (HIV) infection (Sekigawa *et al.*, 2002b). The protective effects by autoimmunity can be seen in systemic lupus erythematosus (SLE) patients, where the efficiency in the antiviral defence against the virus in HIV-infected SLE patient assists in alleviating the symptoms of the disease (Bonsignori *et al.*, 2014). Monoclonal antibodies extracted from an SLE patient also showed neutralization effects against HIV (Alcéna *et al.*, 2013). We also had an unofficial observation that there is very rare to almost none cases of severe dengue among SLE patients. Further understanding of the involvement of autoimmunity in dengue may provide critical information on dengue pathogenesis, especially severe dengue.

1.2. Study objectives

The involvement of autoimmunity in dengue and other virus infections, prompted our interest to determine the involvement of autoimmune response in the pathogenesis of severe dengue. Thus, our study endeavour: i) to verify the association of genetic polymorphism distribution among non-severe dengue, severe dengue and SLE patients using next-generation sequencing technology; ii) to determine the neutralization capability of SLE patients' sera against DENV; and iii) to investigate the role of high mobility group box 1 (HMGB1) protein, a biomarker of autoimmune diseases (Kang *et al.*, 2014a; Lu *et al.*, 2015a; Pisetsky, 2014), in the pathogenesis of dengue.

CHAPTER 2: LITERATURE REVIEW

2.1. Dengue

2.1.1. Dengue history

Major epidemics of a disease that was thought to be dengue was officially reported in 1779 and 1780 in three continents (Asia, Africa and North America) (Hirsch, 1883; Howe, 1977; Rush, 1794). Nevertheless, the earliest record on dengue-like disease has been stated in a Chinese encyclopaedia of disease symptoms and medicines, published by the Chin Dynasty (265-420 AD) describing the manifestation of fever, rash, arthralgia and myalgia, caused by a disease called 'water-poison' associated with flying insect (Gubler, 1998, 2006). The word 'dengue' from a Latin word 'denguis' was first used and popularized during an outbreak in Cuba by Spanish-speaking people (Kuno, 2015). The increase in the transmission of dengue occurred during the World War II, due to the ideal condition for the mosquito breeding and the spread of the virus (Gubler, 1998). The etiologic agent of the disease, DENV, was identified around 1940s (Hotta, 1952; Sabin, 1952). The increase of virus transmission leads to the further spread of dengue in the Southeast Asia and Pacific regions. Severe dengue characterized by dengue hemorrhagic fever (DHF) was first recorded in Manila, Philippines in 1953-1954 (Hammon *et al.*, 1960) and then, spread throughout Southeast Asia, becoming the main cause of hospitalization and fatality among children in the region (Gubler, 1998). Dengue was reintroduced in the Pacific region in 1970s (Gubler, 1993) and during 1980s-1990s, where dengue incidents intensified globally due to the increase in the geographic distribution of the vector and better adaptation of the virus (Gubler *et al.*, 1993; Gubler *et al.*, 1995; Halstead, 1992; Pinheiro *et al.*, 1997).

Currently, DENV was predicted to cause 390 million infections, including 96 million symptomatic cases globally per year, estimated using the formal modelling framework

mapping (Bhatt *et al.*, 2013), suggesting the hazard and the importance of the disease. Among 96 million symptomatic global cases, almost 70% of them was predicted to occur in Asia Pacific region (Bhatt *et al.*, 2013). In Malaysia, dengue cases increased from 7103 cases in 2000 to 46,171 cases in 2010, causing an upsurge in dengue incidence from 32 to 160 per 100,000 (Anker *et al.*, 2011; Arima *et al.*, 2011; Dom *et al.*, 2010; MOH, 2012; Mohd Zaki *et al.*, 2014; WHO, 2012; Yusoff, 2008). However, the dengue incidence drops to 70 per 100,000 in the year 2011 (Mohd Zaki *et al.*, 2014). A national cross-sectional study indicates that 91.6% of adults aged ranging from 35 to 74 years old were DENV seropositive (Azami *et al.*, 2011). The DENV seropositive percentage increased with the age (80% of adults aged 35-44 years old and 94% of adults aged 55-74 years old) (Azami *et al.*, 2011). Seroprevalence study among schoolchildren demonstrated 11% to 14% of children aged 7 to 18 years old were DENV seropositive (AbuBakar *et al.*, 2011; Tiong *et al.*, 2010). A study in one of the regions in Selangor showed that the DENV seropositive rate was 33% among adults <20 years old and 100% among adults more than 60> years old (Chen *et al.*, 2003b).

2.1.2. Clinical presentations of dengue

Clinical manifestation of dengue ranges from asymptomatic to a wide spectrum of symptomatic reactions. Among the estimated 390 million people exposed to the risk of DENV infection, three-quarters of them were predicted to be asymptomatic or clinically inapparent (Bhatt *et al.*, 2013). Asymptomatic people showed huge potential as a reservoir of DENV infection and found to be more infectious to the mosquitoes than those with symptomatic infection (Duong *et al.*, 2015). The intrinsic incubation period is the time between the infection of the virus and the onset of the apparent symptoms in human, which usually takes about 4-10 days (WHO, 2009). Symptomatic dengue is typically characterized by an onset of high fever (>40°C) with one or more of the following signs, including headache, rash, arthralgia, myalgia, vomiting or thrombocytopenia, which

usually subside without any significant complications (Gubler, 1998; Guzman *et al.*, 2010b; Hayes *et al.*, 1992; Waterman *et al.*, 1989).

Severe dengue commonly occurred after the drop of body temperature at the time of defervescence. It is characterized by critical thrombocytopenia (<100,000 platelets mm^{-3}), hemorrhagic manifestation, such as petechiae, purpuric lesion and ecchymosis or plasma leakage signs, including bleeding, pleural effusion, haematocrit >20%, ascites and hypoproteinemia (Eram *et al.*, 1979; Guzman *et al.*, 2010b; Sumarmo *et al.*, 1983). Delayed detection and lack of proper management of the disease may cause the development of dengue shock syndrome (DSS) in the patient due to the blood loss. DSS symptoms include hypotension with pulse pressure of ≤20 mm Hg, cold and clammy skin and restlessness, which may resulted with death within 12 to 36 hours after the shock onset, if the patient is further neglected and no prompt treatment applied (Martina *et al.*, 2009; WHO, 1997)

2.1.3. Classification of dengue

The devastating impacts of dengue outbreaks in Southeast Asia during the late 1960s raised the awareness of the disease (Cohen *et al.*, 1966; Nimmannitya *et al.*, 1969; Pongpanich *et al.*, 1973), which then prompt the establishment of dengue clinical classification published by the World Health Organization (WHO) (WHO, 1975, 1997). According to the guideline, dengue is classified into dengue fever (DF) and dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS). Classic DF is classified as fever, headache, myalgia and arthralgia and hemorrhagic manifestations. DHF is classified into 4 categories; Grade I (thrombocytopenia, hemoconcentration), Grade II (bleeding), Grade III (pulse pressure 20 mmHg, hypotension, cold skin and restlessness) and Grade IV (shock, undetectable blood pressure). However, a few limitations of this guideline have been raised, particularly in the definition of DHF, which claimed to be too rigid and too difficult to apply into primary care (Balmaseda *et al.*, 2005; Bandyopadhyay *et al.*, 2006;

Phuong *et al.*, 2004) and might not be universally representing dengue (Rahman *et al.*, 2002). Furthermore, the system also failed to determine the significant proportion of severe dengue, since severe manifestations as encephalopathy and hepatic malfunction were not included in the DHF (Srikiatkhachorn *et al.*, 2011).

The new 2009 WHO classification guideline generated from the basis finding of a multicentre study (Dengue Control [DENCO study]) classified dengue according to the level of severity: dengue without warning signs (nausea, rash, tourniquet test positive, aches), dengue with warning signs (abdominal pain, continuous vomiting, fluid build-up, lethargy, increasing haematocrit, liver enlargement, decreasing platelets), and severe dengue (plasma leakage, severe bleeding, organ dysfunction) (Figure 2.1) (WHO, 2009). The signs and symptoms listed in the revised guideline are more various and less specific than those listed in the 1997 guideline (Srikiatkhachorn *et al.*, 2011), which are practical and easy to be applied in primary care. Moreover, surveillance data are easier to be standardized using the new 2009 guideline, as well as able to prevent the different classification used in different countries, incidents of unparalleled data sets and underestimation of dengue symptoms (Horstick *et al.*, 2012).

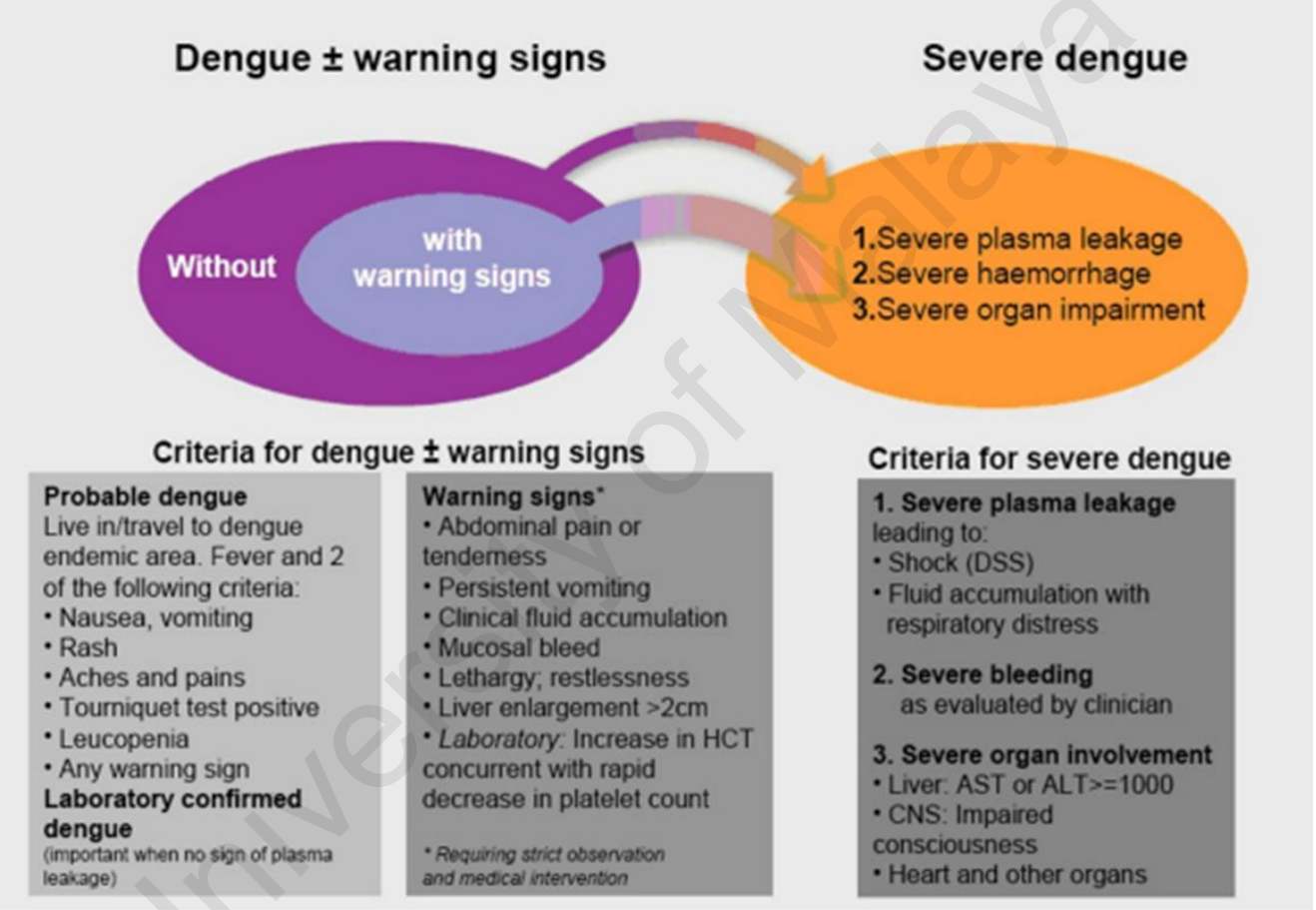


Figure 2.1: WHO 2009 dengue classification. [Source: Dengue guideline for diagnosis, treatment, prevention and control (WHO, 2009)]. Figure from Barniol *et al.*, 2011.

2.2. Dengue Virus

DENV is a single stranded RNA virus and belongs to the genus *Flavivirus* and family *Flaviviridae*. There are four antigenically distinct serotypes of DENV including DENV1, DENV2, DENV3 and DENV4. Each serotype has been identified with distinct genotype and lineage, emphasizing the genetic variations among the serotypes (Holmes *et al.*, 2000; Russell *et al.*, 1967). DENV is transmitted by the bites of the infected female *Aedes* mosquitoes, specifically *Ae. aegypti* and *Ae. Albopictus* (Henchal *et al.*, 1990).

2.2.1. Virus structure

DENV genome consists of a positive-sense strand RNA that is approximately 11 kb in size. The RNA has a single open reading frame (ORF) which is flanked by 5'- and 3'-noncoding regions (NCR), encodes 3 structural proteins: capsid (C), pre-membrane (prM) and envelope (E), that form the viral particles and 7 nonstructural (NS) proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5, which contribute in the viral RNA replication (Henchal *et al.*, 1990). DENV capsid is a highly basic protein of 12 kDa in size. Capsid is important in the nucleocapsid configuration, which is the first stage in assembling virus components. Moreover, the RNA structures essential for viral genome replication exist in the coding region of capsid, hence useful in studying the viral synthesis of dengue (Byk *et al.*, 2016). Previous study showed that capsid interacts with nuclear host proteins such as heterogeneous nuclear ribonucleoproteins (hnRNPs), death-domain associated protein (DAXX), core histones and nucleolin (Balinsky *et al.*, 2013).

The uncleaved precursor of membrane (M) protein, prM, is found in the E-prM heterocomplex of immature virions (Kuhn *et al.*, 2002). The function of prM in the heterodimer complex of E-prM includes chaperone for E protein folding (Konishi *et al.*, 1993; Lorenz *et al.*, 2002) and avoid premature fusion of E protein along the secretory pathway (Guirakhoo *et al.*, 1992; Stiasny *et al.*, 1996). Studies show that prM is involved

in the virus replication of West Nile virus (WNV) (Calvert *et al.*, 2012; Tan *et al.*, 2009), tick-borne encephalitis virus (TBEV) (Yoshii *et al.*, 2012), Japanese encephalitis virus (JEV) (Kim *et al.*, 2008a), and DENV (Hsieh *et al.*, 2014). Moreover, prM/M is also involved in apoptosis (Brabant *et al.*, 2009; Catteau *et al.*, 2003) and interacts with host protein during the virus entry and virion particle gathering (Brault *et al.*, 2011; Duan *et al.*, 2008; Gao *et al.*, 2010).

The E protein is a main target of neutralization antibodies and has been extensively studied in the antiviral research of dengue (Fahimi *et al.*, 2016; Jadav *et al.*, 2015; Ji *et al.*, 2015; Leal *et al.*, 2017; Liu *et al.*, 2016b). The size of the E protein is 53 kDa and comprises of 3 ectodomains, including domain I, domain II and domain III (Christian *et al.*, 2013; Modis *et al.*, 2004). The hydrophobic pocket of E protein lying at the hinge region of domain I and domain II is sensitive to low pH and caused conformational changes once triggered (Leal *et al.*, 2017). E protein plays major role in the entry of the virus, where conformational changes of E protein contribute in the fusion between virus and host cell membranes (Kaufmann *et al.*, 2009; Stiasny *et al.*, 2007).

Most of the nonstructural protein plays major role in virus replication. NS1, a 43-45 kDa glycoprotein, regulates early events of virus replication (Mackenzie *et al.*, 1996; Youn *et al.*, 2013) and shown to be critically important in proliferation of viruses, observed from the total abolishment of replication in NS1-depleted virus genome (Khromykh *et al.*, 2000; Lindenbach *et al.*, 1997). NS1 also plays a role in the pathogenesis of dengue by cross-reacting against human-blood clotting, integrin or adhesion protein and endothelial cells (Falconar, 1997; Lin *et al.*, 2001; Lin *et al.*, 2003), platelets (Chen *et al.*, 2009; Cheng *et al.*, 2009a), complement components (Avirutnan *et al.*, 2010; Avirutnan *et al.*, 2011), and prothrombin (Lin *et al.*, 2012).

NS3 is a multifunctional protein (68 kDa) which functions as protease when it uses NS2B as a co-factor for polyprotein processing (Chambers *et al.*, 1991; Falgout *et al.*, 1991;

Wengler *et al.*, 1991; Yusof *et al.*, 2000). NS3 also endowed with helicase, nucleoside-triphosphatase (NTPase) and 5'terminal RNA triphosphatase activities that are essentials in viral RNA replication (Bartelma *et al.*, 2002; Benarroch *et al.*, 2004; Li *et al.*, 1999).

The largest and the most conserved protein among the Flaviviruses' NS protein, NS5, is a 103 kDa bifunctional protein with methyltransferase and RNA-dependent RNA polymerase domains. NS5 involves in the capping (Dong *et al.*, 2008; Koonin *et al.*, 1993), methylation (Egloff *et al.*, 2002; Egloff *et al.*, 2007) and viral replication (Rawlinson *et al.*, 2006; Tan *et al.*, 1996). NS5 interacts with NS3 to form viral replication complex, which then contributes in the virus genome amplification (Erbel *et al.*, 2006; Johansson *et al.*, 2001; Zhao *et al.*, 2015).

NS2A (22 kDa) and NS4A (16 kDa) and NS4B (27 kDa) are hydrophobic proteins, while NS2B (14 kDa) is a small membrane-associated protein endowed with a central hydrophilic region bordered by two hydrophobic domains. NS2A plays an essential role in the assembly of virus particles (Leung *et al.*, 2008; Mackenzie *et al.*, 1998) and acts as the recognition site of viral protease (Kümmerer *et al.*, 2002). NS2B interacts with NS3 for the activation of NS3 protease activity (Chambers *et al.*, 1991; Chambers *et al.*, 1993; Falgout *et al.*, 1991; Yusof *et al.*, 2000). In addition to being a target site for NS3-NS2B protease activity in the cytoplasm (Lin *et al.*, 1993), NS4A is also involved in the viral polyprotein processing, hence important in the viral RNA replication (Lindenbach *et al.*, 1999; Mackenzie *et al.*, 1998; Miller *et al.*, 2007). Though not clearly defined, NS4B was shown to regulate viral replication via interaction with NS3 helicase domain (Miller *et al.*, 2006) and dissociation of NS3 from single-stranded RNA (ssRNA) (Umareddy *et al.*, 2006).

2.2.2. Virus replication

The cycle of virus replication starts with the infection of permissive host cells. Various types of human cells were revealed to be susceptible to DENV including dendritic cells, monocytes/macrophages, endothelial cells, B cells, T cells, hepatocytes and neuronal cells (Anderson, 2003). Generally, the entry of DENV is attained by receptor-mediated endocytosis (Bartenschlager *et al.*, 2008; Clyde *et al.*, 2006; van der Schaar *et al.*, 2008) via the binding of DENV E protein with different mammalian virus receptors such as dendritic cell-specific intercellular adhesion molecule-3 (ICAM3)-grabbing non-integrin (DC-SIGN) (Navarro-Sanchez *et al.*, 2003; Tassaneetrithep *et al.*, 2003), heparan sulfate (Chen *et al.*, 1997; Germi *et al.*, 2002), C-type lectin domain family-5-member A (CLEC5A) (Chen *et al.*, 2008b; Cheung *et al.*, 2011; Watson *et al.*, 2010), heat shock protein 70 (Hsp70) and heat shock protein 90 (Hsp90) (Reyes-del Valle *et al.*, 2005), glucose-regulated protein 78 GRP78/BiP (Jindadamrongwech *et al.*, 2004), high affinity laminin receptor (Thepparit *et al.*, 2004), TIM and TAM proteins (Meertens *et al.*, 2012), neutral glycosphingolipids (Wichit *et al.*, 2011) and cluster of differentiation 14 (CD14)-associated protein (Chen *et al.*, 1999).

Upon internalization, the acidic pH in the endosome causes the conformational changes of the virus E protein dimers into trimers, which then mediated the membrane fusion between virus glycoprotein and the host cellular membrane. The membrane fusion allows the disassembling of the nucleocapsid, causing the RNA release into the host cell cytoplasm (Heinz *et al.*, 2003). The RNA then serves as mRNA and translated into a single viral polyprotein, which is subsequently cleaved by the host and viral proteases into three structural proteins and seven NS proteins. Host signal peptidase cleaves the N terminal of structural protein (C/prM, prM/E, E/NS1) and the C terminal of NS4A in the endoplasmic reticulum (ER), whereas NS2B/NS3 protease cleaves the C terminal of the NS protein (NS2A/NS2B, NS2B/NS3, NS3/NS4A, NS4B/NS5) (Rice, 1996).

After the translation and the processing of the viral positive-strand, the synthesis of a negative strand intermediate takes place, which used as a template for production of multiples new positive RNA virus strand. The positive-strand continuously binds to the ribosomes to commence the new virus translation cycle or gathers into an immature virion (prM/E heterodimers) via budding into the ER lumen (Bartenschlager *et al.*, 2008; Salonen *et al.*, 2005; Welsch *et al.*, 2009). The maturation of the immature virion achieved as they travel through the secretory vesicles to the Golgi complex. The acidic pH of trans-Golgi network induces the structural reformation of the glycoprotein, which allows the cleaving of prM by endoprotease furin into membrane-associated M and a "pr" peptide (Xu *et al.*, 2005), Study shows that the "pr" peptide binds to the virion until the exocytosis of the progeny virus to stabilize the E protein during travel through the secretory vesicles. This is to avoid the premature conformational changes of the E protein that may lead to membrane fusion (Aruna, 2014). Mature infectious viruses are formed after the "pr" peptide dissociation, which induced by the neutral pH of the extracellular milieu (Aruna, 2014).

2.3. Host immunity

2.3.1. Host immune response in dengue

The infection of DENV started after the inoculation of the virus by the infected mosquito. Dendritic cells (DC), mononuclear phagocyte lineage and macrophages, which are the important players of the innate immune responses are also the early targets of DENV (Gordon, 1998; Halstead *et al.*, 1977b; Halstead, 1988). These cells detect antigen via various types of host pattern recognition receptors (PRRs), particularly toll-like receptors (TLRs), retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene (MDA5) (Figure 2.2).

Activated DCs after DENV infection lead to the secretion of interferon (IFN)-α, tumour necrosis factor alpha (TNF-α), IFN-γ-inducible chemokine ligand 9 (CXCL9), chemokine ligand 10 (CXCL10), chemokine ligand 11 (CXCL 11) and regulatory cytokine, interleukin (IL)-10 (Ho *et al.*, 2001; Libraty *et al.*, 2001; Sun *et al.*, 2006). The maturation markers including human leukocyte antigen-antigen D related (HLA-DR), CD11b and CD83 are also expressed in DENV-activated DCs (Libraty *et al.*, 2001). Moreover, DC-SIGN with a C-type lectin ectodomain plays a major role in the initiation of the contact between DCs and resting T-cells (Geijtenbeek *et al.*, 2002). In addition to induction of innate immune response, DCs are also important in establishing DENV infection, observed from the significant decrease of DENV infection by DC-SIGN blockade (Martin *et al.*, 2004; Sakuntabhai *et al.*, 2005) (Figure 2.2).

Macrophages release IL-6, IL-8, IL-12, IFN-γ-inducible protein (IP-10), IFN-β and TNF-α when infected with DENV and the production of cytokines was found to be positively correlated with the virus load (Alexopoulou *et al.*, 2001; Edelmann *et al.*, 2004; Sun *et al.*, 2013). Natural killer (NK) cells, one of the bone marrow-derived lymphocytes, is involved in innate immunity against DENV. NK cell activity might be essential in the clearing of primary DENV infection (Shresta *et al.*, 2004) via cytolysis of DENV-infected cells and secretion of chemokines (Robertson, 2002). NK cells also induce IFN-γ which activates macrophages and DCs (Navarro-Sanchez *et al.*, 2005) (Figure 2.2).

The binding of antibody and pathogens forms the immune complexes (IC), which usually cleared from the circulation via phagocytosis by macrophages (Sun *et al.*, 2013). NK cells recognize ICs and afterwards, induce antibody-dependent cellular cytotoxicity (ADCC), a killer mechanism that target infected cells (Stokes *et al.*, 1988). Studies show that high ADCC activity associates with lower viremia levels while low activity of ADCC correlates with DHF (Laoprasopwattana *et al.*, 2007). Indeed, there is no connection

between ADCC activities with the severity of dengue (Laoprasopwattana *et al.*, 2007). Complement system can also be activated by ICs, where the opsonisation by complement protein directs the destruction of the infected cells, thus limiting virus replication (Füst, 1978). Complement proteins limit progression of disease by the inhibitory effects of the classical pathway, C1q (Mehlhop *et al.*, 2007). Pre-incubation of C1q and DENV, prior to the infection in human leukaemia monocytic (THP-1) cells showed significant decrease of virus infectivity (Douradinha *et al.*, 2014).

Antigen presenting cells (APCs) targeted by DENV such as monocytes, macrophages and DCs are critical for cell-mediated immunity stimulation. Cell-mediated immunity comprised of $CD4^+$ T cells and $CD8^+$ T cells, where $CD4^+$ T cell functions is to activate other cells, while $CD8^+$ T cell directly kills infected cells via cytotoxicity (cytotoxic T lymphocytes [CTL]). $CD4^+$ T cells are divided into 2 subtypes, type-1 T helper (Th1) and type-2 T helper (Th2). Th1 stimulates production of TNF-α, IFN-γ, and IL-2, whereas Th2 induces IL-4, IL-5, IL-13 and IL-10 (Sun *et al.*, 2013). In addition to Th1 and Th2 cells, T-helper 17 (Th17) cells are associated with autoimmune and inflammatory diseases and essential in sustaining the antiviral response in the cell (Barkhordarian *et al.*, 2015). Th17 cells induced by T-cell receptor (TCR) activates STAT3, IL-6, IL-21, and IL-23 and play important roles in the immune response against bacteria and viruses (Muranski *et al.*, 2013). $CD4^+$ T cells indirectly activate $CD8^+$ T cells by APCs activation (Bevan, 2004), while $CX3CR1^+$ cytotoxic dengue-specific $CD4^+$ T cells is associated with protective immunity against dengue disease (Weiskopf *et al.*, 2015). $CD8^+$ T cells confer protection against dengue reinfection in mice (Zellweger *et al.*, 2015) and shown to cause a decrease in HIV load (McMichael *et al.*, 2000).

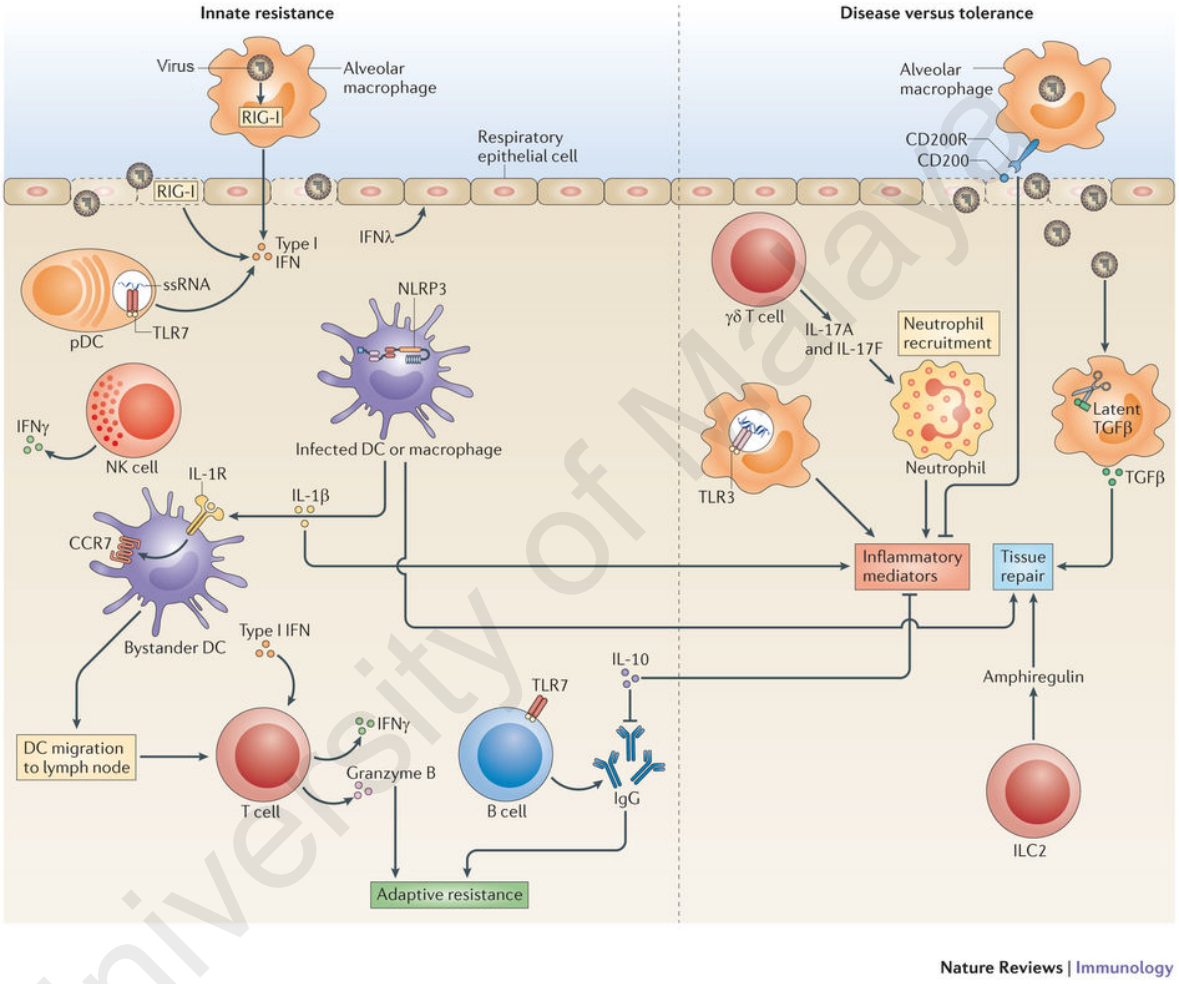


Figure 2.2: Host immune response against virus. Figure modified from Iwasaki *et al*., 2014.

2.3.2. Cross-reactivity in heterologous immunity and virus infection

Heterologous immunity is defined by the development of immunity against one pathogen after the host had been exposed to non-identical pathogens (Clark, 2001; Selin *et al.*, 1998; Welsh *et al.*, 2002). This event does not only occur within those infected with closely related species, but also observed between those infected with unrelated pathogens. Heterologous immunity occurs within various groups of pathogen including parasites, bacteria and viruses or even among the groups such as seen between bacteria and virus, observed from the protective immunity in vaccinia virus infected mice conferred by tuberculosis vaccine bacterium bacilli Calmette-Guerin (BCG) (Mathurin *et al.*, 2009). Cross-protective immunity between different species of viruses was observed in dengue and Zika, where monoclonal antibodies retrieved from dengue-infected patient showed high cross-reactivity against the Zika virus (Priyamvada *et al.*, 2016). Moreover, enterovirus 71 (EV71) vaccine prevents fatality in neonatal mice infected by a lethal dose of coxsackievirus A16 (CA16) (Yang *et al.*, 2016). Cross-protection was also seen among lymphocytic choriomeningitis virus (LCMV), Pichinde virus (PV) and murine cytomegalovirus (MCMV) against each other with different efficacy (Chen *et al.*, 2003a; Mathurin *et al.*, 2009; Selin *et al.*, 1998; Welsh *et al.*, 2010).

Though regarded as less effective than homologous immunity, which usually associated with stronger resistance against specific pathogen, heterologous immunity has been shown to alleviate severe development of infection (Bonsignori *et al.*, 2014; Selin *et al.*, 1998). In other hands, heterologous immunity also showed involvement in the pathogenesis of disease (Chen *et al.*, 2003a; Selin *et al.*, 1998; Walzl *et al.*, 2000). Previous study showed the possible involvement of T-cell-mediated heterologous immunity in the cross-reactive immune response (Kim *et al.*, 2005). T-cell mediated immunity confers efficient antiviral response and virus clearance, however, it may also cause abnormal distortion in the T-cell repertoire (Welsh *et al.*, 2010). T cells can be

highly cross-reactive or "polyspecific" due to its nature to initially scan the peptide-presented on the major histocompatibility complex (pMHC) by binding to the MHC and subsequently develop higher affinity binding around the peptide. The binding is usually enhanced via conformational changes of T cell receptors (TCRs) to accommodate pMHC, a process that allows dramatic variations in the peptide sequences (Lee *et al.*, 2004; Wu *et al.*, 2002), hence the high cross-reactive possibility. This phenomenon may explain the susceptibility of heterologous immunity in initiating cross-reactivity against self-cells/tissues, which is one of the main factors in the autoimmune disease activity. This high autoreactive T cells might also be one of the factors in the protective effect of SLE immune responses against HIV infection mentioned previously (2.3.3), by its cross-reactive capability and efficiently inhibit virus replication at an early stage of infection (Bonsignori *et al.*, 2014; Chang *et al.*, 1999; Diri *et al.*, 2000b; Frei *et al.*, 2015; Laoprasopwattana *et al.*, 2007; Moody *et al.*, 2010; Palacios *et al.*, 2002; Tharakaraman *et al.*, 2013).

2.3.3. Pathogenesis of dengue

Although extensively investigated, the pathogenesis of dengue, especially severe dengue is not completely understood. There are a few hypotheses that have been put forward to elucidate the mechanism of dengue, including viral pathogenesis and immunopathogenesis (Martina *et al.*, 2009; Wan *et al.*, 2013).

Virus variation plays a role in dengue pathogenesis, where different serotypes of DENV show different capacity in causing dengue. Each of dengue serotype causes DENV infection and has been responsible for an outbreak. Nevertheless, DENV2 and DENV3 are most likely to cause severe dengue and death (Guzman *et al.*, 2010a). In other hand, DENV1 was found to be more severe in comparison to DENV2 (Yung *et al.*, 2015). Primary infection of DENV4 showed relatively mild symptoms and illness than DENV1, DENV2, and DENV3 (Nishiura *et al.*, 2007). The cell and tissue tropism may affect the

infectivity of the DENV. Langerhans cells (dermal dendritic cells) and keratinocytes are the initial target of DENV once entered inside the bloodstream (Limon-Flores *et al.*, 2005; Wu *et al.*, 2000). DENV can also be found in the liver, spleen, lymph node, kidney, lung, thymus, brain and bone marrow (Martina *et al.*, 2009). Wide range of cells that is susceptible to dengue suggested broad distribution of DENV receptors. The affinity of DENV with the receptors influenced the infectivity and the virulence of DENV (Wan *et al.*, 2013). Moreover, higher viral load and slower rates of the virus clearance may also contribute to the pathogenesis of severe dengue (Ben-Shachar *et al.*, 2016; Wang *et al.*, 2006).

Antibody dependent enhancement (ADE) has been proposed widely as one of the factors in the pathogenesis of severe dengue, suggesting that the pre-existing sub-neutralization antibodies enhance the virus entry and infection in the mononuclear cell lineage, rather than neutralizing the virus (Brandt *et al.*, 1982; Halstead *et al.*, 1977a, 1977b; Halstead, 1988). The "original antigenic sin" is a phenomenon where the low-affinity memory T cells generated during primary infection, proliferate during the secondary infection of different virus serotype (Halstead *et al.*, 1983; Mongkolsapaya *et al.*, 2003). Both of these occurrences lead to an inefficient clearance of the virus, hence increases virus replication. The expansion of virus-infected cell subsequently initiates cascade activation of T cells, monocytes and endothelial cells, resulting in overproduction of cytokines, chemokines and other mediators, called "cytokine storm'' (Chaturvedi *et al.*, 2000; Pang *et al.*, 2007). The involvement of autoimmunity in the pathogenesis of dengue has been described previously (Lin *et al.*, 2006; Wan *et al.*, 2013). Autoantibodies produced during DENV infection trigger direct and indirect injuries against endothelial cells (Falconar, 1997; Lin *et al.*, 2003), platelets (Lin *et al.*, 2001; Oishi *et al.*, 2003) and coagulatory molecules (Chungue *et al.*, 1994; Markoff *et al.*, 1991). Factors such as genetic composition, gender,

age, and race also play roles in the pathogenesis of dengue (Chakravarti *et al.*, 2016; Egger *et al.*, 2007; Hoh *et al.*, 2014; Sam *et al.*, 2013; Sierra *et al.*, 2007; Thai *et al.*, 2011)

2.4. Autoimmune and autoimmunity

Autoimmunity is associated with the dysregulation of immune responses, a condition where the immune response is misdirected against healthy cells and tissues. Mild autoimmunity is usually harmless, however, in a severe condition, it can lead to illness known as autoimmune disease. Autoimmune diseases include systemic lupus erythematosus (SLE), rheumatic arthritis (RA), Sjögren's syndrome, multiple sclerosis (MS), Graves' diseases, Celiac disease, dermatomyositis, ankylosing spondylitis, polymyositis (Caso *et al.*, 2018).

2.3.2. Antibody immune response in dengue

Primary antibody response is observed in the patient that has never been infected with DENV and no immune response against it. Generally, primary antibody response (Figure 2.3) is slow and at low-titre, where the first antibody isotype to appear is IgM, after 4-5 days of the onset of fever (Wahala *et al.*, 2011). The IgM levels peak at approximately 2 weeks after the onset of the disease and typically reduced to the untraceable level after 3 months (Innis *et al.*, 1989; PAHO, 1994; Wahala *et al.*, 2011; WHO, 2009). IgG response is initiated at low levels about a week after the illness started and peaks several weeks onward. The secondary infection occurs in the patients with previous DENV infection. IgM response in secondary infection is significantly lower in comparison to those with primary infection (Chanama *et al.*, 2004; Gubler, 1996; PAHO, 1994) whereas IgG response initiated rapidly at high levels, which can be detected even during the acute phase and sustained for about 2 weeks later (Gubler, 1997). IgM response lifetime is more variable and some cases untraceable, whereas IgG persists for decades, if not longer (Gubler, 1997; Innis *et al.*, 1989). Flavivirus NS1 is expressed in the serum between days

3 to 8 after infection and highest on the day 5, the day preceding to the onset of the clinical manifestation (Macdonald *et al.*, 2005).

Homotypic immunity gained from a particular DENV serotype infection against the similar serotype can last for a lifetime, whereas heterotypic immunity of the cross-reactive antibodies from other DENV serotype can only last a limited time (approximately 3 to 6 months) (Sabin, 1952). Initial immune response during early convalescent period demonstrated broad cross neutralizing via inhibition of B-cell inhibitory Fcγ receptor (FcγRIIB) on the monocytes, but yet the effect is weak and only temporary (Chan *et al.*, 2011).

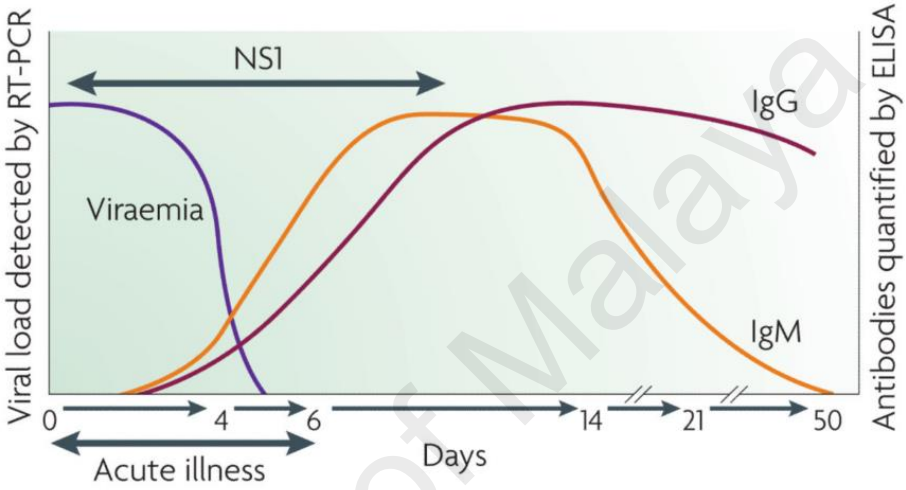


Figure 2.3: Primary antibody response in dengue. During primary dengue infection, isotype IgM is first to appear by days 4 to 5. IgM levels are high for about two weeks after the fever onset and decrease to undetectable amount after 2 to 3 months. Expression of IgG is low during and at the end of the first week of disease onset and increases afterwards. NS1 levels are detected between days 3 and 8 post-infection. Figure from Guzman *et al.*, 2010b.

2.4.1 Autoimmunity and virus infection

Autoimmunity and infection have been linked in multiple studies. Previous studies showed that infections play roles in the pathogenesis of autoimmune diseases by both inducing and inhibiting the development of the diseases (Shamriz *et al.*, 2018). Hepatitis B virus induces production of antiphospholipid antibodies such as anticardiolipin (ACL), antib (2)-glycoprotein I (b2GPI) and lupus anticoagulant antibodies, markers for the development of autoimmune diseases (Barzilai *et al.*, 2007). However, a study using pristine-induced lupus mouse model showed that HBV-infected mice have lower titers of antinuclear antibodies (ANA), lower levels of interleukin-17, tumor necrosis factor-a, and B-cell activating factor than non-infected pristine-induced lupus mice (Liu *et al.*, 2016a). Group B Coxsackieviruses (CVB) increased the risk of type-1 diabetes mellitus (TID) observed by the induction of TID in the infected transgenic nonobese diabetic (NOD) mice with CVB4 (Horwitz *et al.*, 1998). CVB also showed protective features against TID, where NOD mice infected by several strains of CVB exhibited 2 to 10-folds decreased of TID incidents (Tracy *et al.*, 2002). Epstein-Barr virus (EBV) might play a role in the etiology of SLE via molecular mimicry hypothesis, which is the cross-reactivity of pathogen peptides and self-peptides due to structural similarities (Munz *et al.*, 2009). EBV also instigated increase SLE-related autoantibodies in mice (Tu *et al.*, 2018).

Contrary to the involvement of virus infections in the pathogenesis of autoimmune diseases, there were possibilities that the autoimmune disease patients, particularly SLE, might have protective effects against virus infection (Sekigawa *et al.*, 2002a). There were many cases of HIV transmission by blood donation and organ transplantation between 1978 to 1983 due to lack of screening method, however, there was no infection of HIV among SLE patients (Barthel *et al.*, 1993). Moreover, there are no less than 400 people in the U.S.A that have both SLE and HIV-1 based on the prevalence data (Barthel *et al.*,

1993; Fox *et al.*, 1997a). Yet, only about 20 patients that displayed both conditions have been reported (Alonso *et al.*, 2000; Chang *et al.*, 1999; Diri *et al.*, 2000a). The protective effects against HIV by SLE patients were probably achieved via their efficiency in the production of autoantibodies known as broadly neutralizing antibodies (bNAbs) in the early phase of infection (Bonsignori *et al.*, 2014). High IL-16 titer in the serum of SLE patients might also be one of the factors in the protection against HIV infection (Center *et al.*, 1996b; Cruikshank *et al.*, 2000).

2.4.2. Autoimmunity in the pathogenesis of dengue

Autoimmunity is one of the factors involved in the pathogenesis of dengue (Wan *et al.*, 2013). Autoantibodies reactions against endothelial cells, platelets and coagulatory molecules were postulated due to the molecular mimicry between the host cells with NS1, prM and E protein (Chuang *et al.*, 2014; Liu *et al.*, 2011). Cross-reactivity of anti-NS1 antibody against endothelial cells caused damage to the cells by apoptosis reaction via caspase-dependent pathway and cell lysis by complement activation (Lin *et al.*, 2003; Lin *et al.*, 2002). In addition to apoptosis reaction, anti-DENV NS1 antibodies also stimulate inflammatory activation in endothelial cells, suggesting the involvement of anti-NS1 antibodies in vasculopathy of dengue (Lin *et al.*, 2005). Moreover, the binding of DENV2 NS1 to human endothelial cells at the N-terminal region leads to the high affinity of anti-NS1 antibodies towards endothelial cells (Falconar, 1997). Though known to cause damage to the host cell or tissues, previous study showed that NS1 was immunodominant in both mouse vaccination and human infection studies, suggested that the vaccination of NS1 can produce antibodies to regions that are not aimed in natural infection, hence able to provide added protection against severe DENV infection (Hertz *et al.*, 2017). Moreover, immunization of mice with NS1 prevents endothelial permeability and vascular leakage triggered by NS1, proposing the potential of NS1 as a therapeutic antibody for dengue (Beatty *et al.*, 2015).

Study on the samples collected during an outbreak in Taiwan showed that anti-platelet immunoglobulin M (IgM) levels were higher in severe than non-severe dengue patients (Lin *et al.*, 2002). Anti-platelet immunoglobulin G (IgG) showed an association with the thrombocytopenia and the severity manifestation in secondary DENV infection (Oishi *et al.*, 2003; Saito *et al.*, 2004). Anti-NS1 antibodies induced lysis of platelets in the presence of complements and the complement-mediated lysis was increased when platelets were activated by ADP or thrombin (Lin *et al.*, 2008). Cross-reactivity between autoantibodies against the coagulatory molecules such as plasminogen was also observed during DENV infection, which postulated to be correlated with the hemorrhage event in dengue (Chungue *et al.*, 1994; Markoff *et al.*, 1991). Proteomic analysis revealed that the C-terminal amino acid region of NS1 shows homology with the host protein such as endothelial cells and platelets, triggering the autoantibodies reaction between the immune responses and host cells/tissues (Chen *et al.*, 2009; Cheng *et al.*, 2009a; Cheng *et al.*, 2009b).

Previous studies demonstrated that dengue also involved in the pathogenesis of autoimmune diseases, as observed from the lupus flare induction in a patient with DENV infection (de Souza *et al.*, 2010). The development of SLE and lupus nephritis from DENV infection was reported during the outbreak of dengue fever in India (Rajadhyaksha *et al.*, 2012; Talib *et al.*, 2013). Symptomatic DENV infection also shown to evolve into thrombotic thrombocytopenic purpura (TTP) (Deepanjali *et al.*, 2015).

Similar to HIV, cases of dengue manifestation especially severe dengue among autoimmune patients, especially SLE, are very rare. There is, however, one case reported the infection of DENV in the autoimmune (SLE) patient, where the patient developed lupus flare and tested positive for dengue serology (Verdolin *et al.*, 2014). The patient, however, was discharged in an asymptomatic condition (Verdolin *et al.*, 2014).

2.4.3. Systemic Lupus Erythematosus (SLE)

Systemic lupus erythematosus (SLE) is a chronic multisystem autoimmune disease that target almost all organs, including brain, blood, skin, joints and kidney (Tsokos 2011). SLE is characterized by the loss of the immune self-tolerance mechanisms, which along with other factors, could lead to the activation/expansion of innate immune cells and higher production of autoantibodies (La Paglia *et al.*, 2017; Yurasov *et al.*, 2006). The increased production of DNA binding B cells (recognized structure-like DNA) and hyperactivation of B cells/T cells associate with the SLE disease activity (Firestein *et al.*, 2009; Liossis *et al.*, 1996). Moreover, the clearance of the autoantibodies was also inefficient due to low quantity and functionally deficient of Fc and complement receptors (Kimberly, 1987).

The clinical manifestation of SLE is diverse, ranging from minor symptoms such as high fever, achy joints, skin rash (butterfly-shaped rash), extreme fatigue, sensitivity to the sun/light to severe complication of skin, kidney, blood, cardiovascular system and joints. According to the American College of Rheumatology (ACR) Revised Criteria for Classification Criteria of SLE, patients must fulfilled four of the eleven criteria for the formal diagnosis of SLE (Table 2.1) (Tan *et al.*, 1982).

Global SLE prevalence ranges from 40-400 cases per 100,000 populations (Helmick *et al.*, 2008), whereas in Malaysia, a prevalence of 43/100,000 individuals was diagnosed with SLE (Chai *et al.*, 2012). This disease predominantly affects women with the ratio of 9:1 (Weckerle *et al.*, 2011), presenting the important role of gender in disease development of SLE. In addition, genetic composition, geographical influence and environmental factors also contribute in causing SLE (Tsokos 2011).

Table 2.1: 1982 Revised Criteria for Classification of Systemic Lupus Erythematosus (Tan *et al.*, 1982).

No.	Criterion	Definition
1	Malar rash	Fixed erythema, flat or raised, over the malar eminences, tending to spare the nasolabial folds
2	Discoid rash	Erythematous raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring may occur in older lesions may
3	Photosensitivity	Skin rash as a result of unusual reaction to sunlight, by patient history or physician observation
4	Oral ulcers	Oral or nasopharyngeal ulceration, usually painless, observed by a physician
5	Arthritis	Nonerosive arthritis involving 2 or more peripheral joints, characterized by tenderness, swelling, or effusion
6	Serositis	a) Pleuritis-convincing history of pleuritic pain or rub heard by a physician or evidence of pleural effusion b) Pericarditis-documented by ECG or rub or evidence of pericardial effusion
7	Renal disorder	a) Persistent proteinuria greater than 0.5 grams per day or greater than 3 + if quantitation not performed b) Cellular casts-may be red cell, hemoglobin, granular, tubular, or mixed
8	Neurologic disorder	a) Seizures-in the absence of offending drugs or known metabolic derangements; e.g., uremia, or electrolyte imbalance ketoacidosis, b) Psychosis-in the absence of offending drugs or known metabolic derangements, e.g., uremia, ketoacidosis, or electrolyte imbalance
9	Hematologic disorder	a) Hemolytic anemia-with reticulocytosis *OR* b) Leukopenia-less than 4,000/mm3 total on 2 or more occasions *OR* c) Lymphopenia-less than 1,500/mm3on 2 or more occasions *OR* d) Thrombocytopenia-less than 100,000/mm3 in the absence of offending drugs
10	Immunologic disorder	a) Positive LE cell preparation *OR* b) Anti-DNA: antibody to native DNA in abnormal titer *OR* c) Anti-Sm: presence of antibody to Sm nuclear antigen *OR* d) False positive serologic test for syphilis known to be positive for at least 6 months and confirmed by *Treponerna pallidurn* immobilization or fluorescent treponemal antibody absorption test
11	Antinuclear antibody	An abnormal titer of antinuclear antibody by immunofluorescence or an equivalent assay at any point in time and in the absence of drugs known to be associated with "drug-induced lupus" syndrome

2.5. High Mobility Group Box 1 (HMGB1)

2.5.1. Protein characteristics

High Mobility Group Box (HMGB) protein, a non-histone nuclear protein, is highly conserved and the most abundant amongst HMGs protein (Goodwin *et al.*, 1978). HMGB box binds with DNA without any specificity and afterwards, initiate DNA conformational changes (Agresti *et al.*, 2003; Ueda *et al.*, 2010). The acidic C-tails in HMGB1, HMGB2 and HMGB3, but not in HMGB4, could bind with other nuclear protein, including HMG box family to enhance their affinity to various distorted DNA (Kang *et al.*, 2014a). Among the four members of HMGB, HMGB1 (previously known as HMG1, HMG-1 or HMG 1) is the most expressed in comparison to other HMGB protein, at approximately 10^6 particles of HMGB1 per cell (Romani *et al.*, 1979) and also the first to be discovered (Štros, 2010). HMGB1 is also a ubiquitous nuclear protein and exists in nearly all cell types. The structure of HMGB1 comprises of two-folded DNA-binding region, known as A and B boxes with an acidic C-tails (Figure 2.4). Nuclear-emigration signals (NES), which mediated by nuclear exporting chromosome-region maintenance 1 (CRM1) is located in the DNA binding domain (Bonaldi *et al.*, 2003). There are three cysteines, located at position 23 and 45 in box A and position 106 in box B, as well as two nuclear localization sequences (NLSs) located at each box (Yang *et al.*, 2013). There is 100% sequence homology of HMGB1 between mouse and rat, whereas rodent and human display 99% homology of the protein sequence (Ferrari *et al.*, 1994; Gariboldi *et al.*, 1995; Wen *et al.*, 2013).

HMGB1 is vulnerable to the acetylation process that leads to the translocation of HMGB1 out of the nucleus to the cytoplasm and eventually extracellular milieu (Bonaldi *et al.*, 2003; Lu *et al.*, 2012). The translocation of HMGB1 occurs generally through two pathways; actively via activated immune cells or passively via necrotic cells (Scaffidi *et*

al., 2002). HMGB1 has different roles, depending on the location of the protein, whether in the nucleus, cytoplasm or extracellular environment.

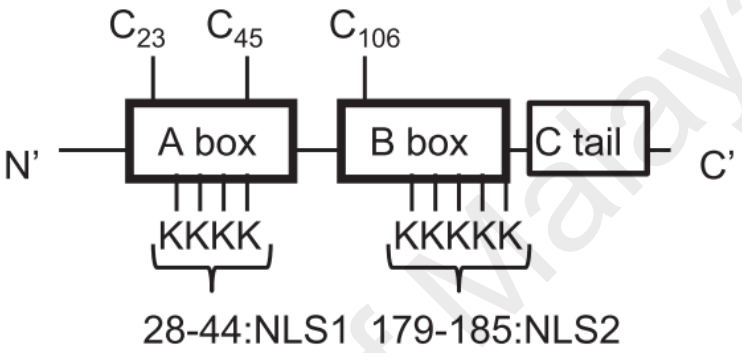


Figure 2.4: HMGB1 protein structure. HMGB1 comprises of two folded DNA-binding domain, A and B boxes and an acidic tail attached to a string of glutamic and aspartic acid. There are three cysteines located at position 23, 45 (A box) and 106 (B box) and two nuclear localization sequences (NLSs) in each DNA-binding box. Figure from Yang *et al*., 2013.

2.5.2. Functions of HMGB1

HMGB1 is initially defined as nuclear protein and plays a major role in the regulation of DNA transcription. Nuclear HMGB1 also involves in the regulation of binding and the bending of DNA, as well as various important events in DNA, including nucleosome stability (Andrews *et al.*, 2011; Cato *et al.*, 2008; Travers, 2003), nucleosome secretion (Huang *et al.*, 2014; Kang *et al.*, 2014b), genome chromatinization (Celona *et al.*, 2011; Krynetskaia *et al.*, 2008), V(D)J recombination (Agrawal *et al.*, 1997; Dai *et al.*, 2005; Grundy *et al.*, 2009; Little *et al.*, 2013), DNA replication (Bonne-Andrea *et al.*, 1986; Hoffmann *et al.*, 1997; Topalova *et al.*, 2008), DNA repair (Kang *et al.*, 2014a; Lange *et al.*, 2008; Livesey *et al.*, 2012), telomere and telomerase (Prochazkova Schrumpfova *et al.*, 2011), gene transcription (Ge *et al.*, 1994), gene transfer (Zayed *et al.*, 2004) and gene delivery (Han *et al.*, 2009; Kim *et al.*, 2008b; Yi *et al.*, 2011). HMGB1 generally increases the transcriptional activity of other proteins via its L-shaped A-box and B-box, which contribute in enhancing the interaction between other proteins and DNA (Lotze *et al.*, 2005). During inactive state, HMGB1 shields the A-box and B-box using long acidic tail, hence preventing abnormal interaction pre-binding of HMGB1 to DNA (Knapp *et al.*, 2004) (Figure 2.5).

HMGB1 was later discovered to have significant functions once released out from the nucleus. Conditions as infection, cytokine and chemokine stimulation, hypoxia, oncogene and heat stimulate the migration of HMGB1 from the nucleus to the cytoplasm (Kang *et al.*, 2014a; Ong *et al.*, 2012). In normal condition, the ratio of HMGB1 in the nucleus and cytoplasm is 30:1 (Kuehl *et al.*, 1984). In the cytoplasm, HMGB1 was found to be majorly involved in the induction of autophagy by acting as Beclin 1-binding protein and cause the dissociation of autophagy inhibitor Beclin 1 with its inhibitory partner, Bcl-2 (Kang *et al.*, 2011; Tang *et al.*, 2010). Cytosolic HMGB1 also potentially involves in the unconventional secretory pathway (Lee *et al.*, 2010). HMGB1 is broadly expressed on

the cell surfaces and those that present on the cell surface displays involvement in the platelet activation (Fuentes *et al.*, 2014; Maugeri *et al.*, 2012), cell differentiation (Passalacqua *et al.*, 1997), adhesion (Parkkinen *et al.*, 1991), and innate immunity (Ciucci *et al.*, 2011).

HMGB1 is considered as one of the cytokines due to the extracellular release of the protein by activation of immune cells, which then mediates systemic inflammatory signals (Wang *et al.*, 2004b). However, the release of HMGB1 occurs noticeably later than other classical pro-inflammatory molecules, about 12-18 hours after the peak of TNF and IL-1 (Wang *et al.*, 2004a; Yang *et al.*, 2004). Active translocation of HMGB1 occurs in the activated immune cells, such as macrophages (He *et al.*, 2012), mature myeloid DCs (Yang *et al.*, 2007), NK cells (Balsamo *et al.*, 2009) and non-immune cells such as endothelial cells (Fiuza *et al.*, 2003; Treutiger *et al.*, 2003). Passive release of HMGB1 occurs in cell death via necrosis, apoptosis, lysosomal and autophagic cell death (Kang *et al.*, 2014a) or cell injury due to chemotherapy, irradiation, hypoxia, hyperthermia, hyper-pressure (Fucikova *et al.*, 2014; Lotze *et al.*, 2005), toxins (Kennedy *et al.*, 2009; Radin *et al.*, 2011), ATP (Kawano *et al.*, 2012), glucose deficiency condition (Lee *et al.*, 2011), and cytolytic cells (Ito *et al.*, 2007a) (Figure 2.5).

Extracellular HMGB1 stimulates activation of immune response via interaction with receptor for advanced glycation end-products (RAGE), TLR2 and TLR4 (Lotze *et al.*, 2005). Activation of the receptor by HMGB1 induces the production of TNF (Agnello *et al.*, 2002; Andersson *et al.*, 2000; Kim *et al.*, 2010), IL-1α (Andersson *et al.*, 2000), IL-1β (Andersson *et al.*, 2000; Kim *et al.*, 2010), IL-6 (Agnello *et al.*, 2002; Andersson *et al.*, 2000; Hou *et al.*, 2011; Kim *et al.*, 2010), IL-8 (Andersson *et al.*, 2000; Dejean *et al.*, 2012; Kim *et al.*, 2010), IL-10 (Wu *et al.*, 2013), IL-11 (Kim *et al.*, 2010), IL-12 (Matsuoka *et al.*, 2010), IL-17 (Kim *et al.*, 2010), macrophage inflammatory protein (MIP)-1α and MIP-1β (Andersson *et al.*, 2000; Wu *et al.*, 2013), chemokines including

CCL5, CXCL1, CXCL2, CCL2, CCL20 and CCL3 (Pedrazzi *et al.*, 2007), adhesion molecules such as ICAM-1, VCAM-1 and E-selectin, G-CSF (Treutiger *et al.*, 2003), antigen CD40 (Matsuoka *et al.*, 2010) and inflammatory related protein tissue factor (TF) (Lv *et al.*, 2009), inducible nitric oxide synthase (iNOS) (Ren *et al.*, 2006) and inhibitor-signal transducer and activator of transcription-1 (SOCS-1) (Li *et al.*, 2011). The underlying factor in the induction of pro-inflammatory molecules by HMGB1 is the activation of various signalling pathways, including NF-κB, ERK (Park *et al.*, 2003), JNK (Kim *et al.*, 2012; Wu *et al.*, 2013), JAK (Li *et al.*, 2011), PI3K/Akt (Hou *et al.*, 2011; Kim *et al.*, 2012), Sirt1 (Kim *et al.*, 2010), HSP27 (Wolfson *et al.*, 2011) and EGR1 (Lv *et al.*, 2009). The inflammatory activation by HMGB1 may lead to the vascular barrier damage (Wolfson *et al.*, 2011), intestinal barrier disruption (Sappington *et al.*, 2002), severe lung injury (Abraham *et al.*, 2000), and thrombosis (Ito *et al.*, 2007b). Conversely, HMGB1 is speculated to promote the repairing of tissue via its role in neurite outgrowth in promoting cell survival (Lotze *et al.*, 2005) (Figure 2.5).

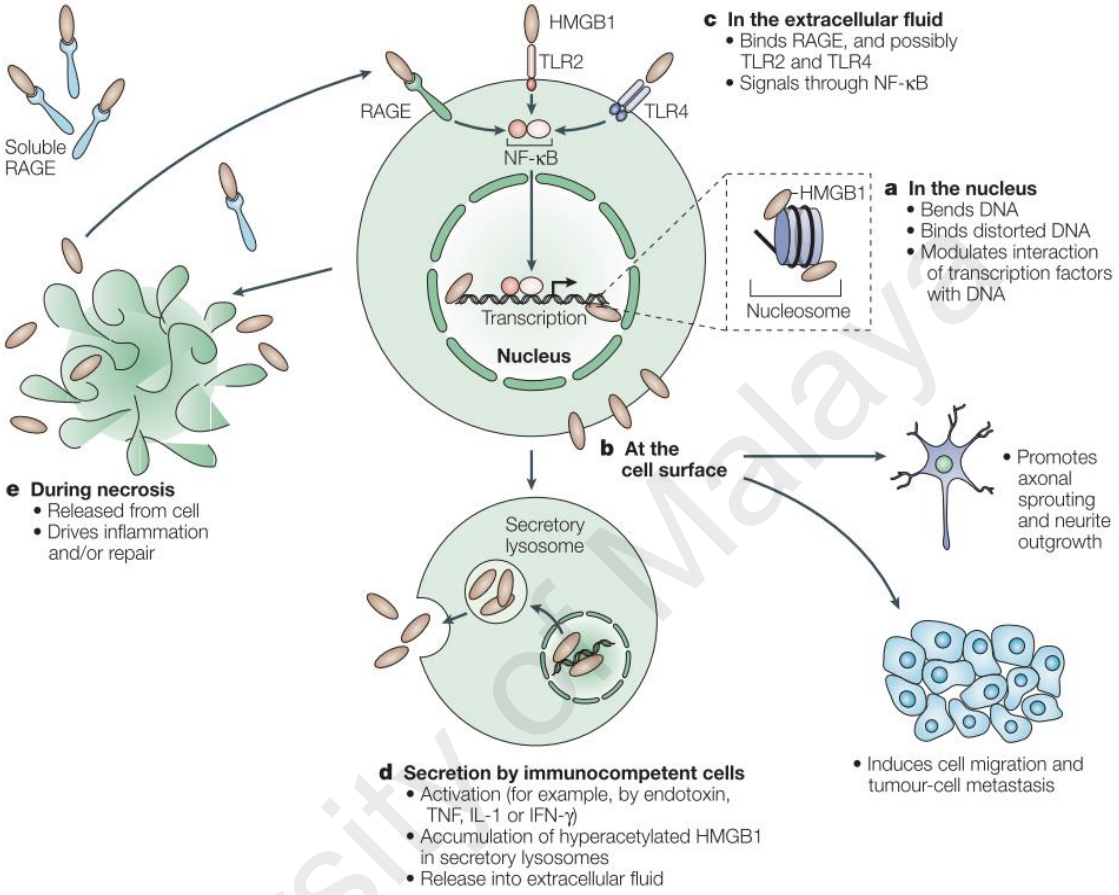


Figure 2.5: Roles of intranuclear and extranuclear of HMGB1. (A) Nuclear HMGB1 is essential for the regulation of DNA transcription. **(B)** HMGB1 on the cell surfaces plays a role in the axonal sprouting and neurite growth, cell migration and adhesion, cell differentiation and innate immunity. **(C)** Extracellular HMGB1 binds to RAGE, TLR2 or TLR4 and induces the activation of NF-κB pathway. **(D)** Secretion of HMGB1 by immune cells leads to the activation of pro-inflammatory cytokines and accumulation of hyperacetylated HMGB1 in the extracellular fluid. **(E)** Release of HMGB1 by necrotic cells induces inflammation or repair processes. Figure modified from Lotze *et al.*, 2005.

2.5.3. The role of HMGB1 in autoimmune diseases

HMGB1, an inflammatory response aggressor is known to be involved in the pathogenesis of autoimmune disease such SLE, rheumatoid arthritis (RA), multiple sclerosis (MS) and myositis (Kang *et al.*, 2014a; Pisetsky, 2014). HMGB1 level in the serum showed positive correlation with the SLEDAI scores and proteinuria, the disease activity markers (Abdulahad *et al.*, 2011).

2.5.3.1. The role of HMGB1 in SLE

HMGB1 is also associated with the level of pro-inflammatory cytokines like TNF-α and IL-6 and the heightened HMGB1-mediated macrophage inflammatory response in SLE (Lu *et al.*, 2015a).

2.5.3.2. The role of HMGB1 in RA

In addition, sites of joint inflammation in RA such as synovial tissues and synovial fluid displayed a high level of HMGB1 (Hamada *et al.*, 2008; Kokkola *et al.*, 2002; Taniguchi *et al.*, 2003) and inoculation of HMGB1 in the normal joint instigated arthritis in 80% of the animals used in a study (García-Arnandis *et al.*, 2010; Pullerits *et al.*, 2003).

2.5.3.3. The role of HMGB1 in MS

HMGB1 also showed potential as a biomarker for MS, observed from the high level of HMGB1 in the peripheral blood mononuclear cells of MS patient in comparison to the controls.

2.5.3.4. The role of HMGB1 in myositis

High cytoplasmic translocation and the release of HMGB1 were observed in myositis (Ulfgren *et al.*, 2004). In contrast, HMGB1 also stimulates regeneration of muscle fiber in myositis (De Mori *et al.*, 2007; Vezzoli *et al.*, 2011).

2.5.4. The role of HMGB1 in virus infections

The function of HMGB1 as a damage-associated molecular pattern molecule (DAMP) and its inflammatory activity after being released into the extracellular milieu leads to the association of the protein with the severity of inflammatory-related disease (Lotze *et al.*, 2005; Sheller-Miller *et al.*, 2017). Another DAMP known to be involved in the pathogenesis of dengue is metalloproteinase-9 (MMP9), a molecule that showed an increased in the plasma of dengue patients in comparison to the healthy individuals (Kubelka *et al.*, 2010) and suggested to induce permeability of in vitro endothelial cells and vascular leakage in mice (Luplerdlop *et al.*, 2006). In addition, galectin-9 (GAL-9) which is also a DAMP showed an increased in the supernatant and decreased in the cells infected by DENV3, proposing the possibilities that dengue virus inhibited GAL-9 expression in the cells (Dapat *et al.*, 2017). Endogenous activators of Toll-like receptor 4 such as myeloid-related protein-8 and -14 (MRP8/MRP14) induced endotoxin-induced lethal shock and known to be the new inflammatory components (Vogl *et al.*, 2007).

Previous studies showed that the level of HMGB1 was significantly upregulated in the sera of dengue patients in comparison to the healthy controls (Allonso *et al.*, 2012; Allonso *et al.*, 2013). DENV-induced necrosis cells stimulate passive release of HMGB1 (Chen *et al.*, 2008a), while another study described the release of HMGB1 under non-necrotic condition in DENV-infected DCs (Kamau *et al.*, 2009). During DENV infection, HMGB1 was translocated from the nucleus to the cytoplasm via monocytic cell p300/CBP-associated factor (PCAF) acetylase complex, which activated by DENV capsid protein (Ong *et al.*, 2012). The relocation of HMGB1 depends on the acetylation process of the protein that leads to the release of HMGB1 into the extracellular milieu, where the binding of HMGB1 and RAGE takes place and possibly contributes to the inflammatory activation that disrupts the vascular barrier (Ong *et al.*, 2012). Besides dengue, the level of HMGB1 was significantly higher in West Nile neuro-invasive disease

(WNND) patients than those with normal West Nile virus (WNV) infection and healthy controls (Fraisier *et al.*, 2015). The significant increase in the level of HMGB1 in Dobrava virus (DOBV) or Puumala virus (PUUV) infection (hemorrhagic fever with renal syndrome, HFRS) and Crimean Congo hemorrhagic fever virus (CCHFV), suggest the potential of HMGB1 as a biomarker for severe virus hemorrhagic fevers (Rus *et al.*, 2016). Concurrently, studies implied that supplying anti-HMGB1 monoclonal antibody leads to the suppression of inflammatory activation in the disease, as observed in the influenza A virus (H1N1)-induced pneumonia in mice (Nosaka *et al.*, 2015). HMGB1-specific antibody also decreased the inflammatory responses in influenza H5N1 virus infection, though not affecting the virus titer (Hou *et al.*, 2014). The clearance of HMGB1 using a highly adsorptive dialysis membrane (HADMs) demonstrated significant attenuation of inflammatory manifestation and fatality rates of H1N1 (Honore *et al.*, 2015).

HMGB1 also plays a role in the virus replication, where it facilitates the replication of Hepatitis C virus (HCV) via interaction with the HCV genome (Yu *et al.*, 2016). The HMGB1-pathogen-associated molecular pattern (PAMP) complex enhances the inflammatory loops and assists in virus replication (Trøseid *et al.*, 2010). In contrary, intracellular HMGB1 decreases HIV transcription via inhibition of long terminal repeat (LTR). Moreover, HMGB1 has been associated with the decreased DENV replication in DCs (Kamau *et al.*, 2009). Furthermore, we reported the role of HMGB1 in the antiviral response against dengue (Zainal *et al.*, 2017).

CHAPTER 3: METHODOLOGY

3.1. Aim 1: To verify the association between single nucleotide polymorphism (SNP) in severe dengue, non-severe dengue and autoimmune patients

3.1.1. Patient Samples

Blood samples from dengue-diagnosed (n=37) and SLE patients (n=18) were collected from UMMC and Putrajaya Hospital, respectively. The clinical records of the dengue-diagnosed patients were evaluated and the classification of non-severe dengue and severe dengue were performed according to the World Health Organization (WHO) guideline, 2009 (WHO, 2009). Severe dengue samples were obtained from 21 dengue patients with symptoms including severe plasma leakage, severe haemorrhage and/or severe organ impairment, whereas non-severe dengue samples were collected from 16 dengue patients with fever, body aches, abdominal pain, nausea, mucosal bleeding, headache and/or epistaxis and gum bleeding. SLE samples were extracted from 18 patients that conformed to 1982 ACR revised criteria for Classification of Systemic Lupus Erythematous (Table 2.1) (Tan *et al.*, 1982). The information on the medication, treatment and the relapse/remission status of SLE patients were not able to be disclosed. The ethical clearance for this study was approved by the UMMC Medical Ethics Committee (Ethics Committee/IRB Reference Number: 962.6) and Medical Research & Ethics Committee, Ministry of Health Malaysia (reference number: NMRR-12-1412-13606).

3.1.2. DNA Extraction

DNA was extracted from the blood samples using DNeasy Blood & Tissue Kit (Qiagen, CA). The blood was added into the ceramic bead tube (Omni International, GA) and subsequently liquefied by Precellys® 24 homogenizer (Bertin 249 Technologies, Bretonneux, France) at 5000 rpm with a cycle of 10 seconds run and 5 seconds break. Next, 400 µl of the liquefied blood was transferred into a new 1.5 ml microcentrifuge

tube containing 40 µl of Qiagen Proteinase K. RNase (8 µl) was added into the mixture and incubated for 2 minutes at room temperature. Afterwards, 400 µl of AL buffer was added into the mixtures and incubated at 56°C for 10 minutes. Ethanol was added into the mixture and mixed. The mixture was transferred into the QIAamp Spin Column and centrifuged at 10,000 rcf for 1 minute at room temperature. The liquid flowed through the column was discarded and the column was washed twice using Buffer AW1 and AW2. Each wash was performed with centrifugation at 10,000 rcf for 1 minute and 20,000 rcf for 3 minutes, respectively. Washed column was transferred to the new tube and 50 µl of nuclease-free water was used for DNA elution by centrifugation at 10,000 rcf for 1 minute. Eluted DNA was collected and kept at -80°C until further use.

3.1.3. Quantification of DNA

DNA quantification was carried out using Qubit Assay Kits (ThermoScientific, Waltham, MA). The standard and samples were diluted at 1:20 dilution in the Qubit working solution. Then, the mixtures were vortexed and incubated for 2 minutes at room temperature. The DNA concentration of each sample was read using Qubit® Fluorometer (ThermoScientific, Waltham, MA).

3.1.4. Ion Ampliseq™ Library Preparation

Library preparation of each sample was performed using Ion Ampliseq™ Library Kits 2.0 (Life Technologies, Carlsbad, CA) as previously described (Aloisio *et al.*, 2016). Preparation was carried out strictly as the protocol provided by the manufacturer. The amplification of genomic DNA targets was initiated by adding 5X Ion Ampliseq Hi-Fi Mix and 2X Ion Ampliseq Primer Pool into the extracted DNA. Ion Ampliseq Primer Pool consists of 110 genes (Table 3.1) that are known to be related with dengue and autoimmune response. The mixture was then loaded into the thermal cycler (Veriti 96-well thermal cycle; Applied Biosystems, CA) and run at the following condition:

activation of enzyme at 99°C for 2 minutes, 15 cycles of denaturing at 99°C for 15 seconds and annealing at 60°C for 4 minutes. Next, the digestion of the primer sequence was performed by addition of FuPa reagent into the samples and followed by incubation in the thermal cycler at the following program: 50°C for 10 minutes, 55°C for 10 minutes and 60°C for 20 minutes. The ligation of adapters to the amplicons was carried out by the addition of adapters, followed by the addition of DNA ligase. Subsequently, the mixture was incubated in the thermal cycler at 22°C for 30 minutes and 72°C for 10 minutes. Following adapter ligation, purification of the unamplified library was implemented using 70% ethanol. Afterwards, library amplification was performed by the addition of PCR SuperMix High Fidelity and Library Amplification Primer Mix. The mixture was loaded into the thermal cycle and ran as follows: 98°C for 2 minutes, 5 cycles of 98°C for 15 seconds and 64°C for 1 minute. The final products were then quantified using Qubit Assay Kits as previously described (3.1.3).

3.1.5. Ion Sphere Particles (ISPs) Amplification and Enrichment

Amplification and enrichment of ISPs were carried out using Ion PGM™ Template Kit (Life Technologies, Carlsbad, CA). ISP is a small polystyrene bead that binds to the adapter ligated to the amplicons. This technology allowed the clonal amplification of the library prepared previously. Clonal amplification was performed by the addition of Ion PGM Enzyme™ Mix and Ion PGM™ ISPs into the library. The mixture will be added into the OneTouch Reaction Filter and then inserted into Ion OneTouch™ 2 Instrument (Life Technologies, Carlsbad, CA) for the clonal amplification. Upon completion of ISP amplification, the product was then subjected to ISP enrichment, where the blank ISPs that unattached to the amplicons were removed. The ISP enrichment was carried out using Ion OneTouch™ ES (Life Technologies, Carlsbad, CA).

Table 3.1: Target genes in Ion AmpliSeq Panel.

Target Genes										
Autoimmune diseases associated genes					Dengue and Autoimmune diseases associated genes					
ADAD1	DBC1	TMEM39A	CD58	PTPN22	IL1B	OAS1	IL18	ANGPT1	CBLL1	PLCE1
RSBN1	IL1-ALPHA	CBLB	cIAP2	NKX2-3	BLIMP1	KLC1	HLA-L	ABCB1	IL-7	IL21
FAM69A	RPL5	EVI5	IL-23R	PDE4B	LGALS3BP	CXCL10	CXCL1	IL-8	IFNG	IRF5
MST1	BSN	PTGER4	IL7R	HLA-DQA1	LILRB1	IL17A	C5A	CD40	MxA	IL-3
PSORS1C1	ALK	ANAPC4	NELL1	PADI4	TGFB1	SYNGR1	NPR2	CCL4	CCL3	GM-CSF
NEIL2	OGG1	LAG3	MMEL1	ATG16L1	RANTES	CCL8	CCL2	HLA-DRB1	RIG-I	IL-5
STAT4	ITGAV	PLSCR1	SLC22A5	OCTN1	TNF	ITGAM	ABCF1	XBP1	PTAFR	IL-13
TRAF1	PHF19	ANKRD1			LGALS9	MIF	DR4	DR5	IL-6	IL-4
					APOB	TLR7	PTPN2	CLEC16A	ICAM1	TNFAIP3
					KLRB1	C3AR	CD137	IL2RA	PD-L1	IL-15
					SLC9A3R2	ISG15	IRF7	IL10	CTLA4	IRGM
					TNFSF10	MDA-5	FCGR2B	FCGR2A	IL12B	FCRL3

3.1.6. Ion PGM™ Hi-Q Sequencing

The sequencing was carried out using Ion PGM ™ Hi-Q Sequencing kit (Life Technologies, Carlsbad, CA). Sequencing Primer and Ion PGM ™ Hi-Q ™ Sequencing Polymerase were added into the sample. The final sample mixture was loaded into the Ion 318™ Chip (Life Technologies, Carlsbad, CA). Next, the loaded chip was placed onto the Ion PGM™ Sequencer (Life Technologies, Carlsbad, CA) that previously washed and initialized.

3.1.7. Sequencing Data Analysis

Ion Torrent's specific software, Torrent Suite with the plug-in of the Variant Caller program (Life Technologies, Carlsbad, CA) was performed on the sequencing data. The process comprised of alignment with the human genome build 19 reference genome (hg19), base calling and removal of the poor signal reads. Next, the result of the analysis was further annotated using the free online software, Ion Reporter (Life Technologies, Carlsbad, CA). The SNPs with less than 100X coverages, located in the intronic region, showed less than 0.15 allele ratio of variant to reference and caused synonymous effect were excluded. Variants that were known, had an entry in SNP database (dbSNP) while those without an entry considered as novel variant. Visual assessment on the data was performed using Integrative Genomics Viewer (IGV) Version 2.3 (Broad Institute, Cambridge, MA) to verify the variants called and to detect the short reads with mispriming events.

Potential binding sites of exonic splicing enhancer (ESE) were investigated using online prediction tool, ESEfinder 3.0. (http://krainer01.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi?process=home) and the putative transcription factor binding sites were explored using online analysis software, PROMO (http://alggen.lsi.upc.es/cgibin/promo_v3/promo/promoinit.cgi?dirDB= TF_8.3).

3.1.8. Statistical Analysis

Statistical analysis for this study was completed using IBM SPSS Statistics (IBM Corporation, New York) and GraphPad Prism 5.0 software. The SNPs frequencies between the experimental groups were compared using Fisher's exact test and the association was considered significant with two-sided *P* value < 0.05, assessed by the odd ratio (OR) with corresponding 95% confidence intervals (CIs).

3.2. Aim 2: To determine the neutralization capability of SLE patients' sera against DENV

3.2.1. Ethical Clearance

The approval of the samples collected and experiments in this study were granted by the Medical Research & Ethics Committee, Ministry of Health Malaysia (reference number: NMRR-12-1412-13606) and University Malaya Medical Centre (UMMC) Medical Ethics Committee (Ethics Committee/IRB Reference Number: 962.6). Written informed consent was attained from each volunteer in this study.

3.2.2. Patients Samples

Blood samples were collected from SLE patients in Hospital Putrajaya, Malaysia for a total of 132. The participants were selected based on 1982 ACR revised criteria for Classification of Systemic Lupus Erythematous. Samples of healthy controls and dengue-diagnosed patients were obtained from TIDREC and UMMC, respectively. Blood samples were centrifuged at 2000 rcf for 10 minutes and the serum layer was collected. The sera collected were kept at -80°C until further used. The presence of dengue antibodies in all serum samples was screened using dengue IgG or IgM captures ELISA (antigen E protein) (Standard Diagnostics, Inc., Gyeonggi-do, Korea). There were 3 groups of patient samples studied, including SLE (dengue IgG and IgM negative) (n=82) patients as case study, and healthy (no known SLE and dengue IgG negative) (n=24) and dengue-positive (no known SLE and dengue IgG positive) (n=15) patients as healthy and positive controls, respectively. The history of dengue or other virus infection of SLE patients was not disclosed. ELISA test showed that among 132 SLE samples collected, only 82 (62%) of them were negative for dengue IgG and IgM. Anti-JEV IgG were detected using antibody capture IgG ELISA (antigen glycoprotein E) (Euroimmune AG, Lubeck, Germany) and the methods were performed as per the instructions of the

manufacturer. As previously described (Olver *et al.*, 2010), IgG from the sera was depleted using ProteoExtractAlbumin/IgG Removal Kit (Calbiochem, San Diego, CA) in accordance with the manufacturer's protocol. The quantity of sera tested in each experiment, however, were not equal to each other due to the limitation in sample collection.

3.2.3. Cell Cultures

African green monkey kidney epithelial cells (Vero) used in this study were obtained from our collaborator in Sweden (Ammerman *et al.*, 2008). Cells were cultured in the Dulbecco's modified Eagle medium (DMEM; Life Technologies, Carlsbad, CA) supplemented with 1% penicillin/streptomycin and 10% heat-inactivated fetal bovine serum (FBS; Bovogen, Australia) and kept at 37°C with 5% CO_2. Subpassaging was performed regularly once the cells become confluent.

3.2.4. Viruses and Stock Preparation

There are 3 serotypes of DENV used in this study; DENV1, DENV2 and DENV3. All serotypes were obtained from the WHO Collaborating Centre at University of Malaya (WHOCC, UM). Virus stocks of each serotype were prepared by infecting Vero cells monolayer (80% confluence) with virus inoculum at 1:40 dilution in a serum-free medium. The adsorption of virus to the cells was performed for 1 hour with mild rocking at room temperature. Next, DMEM supplemented with 2% FBS were used to replace the serum-free medium and the cell cultures were incubated at 37°C in a 5% CO_2 condition for 7 days. The supernatant of the cell cultures were harvested and transferred to a new Falcon Tube. The tube was then centrifuged at 2000 rcf for 10 minutes to separate the dead cells and debris from the supernatant. The supernatant was filtered using 0.2 µm syringe filter (Sartorius Stedim Biotech, Germany) to sterile the supernatant containing

the viruses. The supernatant was aliquoted into small tubes and kept at -80° C until further use.

3.2.5. Virus Titration Assay

Virus titer was determined by the foci-forming assay as previously reported (Wong *et al.*, 2007). Vero cells were seeded in the 24-well plate at the density of 7.5 x 10^4 per well in the DMEM supplemented with 10% FBS. Cells were incubated overnight at 37°C in a 5% CO_2 incubator. The virus was diluted at 10-fold dilution in a serum-free medium and then added to the cells. The adsorption of the virus was carried out by mild rocking at room temperature for 1 hour and 30 minutes. Afterwards, infecting fluid was removed and overlay medium containing DMEM supplemented with 2% FBS and 1.5% carboxylmethylcellulose (CMC) (Sigma Aldrich Chemical, St. Louis, MO) was added. Cells were incubated at 37°C with 5% CO_2 for 96 hours (DENV1, DENV3) or 72 hours (DENV2). Virus detection was performed using foci staining assay.

2.4.3. Foci Staining Assay

After the incubation, the overlay medium was removed and the cells were washed thrice with phosphate-buffered saline (PBS). Fixation of the cells was performed using 4% paraformaldehyde (Sigma Aldrich Chemical, St. Louis, MO) diluted in PBS for 20 minutes and subsequently washed with PBS. Cells were permeabilized using 1% Igepal CA-630 (Sigma Aldrich Chemical, St. Louis, MO) diluted in PBS for 15 minutes at room temperature. Again, the cells were washed thrice with PBS and then blocked with 3% skimmed milk diluted in PBS for 2 hours at room temperature. Next, cells were washed thrice with PBS prior to addition of primary antibody (1:500 of human serum diluted in 1% skimmed milk) and incubated at 37°C for 1 hour. The previous solution was discarded and the cells were washed with PBS. Secondary antibody (1:250 of peroxidase-conjugated goat anti-human-IgG serum; Sigma Aldrich Chemical, St. Louis, MO) was

added to the cells and kept at 37°C for 1 hour. For the colorimetric detection, the immunostained cells were subjected to 3, 3'-diaminobenzidine (DAB) substrate (Thermoscientific, Waltham, MA) and the chromogenic development was observed in room temperature within 15 minutes. The foci formed were counted to determine the infectivity virus titer and expressed as foci-forming unit/ml (FFU/ml).

3.2.7. Foci Reduction Neutralization Test (FRNT)

As previously described (Teoh *et al.*, 2013), the neutralization capability of sera against DENV was examined using foci reduction neutralization test (FRNT). First, Vero cells were seeded in the 24-well plates at 7.5 x 10^4 cells per well and incubated overnight at 37°C with 5% CO_2. The patients' sera were heat-inactivated for 30 minutes at 56°C and subsequently diluted into 4-fold dilution (1:20 to 1:5160) in FBS-free medium. DENV were diluted to 100 FFU per well in the FBS-free medium. Next, the virus was added to the diluted serum and incubated at 37°C for 1 hour. The mixture of serum and viruses were added to the Vero cells seeded earlier and incubated at room temperature with gentle rocking for 1 hour. The mixture was removed and overlay medium was added to the cells. Cells were kept at 37°C in a 5% CO_2 condition. The detection of virus was performed using foci staining assay as described previously (3.2.5). The ability of sera to neutralize DENV was determined by the neutralizing antibody titer with a 50% reduction of foci (FRNT50).

3.2.8. Statistical Analysis

Virus reduction percentage of each group (healthy control, SLE and dengue serology positive) was expressed as mean ± the standard error. The statistical significance of variances was determined using Kruskal-Wallis test alongside Dunn's test with 95% confidence intervals. The differences were indicated as significant when $P < 0.05$.

3.3. Aim 3: To investigate the role of HMGB1 in the antiviral mechanism of RESV against DENV

3.3.1. Cell Culture

Human hepatocellular carcinoma cells (Huh7), African green monkey kidney epithelial cells (Vero), Human embryonic kidney 293 with large T antigen cells (293T) and *A. albopictus* larval cells (C6/36) were purchased from the American Type Culture Collection (ATCC; Manassas, VA). Huh7 cells, Vero cells and 293T cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Life Technologies, Carlsbad, CA) supplemented with 1% penicillin/streptomycin and 10% heat-inactivated fetal bovine serum (FBS) (Biological Industries, Kibbutz Bet Haemek, Israel). C6/36 cells were maintained in Eagle's Minimum Essential Medium (EMEM) (HyClone, USA) supplemented with 10% FBS (Biological Industries, Kibbutz Bet Haemek, Israel). Huh7 cells, Vero cells and 293T cells were maintained at 37°C in a 5% CO_2 humidified incubator, whereas C6/36 cells were kept at 28°C without CO_2.

3.3.2. Drug

Resveratrol (RESV) (purity >99%, molecular formula; $C_{14}H_{12}0_3$, molecular weight; 228.24) was purchased from Sigma-Aldrich Chemical. RESV was dissolved in dimethyl sulfoxide. Stock solution was diluted in DMEM supplemented with 2% FBS to obtain a desired final concentration.

3.3.3. shRNA-mediated Knockdown Gene Expression

Knockdown of HMGB1 (shHMGB1) and Sirt1 (shSirt1) gene expression were achieved using the shRNA method (Tsai *et al.*, 2009). Lentiviral shRNA plasmid targeting the gene of interest was purchased from the National RNAi Core Facility, Academia Sinica, Taiwan. 293T cells were used to obtain the lentiviruses expressing shRNA from the lentiviral vector and packaging plasmid. Lentiviral shRNA plasmid, pR plasmid and

pMD2.G plasmid were mixed with GeneJammer Transfection Reagent. Then, the mix was added onto the 293T cells. Cells were incubated for 3 days and the supernatant was harvested. The supernatant was then centrifuged at 5000 rpm, 4°C for 5 minutes and subsequently filtered (0.22 µM). Filtered supernatant was centrifuged at 20,000 rcf, 4°C for 4 hours. The supernatant was removed and the pellet of lentiviruses was suspended by vortexing. The lentiviruses at a multiplication of infection (MOI) of 3 and 8 µg/ml of polybrene were added onto the target cells Huh7 and incubated at 37°C overnight. After 24 hours, the medium was replaced with selective medium containing puromycin (Sigma-Aldrich Chemical) at a final concentration of 3 µg/ml.

3.3.4. Virus Stock Preparation

DENV serotype 2 strain PL046 was used in this study. Propagation of the virus was carried out in C6/36 cells (Igarashi, 1978). The virus was diluted at 1:200 dilution in FBS-free medium and then added to the C6/36 cells. The adsorption process was allowed for one hour by gentle rocking at room temperature. Next, the medium was replaced with EMEM supplemented with 2% FBS and incubated for 7 days at 28°C in a 3% CO_2 humidified incubator. Supernatant containing the viruses was harvested, centrifuged at 10,000 rcf for 10 minutes and filtered using 0.2 µM syringe filter (Sartorius Stedim Biotech, Germany) to remove cell debris. The filtered supernatant containing the viruses were kept at -80°C.

3.3.5. Virus Infection

Huh7 cells (wild type and HMGB1 knockdown and Sirt1 knockdown) were seeded at 1 x 10^5 cells per well in 12-well plate and incubated at 37°C overnight. Then, Huh7 cells were mock-treated or treated with RESV at the final concentration of 50 or 80 µM for 4 hours. The medium was removed and the cells were washed using FBS-free medium twice. Cells were then infected with the DENV serotype 2 at an MOI of 1 or 10 and

incubated in the 37°C incubator for 1 hour (Lin *et al.*, 2000). The supernatant was discarded and the cells were washed using FBS-free medium twice followed by the addition of 1 ml of DMEM supplemented with 2% FBS in each well. The plate was incubated at 37°C for 24 hours and both supernatant and cell cultures were harvested for the virus titer analysis using focus forming assay, determination of protein expression by western blotting, quantification of DNA by quantitative real-time PCR, and observation of protein distribution using immunofluorescence assay (IFA), respectively.

3.3.6. Virus Titer Analysis

Virus titer in cell culture supernatant was determined by focus forming assay and Vero cells were used to carry out this assay (Teoh *et al.*, 2013). Vero cells were seeded at 1 x 10^5 in a 24-well plate (Falcon) and cultured overnight at 37°C in a 5% CO_2 condition. The medium was discarded and the cells were inoculated with serially-diluted supernatant containing viruses harvested from the cell culture previously. The infected cells were gentle-rocked for 1 hour at room temperature for virus adsorption. Afterward, the supernatant was removed and 1 ml of overlay medium comprising of DMEM supplemented with 2% FBS and 1.5% CMC (Sigma Aldrich Chemical) was added. The cell cultures were subsequently incubated at 37°C in a 5% CO_2 condition for 72 hours. Afterwards, foci staining method was performed as explained hitherto (3.2.6). The foci formed were counted to determine the infectivity virus titer and expressed as foci-forming unit/ml (FFU/ml).

3.3.7. Protein Extraction

All of the cell cultures were harvested at 24 hours post infection unless indicated otherwise. Following the removal of the supernatant, cell cultures were washed twice with PBS. Then, the extraction of cells' cytoplasm and nuclear protein or total protein was carried out.

3.3.7.1. Cytoplasm and Nuclear Protein Extraction

The extraction of cytoplasm and nuclear protein were performed using NE-PER Nuclear and Cytoplasmic Extraction Reagents (ThermoScientific, Waltham, MA). The washed cells were harvested using trypsin-EDTA, transferred into a clean microcentrifuge tube and subsequently centrifuged at 500 x g for 5 minutes. Cells were suspended in PBS and centrifuged at 500 x g for 2 minutes. The supernatant was discarded and the cell pellet was left to dry. Ice cold CER I (ThermoScientific, Waltham, MA) was added onto the cell pellet and the tube was vortexed at the highest setting for 15 seconds. Then, the cells were incubated on ice for 10 minutes. Next, CER II (ThermoScientific, Waltham, MA) was added to the cells. The tube containing the cells was vortexed at the highest setting for 5 seconds and incubated on ice for 1 minute. After incubation, the tube was vortexed at the highest setting for 5 seconds and centrifuged for 10 minutes at the maximum speed. Supernatant comprising the cytoplasmic extract was quickly transferred to a clean pre-chilled tube. The insoluble fraction left in the tube was washed with PBS twice and centrifuged at the maximum speed for 10 minutes. The fraction was suspended in ice-cold NER (ThermoScientific, Waltham, MA) using pipettes and then vortexed at the highest setting for 15 seconds. The tube was incubated on ice and vortexed for 20 seconds every 10 minutes, for a total of 40 minutes. The tube was finally centrifuged at the maximum speed for 10 minutes and the supernatant containing the nuclear extract was immediately transferred to a clean pre-chilled tube. Both cytoplasmic and nuclear extracts were stored at -80°C until use.

3.3.7.2. Total Protein Extraction

Cells were washed twice using PBS and subjected to 100 µl of lysis buffer (10 mM Tris-HCl, 50 mM NaCl, 5mM EDTA, 10 mM NaN_3, 10 mM NaF, 10 mM $Na_4O_7P_2$, 1% Triton X-100, pH 7.5). Cells were then scraped completely from the well and transferred to a clean tube. Protein extracts were kept at -80°C until use.

3.3.8. Western Blot Analysis

3.3.8.1. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

SDS-PAGE was carried out using the Mini-PROTEAN Tetra Electrophoresis System (BioRad, Hercules, CA). The separating and stacking gels (amount for 2 gels) were prepared according to the Table 3.2. The mixture of separating gel was poured between the glass plates of electrophoresis system and allowed to polymerize for about 40 minutes. Next, the mixture of stacking gel was poured on top of the separating gel and a comb was swiftly added to create the wells for sample loading. The gels were left at room temperature for 20 minutes to polymerize. The protein extracts were mixed with 25% of sample buffer comprising of 250 mM Tris-HCl, 500 mM dithiothreitol (DTT), 10% SDS, 0.1% bromophenol blue and 50% glycerol. The mixtures were then heated at 100°C for 10 minutes and left to cool in room temperature. Once the gel hardened, the gels were assembled onto the electrophoresis system and afterward, the samples were loaded by pipetting into the wells of stacking gel. The chamber of the electrophoresis system was then filled with electrophoresis buffer consisting of 25 mM Tris, 192 mM glycine and 0.1% SDS. The electrophoresis was performed at the constant voltage of 100 volts for 100 minutes.

Table 3.2: Preparation of separating and stacking gel for SDS-PAGE.

Solution components	Separating Gel (12%)**	Separating Gel (15%)**	Stacking Gel (5%)
30 % Acrylamide Mix	6 mL	7.5 mL	1.3 mL
1.5 M Tris (pH 8.8)	3.8 mL	3.8 mL	1.0 mL
10% SDS	0.15 mL	0.15 mL	0.08 mL
10% Ammonium Persulfate	0.15 mL	0.15 mL	0.08 mL
Distilled water	4.9 mL	3.4 mL	5.5 mL
***TEMED**	0.006 mL	0.006 mL	0.008 mL

*TEMED: tetramethylethylenediamine

**The different percentage of separating gel was used for different size of protein

3.3.8.2. Protein transfers onto the membrane

After the completion of electrophoresis run, the gel containing the separated protein was soaked in the mixture of 80 ml of blotting buffer consisting of 250 mM Tris and 192 mM glycine with 20 ml of methanol for a few minutes. Next, the nitrocellulose membrane and the blotting paper were immersed in the blotting buffer for a few minutes before stacked together with the gel for the transferring process. The protein transfer from the gel onto the nitrocellulose membrane was carried out using HEP-1 Panther semi-dry electroblotter system (ThermoScientific, Waltham, MA) for 3 hours at a constant 45 volts.

3.3.8.3. Detection of the protein

Once the transferring process completed, the membrane was blocked in the 5% skimmed milk diluted in the PBS for 1 hour. The membrane was then washed 3 times with 0.05% Tween-20 PBS. Following the washing, the membrane was soaked in the primary antibody overnight at 4°C. The primary antibodies used in this study were rabbit anti-HMGB1 antibody (1:2500 dilution; Abcam, Cambridge, UK), rabbit anti-NS3 antibody (1:5000 dilution; GeneTex, San Antonio, TX), rabbit anti-MxA (1:1000 dilution; Abcam, Cambridge, UK), rabbit anti-ISG15 (1:1000 dilution; Cell Signaling Technology, Danvers, MA), rabbit anti-Sirt1 (1:1000 dilution; Cell Signaling Technology, Danvers, MA), mouse anti-PARP-1 (1:1000 dilution; Santa Cruz Biotechnology, CA) and mouse anti-Actin (1:10000 dilution; Abcam, Cambridge, UK). After the exposure of the primary antibody, the membrane was washed 3 times with 0.05% Tween-20 PBS. Next, the membrane was immersed in the secondary antibody, horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1:5000 dilution; Cell Signaling Technology) or horseradish peroxidase (HRP)-conjugated anti-mouse IgG (1:5000 dilution; Cell Signaling Technology) at room temperature for 1 hour. The membrane was then washed 3 times with 0.05% Tween-20 PBS and subsequently immersed in the Immobilon Western

Chemiluminescent HRP Substrate for 10 minutes (Millipore, Saint Louis, MO). Then, the membrane was prepared for protein visualization by photographic film exposure.

3.3.9. Quantitative Real-Time PCR (qRT-PCR)

The total RNA of the harvested cells was extracted using the TRIzol solution (ThermoScientific, Waltham, MA), followed by Direct-Zol miniprep kit (Zymo Research, Irvine, CA) (Cheng *et al.*, 2016). Once the TRIzol solution was added, the mixture was vortexed briefly and centrifuged for 1 minute at 12,000 x g. The supernatant of the mixture was then collected and transferred to the new microcentrifuge tube. An equal amount of 95-100% ethanol was added to the supernatant and next vortexed briefly. The mixture was transferred to Zymo-Spin™ IIC Column in a collection tube and afterward centrifuged for 1 minute at 12,000 x g. Direct-zol™ RNA Prewash was dispensed into the column and again centrifuged for 1 minute at 12,000 x g. Then, RNA Wash Buffer was added into the column and centrifuged for 1 minute at 12,000 x g. The column was transferred into a new RNase-free tube and the RNA was eluted by the addition of 50 µl of DNase/RNase-free water directly to the column, followed by centrifugation at 12,000 x g for 1 minute. Reverse transcription of RNA into cDNA was performed using PrimeScript RT reagent kit (TaKaRa Bio. Inc., Shiga, Japan). The reaction mixture (10 µl) contained 1X PrimeScript Buffer, PrimeScript RT Enzyme Mix 1, Oligo dT Primer (50 µM), Random 6 mers (100 µM) and RNA template (1 µl). The reverse transcription was carried out at 37°C for 15 minutes and 85°C for 5 seconds. The real-time PCR was accomplished with Fast SYBR Green PCR master mix (Applied Biosystems, CA). The real-time PCR mixture (20 µl) consisted with 1X SYBR Green Master Mix, 10 µM of each primer and cDNA (4 µl). The real-time PCR was performed at 95°C for 20 seconds and preceded into 40 cycles of both denaturation at 95°C for 3 seconds and annealing/extension at 60°C for 30 seconds. The real-time PCR was conducted in StepOnePlus PCR system (Applied Biosystems, CA). Primers used in real-

time PCR are listed in Table 3.3. The mRNA levels were depicted as fold induction obtained from the value comparison to uninfected cells and normalization to intracellular β-actin mRNA levels.

Table 3.3: List of primer sequences used in this study.

Primer	Forward	Reverse
IFN-β	TAGCACTGGCTGGAATGAGA	TCCTTGGCCTTCAGGTAATG
ISG54	ATGTG CAACC TACTG GCCTA T	TGAGA GTCGG CCCAT GTGAT A
ISG56	GGGCA GACTG GCAGA AGC	TATAG CGGAA GGGAT TTGAA AGC
MxA	ACCAC AGAGG CTCTC AGCAT	CTCAG CTGGT CCTGG ATCTC
β-Actin	AAGGA GAAGC TGTGC TACGTCGC	AGACA GCACT GTGTT GGCGT ACA
MxA-I	GCAGC CATCT CAAAG TATGC	AGGAG CAGAA GCTGA AATCC
MxA-II	TGGAG AGGAA CAGCA GAGG	GCATT CAGCA CATGA TCG

3.3.10. Immunofluorescence Analysis (IFA)

For IFA analysis (Tsai *et al.*, 2014), cells were seeded on the circular cover glass before the RESV treatment and DENV infection. After 24 hours of infection, the supernatant was removed and the cells on the circular cover glass were transferred to a new plate. Cells were fixed using 4% paraformaldehyde for 20 minutes at room temperature. Cells were then washed with PBS for 3 times. Permeability buffer consisting of 1% BSA, 0.05% NaN_3 and 0.1% Saponin in PBS was prepared and used as the dilution solution for the antibodies used in this study. Primary antibodies, including rabbit anti-HMGB1 antibody (1:1000 dilution; Abcam) and mouse anti-E antibody (1:500 dilution; GeneTex, San Antonio, TX) were diluted in permeability buffer and added onto the cells. Cells were incubated overnight with the primary antibody at 4°C. Next, the cells were washed 3 times using PBS and afterwards incubated with secondary antibody (1:250 dilution) for 1 hour at room temperature. Secondary antibodies used in this study were donkey anti-rabbit-IgG Alexa Fluor 594 and goat anti-mouse-IgG Alexa Fluor 488 (Life Technologies, Carlsbad, CA). Nuclei counterstaining was performed by incubation of cells in 4' 6'-diamino-2-phenylindole dihydrochloride (DAPI) at a dilution of 1:1000 for 15 minutes. Cells were washed 3 times with PBS and mounted onto the glass slides using Fluoromount-G™ Slide Mounting Medium (Electron Microscopy Sciences, Hatfield, PA). Cells were left to dry for about 10 minutes in the oven (56°C) before ready to be viewed with BX51 Olympus fluorescence microscope (Olympus, Melville, NY).

3.3.11. Chromatin Immunoprecipitation (ChIP) Assay

ChIP assay was performed as described previously (Cheng *et al.*, 2016). Cross-linking between HMGB1 protein and DNA was induced with fixation of Huh7 cells with 1% paraformaldehyde for 15 minutes. The cross-linked chromatin was extracted and fragmented via sonication to generate an average size of 500 bp DNA. Next, the immunoprecipitation was performed where the fragmented DNA was incubated with the

specific antibodies recognizing HMGB1 or control rabbit IgG at 4°C for 12 to 16 hours. Next, proteinase K was used to reverse the cross-linked between the protein and DNA. The chromatin proteins were removed and the DNA fragments were purified. The identification of the DNA fragments was performed by PCR amplification using the primers amplifying specific regions on the genomic loci of target genes (MxA-I and MxA-II; Table 3.3). The PCR products were then resolved using agarose gel electrophoresis. Gel containing 2% (w/v) of agarose in 1 x Tris base, acetic and EDTA (TBE) was boiled and allowed to cool. Once cooled, SYBR-Safe DNA Gel Stain (ThermoScientific, Waltham, MA) was added into the solution. The gel was then poured into the gel tank and allowed to set. Prior to loading, the genomic DNA was mixed with 6 x loading buffer. GeneRuler 1 kb DNA (ThermoScientific, Waltham, MA) was used as the molecular weight standard. Gel electrophoresis was performed in 1 x TAE at 100 V for 1 hour. Genomic DNA was visualized using UVP Biospectrum Imaging System (UVP, Upland, CA) and then characterized according to the molecular weight.

3.3.12. Statistical Analysis

Statistical analysis of the results was performed using GraphPad Prism, version 5 (GraphPad Software Inc., San Diego, CA). Data were presented as mean ± the standard error and compared using one-way analysis of variance (ANOVA), followed by Tukey's multiple-comparison test with 95% confidence intervals (C.I.) to determine the significance of the variance. Differences were considered significant when the *P* value was < 0.05.

CHAPTER 4: RESULTS

4.1. Association of SNPs among non-severe, severe and SLE patients

As shown in Table 4.1, our study involved a total of 55 patients' blood sample, including 16 non-severe dengue, 21 severe dengue and 18 SLE patients. Non-severe dengue and severe dengue patients were characterized according to the WHO 2009 guideline (WHO, 2009). Dengue infected patients with warning signs such as fever, body aches, abdominal pain, nausea, mucosal bleeding, headache and/or epistaxis and gum bleeding were indicated as non-severe dengue. Primary dengue infected patients were excluded for the recruitment of non-severe dengue patients in this study, to prevent the possibility that they might develop severe dengue if re-infected with DENV. Primary and secondary infected patients were distinguished by using IgG to IgM ratio from enzyme-linked immunosorbent assay (ELISA), where IgG/IgM ratio of ≥1.14 was used as the cut-off point for secondary infection (Cucunawangsih *et al.*, 2015). Patients with severe plasma leakage, severe haemorrhage and/or severe organ impairment were described as severe dengue. SLE patients were selected by ACR 1982 revised criteria for classification of systemic lupus erythematous (Tan *et al.*, 1982). Patients with 4 or more of the 11 criteria (Table 2.1) were confirmed as SLE patients. The ratio of male and female among non-severe dengue and severe dengue was equivalent. In SLE patients, the female proportion was greater than male by the ratio of 9:1, a ratio expected of SLE population (Weckerle *et al.*, 2011). The age ratio among those between 17-35 years old and 35 years old and older was similar between the non-severe dengue, severe dengue and SLE patients. The ratio of Malay and Chinese was equal in both non-severe and severe dengue but lower in Indian and others. The ethnic composition of SLE patients showed highest in Malay, followed by Chinese and Indian.

The Ion Torrent sequencing method that was utilized in our study is one of the next generation sequencing (NGS) strategies, where the region of interest in the gene is

enhanced for sequencing. Due to the shorter target of gene region, this technology allowed more depth coverage of sequencing, which is important in the detection of accurate variants at a lower cost, in comparison to the conventional whole genome sequencing (Hagemann *et al.*, 2015). Ion AmpliSeq is a more advanced approach in target sequencing, where a parallel sequencing of multiple samples targeting a massive amount of particular gene locus can be achieved simultaneously (Seo *et al.*, 2013). This technology comes with a multiplex primer panel comprising of several primers sets flanking the targeted regions of interest with a barcode system for the identification, that allow thorough and rapid genotyping. In this study, the multiplex primer panel was designed for the 110 genes that commonly related to dengue and autoimmune diseases (Table 3.1).

Table 4.1: Demographic of dengue and SLE patients.

	Non-severe dengue n (%)	Severe dengue n(%)	SLE n (%)	p-value (Non-severe: Severe; Non-severe: SLE; Severe: SLE)	OR, 95% CI (Non-severe: Severe; Non-severe: SLE; Severe: SLE)
Gender (Male:Female)	10:6 (60:40%)	10:11 (52:48%)	2:16 (11:89%)	0.7388 0.003 0.0082	1.515; 0.4023 to 5.707, 13.33; 2.237 to 79.47, 8.800; 1.605 to 48.25
Race (Malay: Chinese: Indian:Others)	8:3:2:3 (50:19:13:18%)	7:7:2:5 (33:33:10:24%)	12:4:2:0 (67:22:11%)	N/A	N/A
Median Age (17-35:35-older)	11:5 (69:31%)	13:8 (70:30%)	8:10 (45:56%)	0.7386 0.1854 0.3433	1.354; 0.3419 to 5.361 2.750; 0.6727 to 11.24 2.031; 0.5642 to 7.313

n = number of subjects/patients; OR = Odd ratio; 95% CI = 95 percent confidence interval; SLE = Systemic Lupus Erythematosus; N/A = not available

A total of 1793 amplicons ranging from 125 to 225 bp was targeted with a total of 259.93 kb covered. We identified 189 variants in non-severe dengue patients, 208 variants in severe dengue patients and 184 variants in SLE patients. These variants were those with more than 100x coverages and no less than 0.15 allele ratio (De Leeneer *et al.*, 2011) to ensure the NGS metric quality. In addition to that, these variants were also filtered to only variants situated in exonic region and regulatory region (5-prime untranslated region [5' UTR] and 3-prime untranslated region [3' UTR]) with the omission of intronic region. The average coverage achieved in all samples was 478X. Variants that were registered in SNP database (dbSNP) are considered as known variants whereas those without an entry were regarded as novel. From the total variants of each group, 16, 34 and 29 novel variants were detected in non-severe dengue, severe dengue and SLE patients, respectively. Variants with less than 1% value of minor allele frequency (MAF) were deemed as rare. From the total variants in each patient group, 25 variants among the non-severe dengue, 28 variants among the severe dengue and 31 variants among the SLE patients were rare variants (MAF <0.01), whereas the remaining variants were common variants (MAF >0.01). The summary of the sequencing was described in Table 4.2.

Among all of the variants located in the exonic regions, frame-shift mutation at the chr3:49679737 and chr6:30558477 were the variants with significant distribution among non-severe dengue, severe dengue and SLE patients. The frequencies of both variants were summarized and statistically compared between non-severe dengue, severe dengue and SLE patients in Table 4.3. Frame-shift deletion at the chr3:49679737 of BSN (bassoon) gene was a novel variant. The variant was distributed at a significantly higher frequency among severe dengue patients in comparison to the non-severe dengue ($p<0.0001$ OR=48.00 [95% CI=5.008-460.1]) and SLE ($p<0.0001$ OR=111.0 [95% CI=5.690-2166]) patients, suggesting that this variant might have a role in patient predisposition to severe dengue. Variant chr3:49679737 was distributed similarly among

non-severe dengue and SLE patients (p=0.4706, OR=3.581 [95% CI=0.1358-94.38]). Another exonic variant, frame-shift insertion at the chr6:30558477 of ABCF1 (ATP binding cassette subfamily F member 1) gene known as rs76018112, was detected at significantly lower frequency in severe dengue in comparison to the non-severe dengue (p=0.041, OR=0.1731 [95% CI=0.038-0.795]) and SLE patients (p=0.0007, OR=0.044 [95% CI=0.005-0.396]), implying the possibility of this variant as a protective polymorphism against the development of severe dengue. Both non-severe dengue and SLE patients showed no differences in the distribution of variant rs76018112 (p=0.3226, OR=3.923 [95% CI=0.5121-53.48]). SLE patients showed significantly contrast distribution of both variants against severe dengue patients and similar distribution with the non-severe dengue patients, implying the possible protective mechanism by SLE patients against severe dengue progression.

Two of the variants located in the regulatory region, rs1557370 and rs945635 showed significant distribution differences among non-severe dengue, severe dengue and SLE patients. Summary on the distribution of both variants were depicted in Table 4.4. Variant rs1557370 is a SNP mutation in Mx1 (MX dynamin like GTPase 1) gene, which located at the chr21:42830690 of the 3'UTR region. Variant rs1557370 was detected at significantly lower frequency in the severe dengue patients in comparison to the non-severe dengue (p=0.0124 OR=0.1296 [95% CI=0.027-0.624]) and SLE patients (p=0.0154 OR=0.1333 [95% CI=0.029-0.620]), proposing the association of the variant with the protective effects against acute dengue. No significant differences in the distribution of rs1557370 observed among non-severe dengue and SLE patients (p=1.0000 OR=0.9722 [95% CI=0.2503-3.776]). The distribution of variant rs945635, another SNP located in the 5'UTR at chr1: 157670290 of FCRL3 (Fc receptor-like protein 3) was significantly lower among severe dengue patients in comparison to non-severe dengue (p=0.0390 OR=0.2400 [95% CI=0.060-0.96]) and SLE patients (p=0.0104

OR=0.1538 [95% CI=0.038-0.624]), suggesting the protective association of the variant against severe dengue. Similar rs945635 distribution was observed between SLE patients and non-severe dengue patients (p=0.7166 OR=1.560 [95% CI=0.3676-6.620]). Again, SLE patients showed contrast distribution of both variants against severe dengue patients and similar distribution with non-severe dengue patients, proposing the possible protective effects possessed by autoimmune disorder patients against severe dengue.

Table 4.2: Summary of Ion Torrent sequencing of SLE, severe dengue and non-severe dengue patients.

Sequencing Summary			
	Non-severe dengue	Severe dengue	SLE
No. of genes tested	112		
No. of amplicons	1793		
Genomic sequence covered	259.93kb		
Samples number (n)	16	21	18
No. of total variants *	213	242	216
No. of exonic variants	189	208	184
No. of regulatory variants	24	34	32
No. of novel SNPs covered	16	34	29
No. of known dbSNPs covered	173	174	155
No. of rare variants (MAF< 1%)	25	28	31
No. of common variants (MAF>1%)	164	180	153

n = number of subjects/patients; SLE = Systemic Lupus Erythematosus; MAF = minor allele frequency; SNP = single nucleotide polymorphism; dbSNP = single nucleotide polymorphism database

Table 4.3: Frequency of exonic variants among non-severe dengue, severe dengue and SLE patients.

Gene		Non-severe dengue n (%)	Severe dengue n (%)	SLE n (%)
BSN (novel) (chr3:49679737)				
WT allele	AG	15 (94)	5 (24)[a]	18 (100)
Variant allele	A	1 (6)	16 (76)[b]	0
ABCF1 (rs76018112) (chr6:30558477)				
WT allele	G	3 (1)	12 (57)[c]	1 (6)
Variant allele	GA	13 (81)	9 (43)[d]	17 (94)

n = number of subjects/patients; SLE = Systemic Lupus Erythematosus; WT = wild type; BSN = bassoon; ABCF1 = ATP-binding cassette sub-family F member 1; OR = Odd ratio; 95% CI = 95 percent confidence interval

[a]Decreased in severe dengue in comparison to non-severe dengue and SLE (p<0.0001 OR=0.021, 95% CI=0.002-0.200; p<0.0001 OR=0.010, 95% CI=0.000-0.176)
[b]Increased in severe dengue in comparison to non-severe and SLE (p<0.0001 OR=48.00, 95% CI=5.008-460.1; p<0.0001 OR=111.0, 95% CI=5.690-2166)
[c]Increased in severe dengue in comparison to non-severe dengue and SLE (p=0.041 OR=5.778, 95% CI=1.258-26.54; p=0.0007 OR=22.67, 95% CI= 2.525-203.5)
[d]Decreased in severe dengue in comparison to non-severe dengue and SLE (p=0.041 OR=0.1731, 95% CI=0.038-0.795; p=0.0007 OR=0.044, 95% CI=0.005-0.396)

Table 4.4: Frequency of regulatory variants among non-severe dengue, severe dengue and SLE patients.

Gene		Non-severe dengue n (%)	Severe dengue n (%)	SLE n (%)
MX1 (rs1557370) (chr21:42830690)				
WT allele	A	7 (44%)	18 (86%)[a]	8 (44%)
Variant allele	G	9 (56%)	3 (14%)[b]	10 (56%)
FCRL3 (rs945635) (chr1:157670290)				
WT allele	C	6 (38%)	15 (71%)[c]	5 (28%)
Variant allele	G	10 (62%)	6 (29%)[d]	13 (72%)

n = number of subjects/patients; SLE = Systemic Lupus Erythematosus; WT = wild type; MX1 = Interferon-induced GTP-binding protein; FCRL3 = Fc receptor-like protein 3; OR = Odd ratio; 95% CI = 95 percent confidence interval

[a]Increase in severe dengue in comparison to non-severe dengue and SLE (p=0.0124 OR=7.714, 95% CI=1.602-37.15; p=0.0154 OR=7.500, 95% CI=1.614-34.85)
[b]Decreased in severe dengue in comparison to non-severe and SLE (p=0.0124 OR=0.1296, 95% CI=0.027-0.624; p=0.0154 OR=0.1333, 95% CI=0.029-0.620)
[c]Increased in severe dengue in comparison to non-severe dengue and SLE (p=0.0390 OR=4.167, 95% CI=1.042-16.67; p=0.0104 OR=6.500, 95% CI= 1.602-26.37)
[d]Decreased in severe dengue in comparison to non-severe dengue and SLE (p=0.0390 OR=0.2400, 95% CI=0.060-0.96; p=0.0104 OR=0.1538, 95% CI=0.038-0.624)

Table 4.5 showed the information obtained using free online annotation software, Ion reporter (Life Technology) on the significant variants found in this study. The frequency of the presence of allele was depicted as minor allele frequency (MAF), where the variants with value less than 0.01 was considered rare variants, while those with value more than 0.01 was determined as common variants. The phylogenetic P-values (PhyloP) determined the conservation of the polymorphism, where the negative score depicted variants at the fast-evolving sites and positive score showed polymorphisms at the conserved sites. Novel chr3:49679737 and rs76018112 variants in the exonic region of BSN and ABCF1 gene, respectively, are both homozygous mutation (novel; AG – A/A, rs76018112; G – GA/GA). Novel variant at the chr3:4967973 is a rare frame-shift deletion polymorphism (MAF<0.01) and was predicted to affect Alanine (Ala) protein. Variant rs76018112, also a rare polymorphism, was a frame-shift insertion mutation and known to affect the termination protein (Ter). Both variants showed positive PhyloP, suggesting that the polymorphisms were at the conserved site. SNP rs1557370 and SNP rs945635 were both located in the regulatory region, 3'UTR and 5'UTR, respectively. Both of SNPs were heterozygous mutation (rs1557370; A – A/G; rs945635; C – C/G) and considered as the common variants (MAF>0.01). The PhyloP values of SNP rs1557370 and SNP rs945635 were 1.15 and -1.11, which indicate that SNP rs1557370 was at the conserved site, while SNP rs945635 was at fast evolving site.

In order to associate the variants with the phenotype of the patients, we examined the possible effect of the variants on the binding of exonic splicing enhancers (ESEs). ESE is important in guiding accurate splicing of pre-mRNA into messenger RNA (mRNA). The potential binding sites of ESEs, around the variant site was analysed using an online prediction analysis software, ESEfinder 3.0 (http://krainer01.cshl.edu/cgi-bin/tools/ESE3/esefinder. cgi?process =home). About 40 bp selected from the sequences proximal to the variant location (20 bp upstream and downstream of the variant site) was

analysed and the potential binding sites of ESEs including serine/arginine-rich splicing factor1 (SRSF1), SRSF1 (IgM-BRCA1), SRSF2, SRSF5, and SRSF6 were revealed. The potential binding sites of ESEs between the wild-types and the variants were compared, and the differences were highlighted. Results showed that the novel variant at the chr3:49679737 caused reduction of SRSF1 and SRSF1 (IgM-BRCA1) binding score and completely diminished the binding of SRSF2, within 8 bp of the variant site (Figure 4.1). Variant rs1557370 decreased the binding of SRSF1 and completely reduced the binding of SRSF6 (Figure 4.2), within 8 bp of the SNP position. SNP rs945635 reduced SRSF1 binding score and increased SRSF1 (IgM-BRCA1), within 7 bp of the SNP position (Figure 4.3). No differences in the potential binding sites of the ESEs can be observed between the wild-type and the mutant of rs76018112. These findings indicate that novel chr3:49679737 variant, rs1557370 and rs945635 may affect the normal splicing of the ESEs, which then responsible for the different pathological process of the diseases.

The effects of the variants on the transcription factor binding sites were also studied using an online analysis software, PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoi nit.cgi? dirDB=TF_8.3). About 20 bp proximal to the variant site (10 bp upstream and downstream of variant location) were selected and subsequently analysed for the putative transcription factor binding site. The possible transcription factor binding sites of the wild-type and mutant of the variants were compared, and the differences were highlighted. PROMO predicted that novel variant at chr3:49679737 caused disruption of retinoid acid receptor beta: retinoid X receptor alpha (RAR-beta:RXR-alpha), B-cell specific activator protein (Pax5), and tumour protein 53 (p53) and added activating-protein 2 alpha (AP2-alpha) transcription binding site (Figure 4.4). Variant rs76018112 added nuclear factor of activated T-cells 2 (NF-AT2) transcription factor binding site (Figure 4.5). SNP rs1557370 did not cause any changes in the possible transcription binding sites, whereas rs945635 added transcription binding site namely retinoblastoma-

associated protein 1 (E2F-1) (Figure 4.6). These results indicate that the novel variant at chr3: 49679737, rs76018112 and rs945635 may affect the transcription factor binding sites, which probably influenced the phenotype differences.

Table 4.5: Summary of the significant variants.

Gene	Ref	Alt	Region	Location	Variant Type	dbSNP	Protein Change	MAF	PhyloP
BSN	AG	A/A	exonic	chr3:49679737	INDEL (frameshiftDeletion)	novel	p.Ala225fs	N/A	2.44
ABCF1	G	GA/GA	exonic	chr6:30558477	INDEL (frameshiftInsertion)	rs76018112	p.Ter846fs	N/A	2.55
MX1	A	A/G	utr_3	chr21:42830690	SNV	rs1557370	N/A	0.208	1.15
FCRL3	C	C/G	utr_5	chr1:157670290	SNV	rs945635	N/A	0.444	-1.11

Ref = reference gene; Alt = alternative gene; dbSNP = single nucleotide polymorphism database; MAF = minor allele frequency; PhyloP = Phylop score; INDEL = insertion/deletion; SNV = single nucleotide variation; BSN = bassoon; ABCF1 = ATP-binding cassette sub-family F member 1; MX1 = Interferon-induced GTP-binding protein; FCRL3 = Fc receptor-like protein 3; utr_3 = untranslated region 3; utr_5 = untranslated region 5; N/A = not available

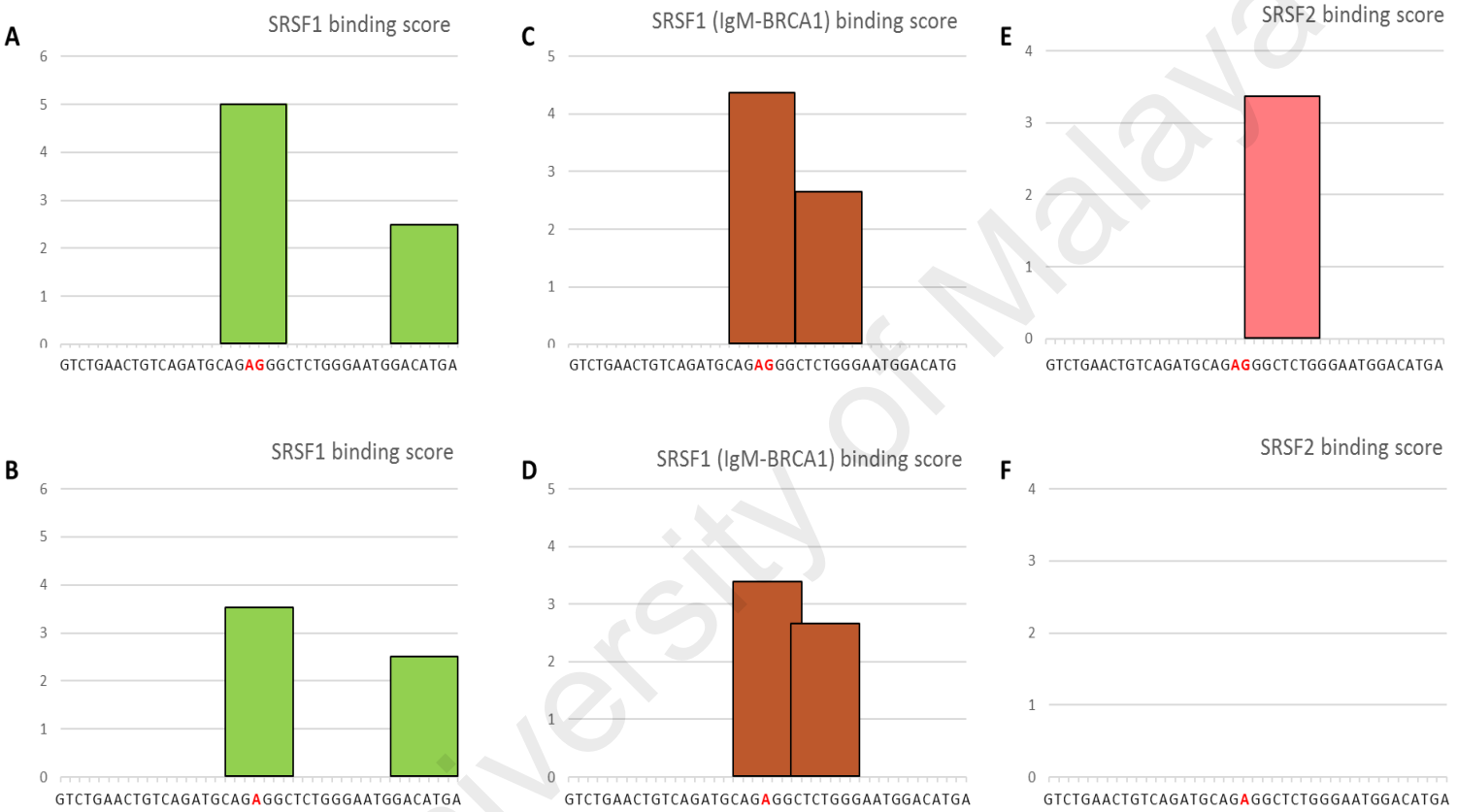


Figure 4.1: In silico analysis of potential disruption of BSN variation at chr3:49679737 on exonic splicing enhancers (ESEs). Prediction of binding score was performed using online analytical tools, ESEfinder (http://krainer01.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi? process=home). The X-axis of the graph showed the 40 base pairs around BSN variant location, whereas, Y-axis showed the binding score prediction. The relative score of splicing enhancer binding motif for following splicing factor; SRSF1 in BSN WT (allele AG) genotype sequence (**A**), SRSF1 in BSN mutant (allele A) genotype sequence (**B**), SRSF1 (IgM-BRCA1) in BSN WT (allele AG) genotype sequence (**C**), SRSF1 (IgM-BRCA1) in BSN mutant (allele A) (**D**), SRSF6 in BSN WT (allele AG) (**E**), SRSF6 in BSN mutant (allele A) (**F**)

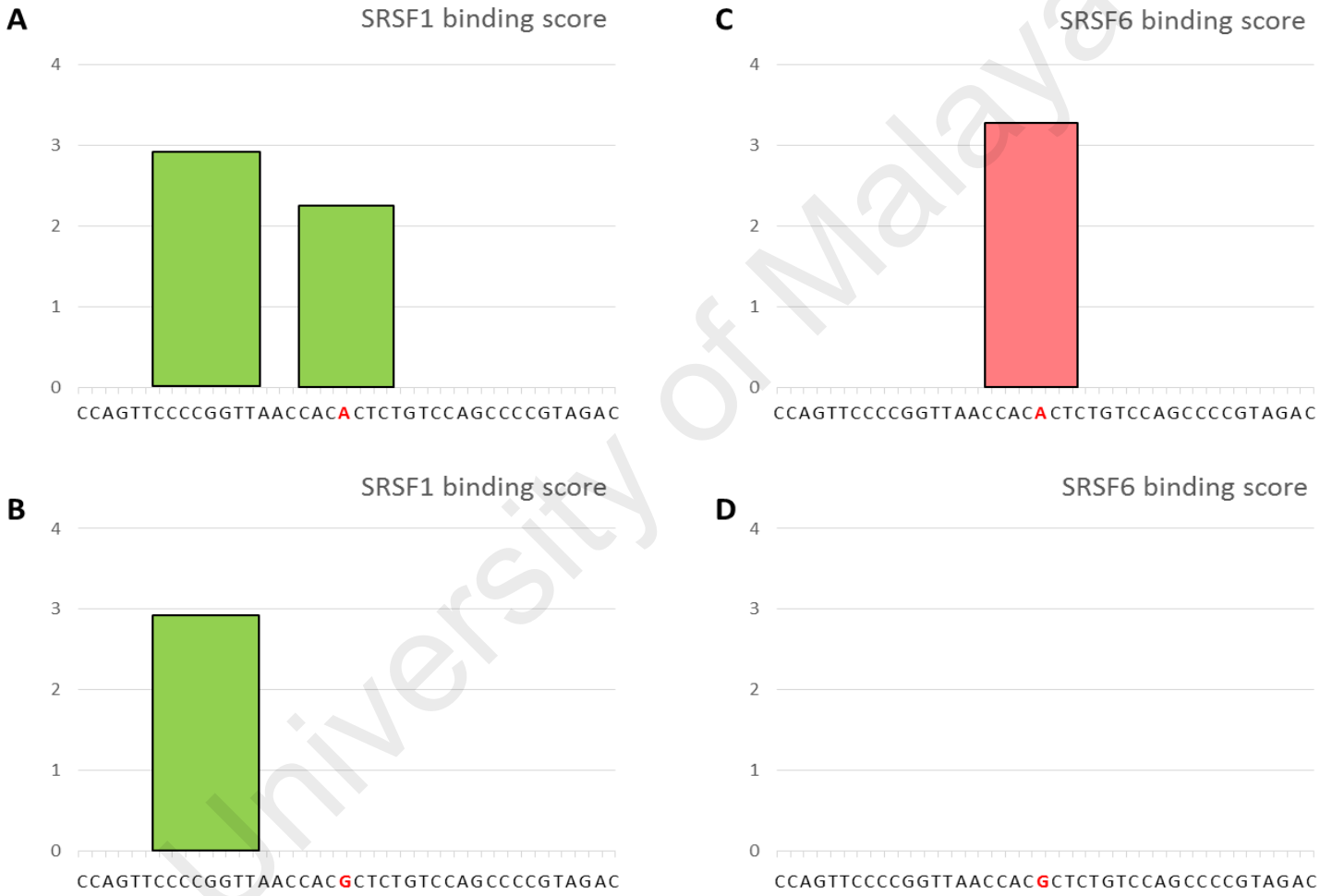


Figure 4.2: In silico analysis of potential disruption of Mx1 variation (rs1557370) on exonic splicing enhancers (ESEs). Prediction of binding score was performed using online analytical tools, ESEfinder (http://krainer01.cshl.edu/cgi-bin/tools/ESE3/esefinder .cgi?process=home). The X-axis of the graph showed the 40 base pairs around BSN variant location, whereas, Y-axis showed the binding score prediction. The relative score of splicing enhancer binding motif for following splicing factor; SRSF1 in Mx1 WT (allele A) genotype sequence (**A**), SRSF1 in Mx1 mutant rs1557370 (allele G) genotype sequence (**B**), SRSF6 in Mx1 WT (allele A) genotype sequence (**C**), SRSF6 in Mx1 mutant rs1557370 (allele G) genotype sequence (**D**).

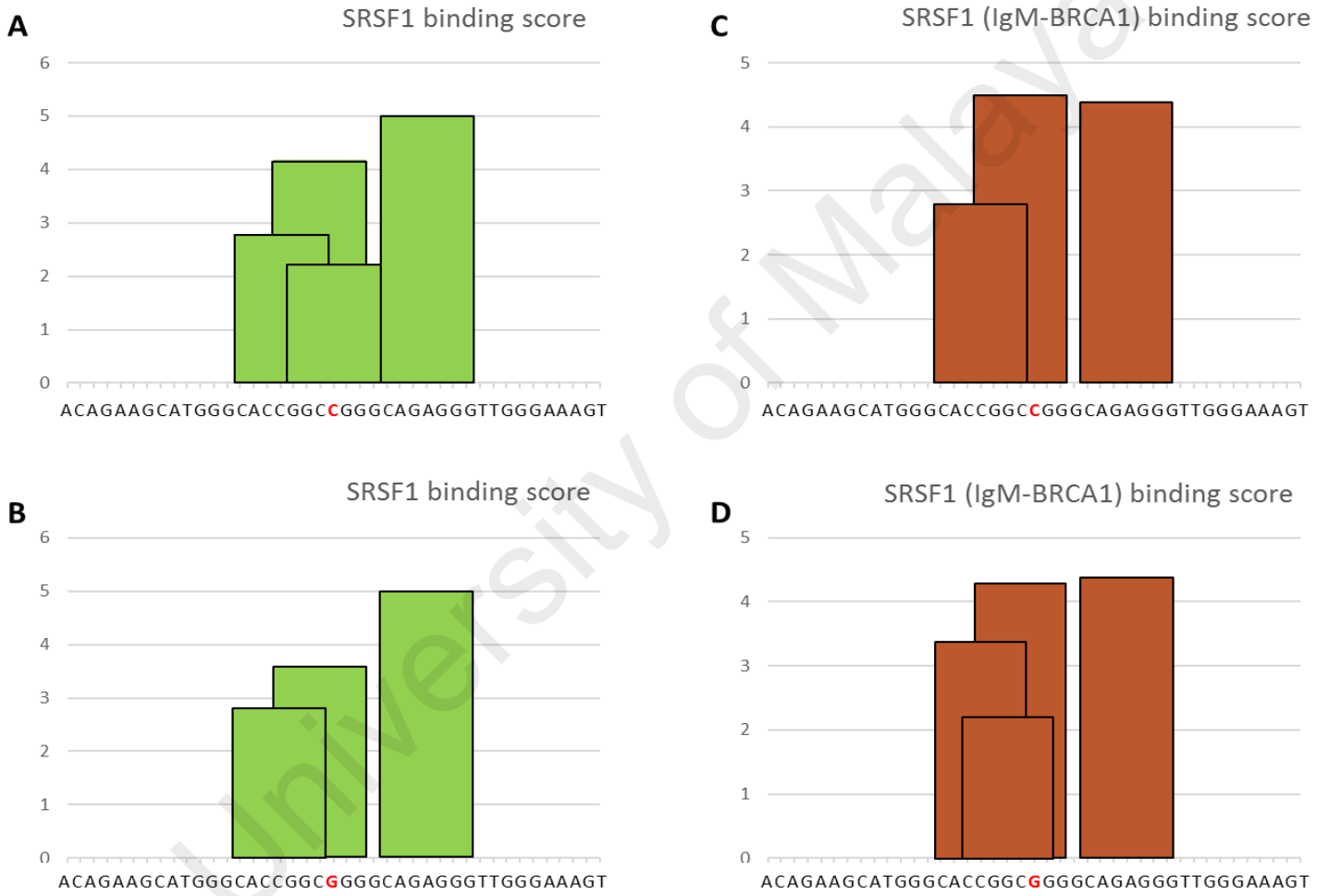
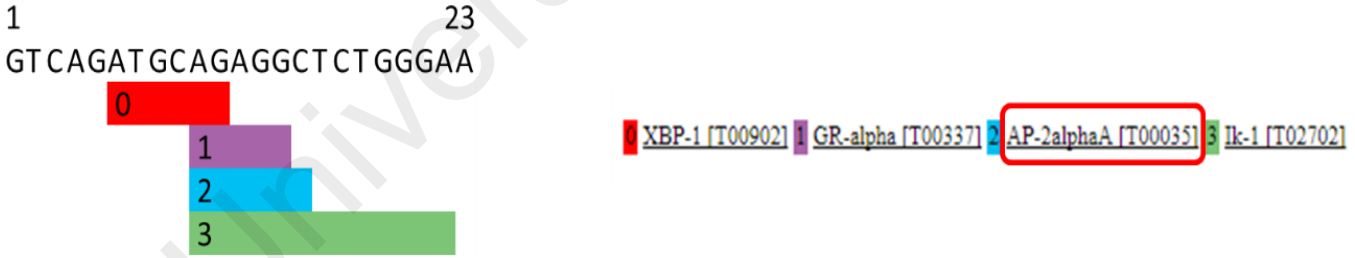


Figure 4.3: In silico analysis of potential disruption of FCRL3 variation (rs945635) on exonic splicing enhancers (ESEs). Prediction of binding score was performed using online analytical tools, ESEfinder (http://krainer01.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi? process=home). The X-axis of the graph showed the 40 base pairs around BSN variant location, whereas, Y-axis showed the binding score prediction. The relative score of splicing enhancer binding motif for following splicing factor; SRSF1 in FCRL3 WT (allele C) genotype sequence (**A**), SRSF1 in FCRL3 mutant rs945635 (allele G) genotype sequence (**B**), SRSF1 (IgM-BRCA1) in FCRL3 WT (allele C) genotype sequence (**C**), SRSF1 (IgM-BRCA1) in FCRL3 mutant rs945635 (allele G) genotype sequence (**D**).

A

BSN wild-type AG (novel)

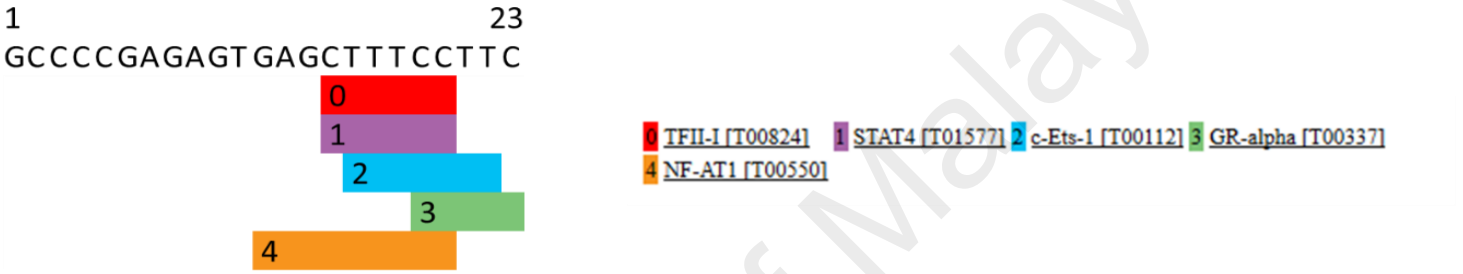
B



BSN mutant A (novel)

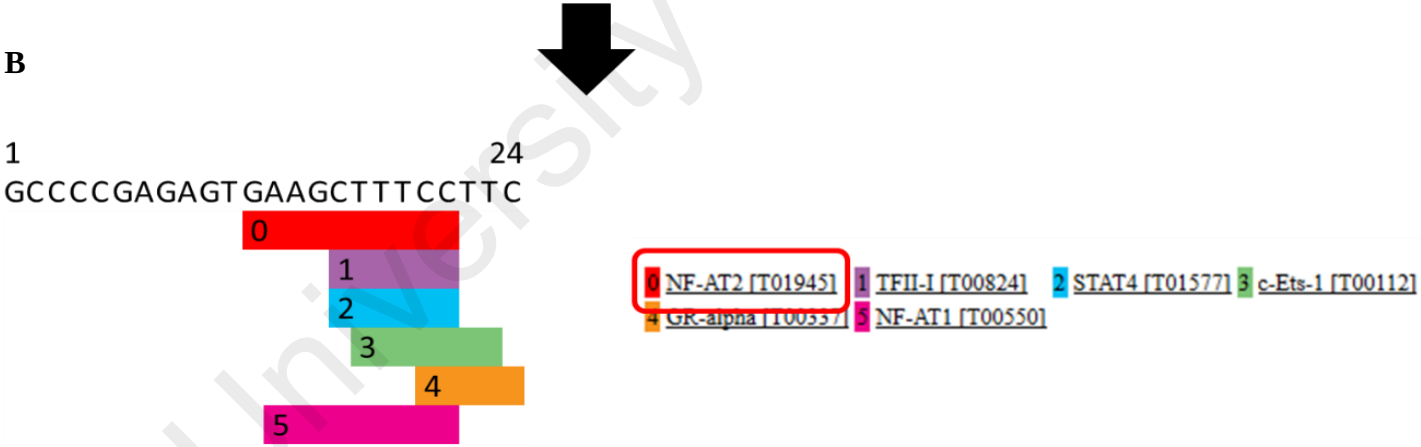
Figure 4.4: Prediction of the variant effects of BSN on the transcription factor binding sites by in silico analysis. Prediction of the transcription factor binding sites was performed using online analytical tools PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo__/promoinit.cgi?dirDB=TF_8.3). The putative transcription binding sites in the BSN wild-type AG (**A**). The putative transcription binding sites in the BSN mutant A (**B**). The red underlines and boxes showed the diminished and added transcription factors respectively, observed between the wild-type and mutant sequence.

A



ABCF1 wild-type G (rs76018112)

B



ABCF1 mutant GA (rs76018112)

Figure 4.5: Prediction of the ABCF1 variant effects on the transcription factor binding sites using by in silico analysis. Prediction of the transcription factor binding sites was performed using online analytical tools PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/ promoinit.cgi?dirDB=TF_8.3). The putative transcription binding sites in the ABCF1 wild-type G (**A**). The putative transcription binding sites in the ABCF1 mutant GA for rs76018112 (**B**). The red box showed the added transcription factor observed in the mutant sequence.

A

FCRL3 wild-type C (rs945635)

B

FCRL3 mutant G (rs945635)

Figure 4.6: Prediction of the FCRL3 variant effects on the transcription factor binding sites using by in silico analysis. Prediction of the transcription factor binding sites was performed using online analytical tools PROMO (http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/ promoinit.cgi?dirDB=TF_8.3). The putative transcription binding sites in the FCRL3 wild-type C (**A**). The putative transcription binding sites in the FCRL3 mutant G for rs945635 (**B**). The red box showed the added transcription factor observed in the mutant sequence

4.2. Cross-neutralization of autoantibodies in SLE patients' sera against DENV

A total of 82 SLE patients (dengue IgG and IgM negative), 15 dengue-positive patients (no known SLE and dengue IgG positive) as a control for neutralization of DENV and 24 healthy individuals (no known SLE and dengue IgG negative) as a control for non-neutralization, participated in this study (Table 4.6). There were no difference in age among SLE and dengue-positive patients with the average of 36 and 34 years old, respectively, while age of healthy individuals was higher with the average of 51 years old. The ratio of male and female among dengue-positive was 2:1 (male: female), whereas female showed a majority against male among healthy individuals with the ratio of 1:2 (male: female). The gender proportion of SLE participants was dominated by female with the ratio of 1:10 (male: female), in accordance with the gender population of SLE (male: female; 1:9) (Weckerle *et al.*, 2011).

Neutralization of DENV by the sera of SLE patients was observed using focus reduction neutralization assay (FRNT), a modified method of plaque reduction neutralization assay (PRNT) (Vaidya *et al.*, 2010). Sera of SLE, dengue-positive and healthy donors were diluted into 1/20, 1/80, 1/320 and 1/1280 and then examined against DENV1 (Figure 4.7). At the lowest dilution of sera, 1:20, the percentage of sera that showed 50% or more DENV1 reduction (FRTN50) in SLE, dengue-positive and healthy donors' sera were 97%, 100% and 54%, respectively (Figure 4.7A). Percentages of FRNT50 sera at the dilution of 1:80 were 90% of SLE patients, 100% of dengue-positive and 33% of healthy individuals (Figure 4.7B). At the dilution of 1:320, both SLE and dengue-positive patients retained high percentage of FRNT50 sera against DENV1, 69% and 93%, respectively, whereas only 13% of healthy donors can neutralize more than 50% DENV1 (Figure 4.7C). At the highest serum dilution, 1:1280, the percentage of SLE and dengue-positive patients with FRNT50 sera against DENV1 were 13% and 31%, respectively, while none of the healthy donors showed more than 50% reduction against DENV1 (Figure 4.7D).

Results showed that SLE patients' sera have the capability to neutralize DENV1, observed from the significantly high virus inhibition percentage of SLE-sera than healthy donors at all serum dilutions. The neutralization capacity of SLE and not unexpectedly dengue-positive sera against DENV1 were a clear concentration-dependent.

Table 4.6: Demographics of patients and healthy sample donors used for the study.

		SLE patients	Dengue positive patients	Healthy individuals
No. of subjects		82	15	24
Age	Mean	36	34	51
	SD	11	14	15
Gender	Male	7	10	8
	Female	75	5	16

Number of subjects and age and gender of the SLE patients, dengue serology positive patients and healthy individuals. Data represented as mean ± and standard deviation. SLE = Systemic Lupus Erythematosus; SD = standard deviation

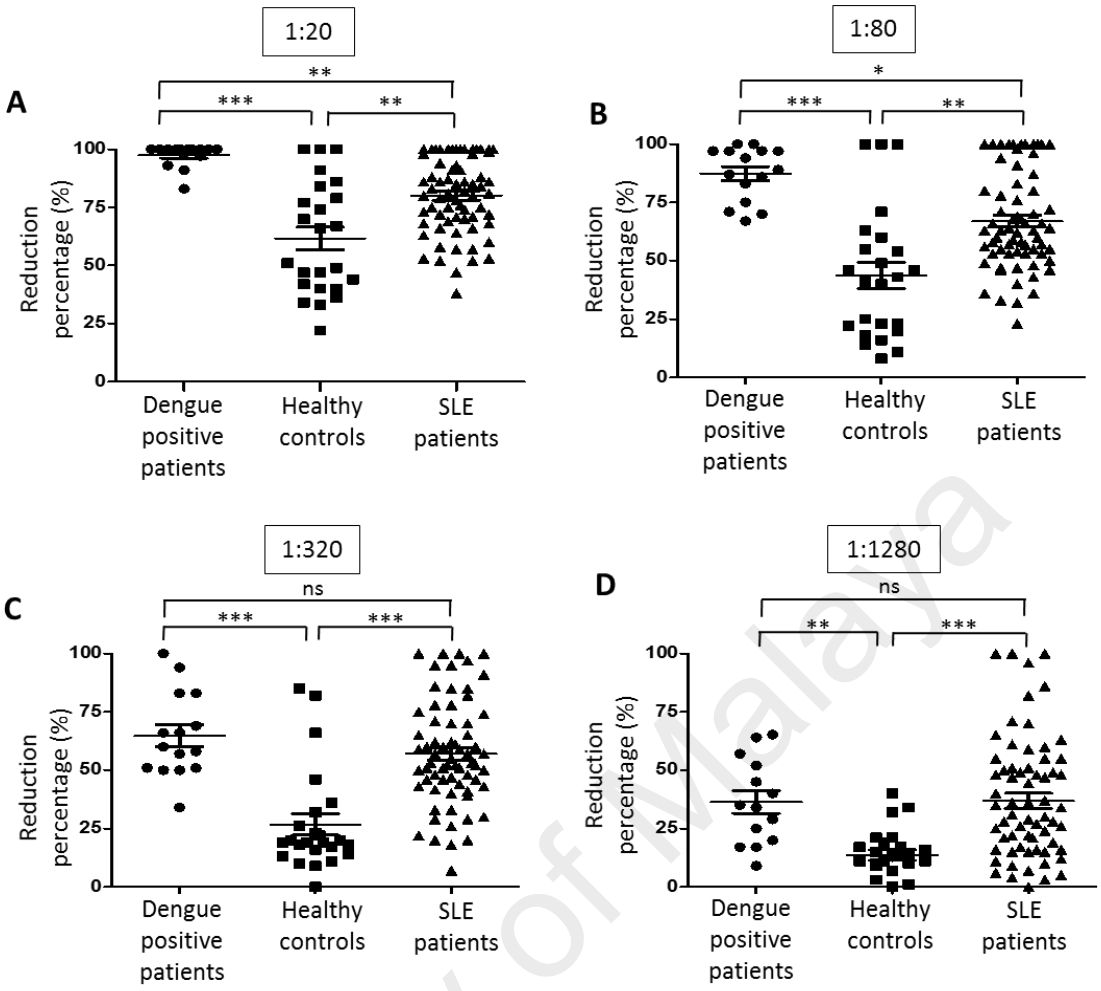


Figure 4.7: SLE-serum neutralizing activity against DENV1. Heat-inactivated dengue positive patients (no known SLE and dengue IgG positive) (n=15), healthy controls (no known SLE and dengue IgG negative) (n=24) and SLE patients' (dengue IgG and IgM negative) (n=67) sera were diluted into 1/20 (**A**), 1/80 (**B**), 1/320 (**C**), 1/1280 (**D**) and incubated with DENV1 for 1 h. The incubated solution was then added to Vero cells and the virus titers were determined using focus forming assay. Neutralization potency was verified with 50% virus inhibition. Values represent the mean ± SEM percentage of DENV1 reduction compared between dengue serology positive sera, dengue serology negative sera and SLE patients' sera. Significant differences between the groups are shown as ns: not significant, *P<0.05, **P<0.01, ***P<0.001 by Kruskal-Wallis test alongside Dunn's test with 95% confidence intervals.

The neutralizing capacity of SLE-sera was also investigated against DENV2. At the dilution of 1:20, percentages of FRNT50 sera against DENV2 in SLE, dengue-positive and healthy donors were 91%, 100% and 74%, respectively (Figure 4.8A). At serum dilution 1:80, 74% of SLE and 100% of dengue-positive patients' sera showed 50% or more reduction of DENV2, whereas only 47% of healthy donors' sera showed FRNT50 (Figure 4.8B). At higher serum dilution, 1:320, percentages of SLE patients, dengue-positive and healthy donors' sera inhibiting 50% or more DENV2 were 61%, 100% and 32%, respectively (Figure 4.8C). Percentages of FRNT50 sera in SLE and dengue-positive patients were 50% and 25%, whereas only 5% of healthy donors showed 50% or more inhibition of DENV2 at serum dilution 1:1280 (Figure 4.8D). Equivalent with the neutralization capability of SLE against DENV1, SLE patients' sera also showed neutralization against DENV2, observed from significantly higher percentage of virus inhibition by SLE-sera than that of healthy donors at the serum dilution of 1:320 and 1:1280. The neutralization effect against DENV2 by SLE-sera was also in a concentration-dependent manner.

Neutralization of DENV3 by SLE patients' sera was also observed. At the lowest serum dilution, 1:20, the percentages of SLE, dengue-positive and healthy donors' sera with the capacity to neutralize 50% or more DENV3 were 91%, 100% and 81% (Figure 4.9A). At a higher dilution of serum, 1:80, SLE patients and dengue-positive sera that showed 50% or more inhibition against DENV3 were 74% and 100%, respectively, while only 27% of dengue-negative patients depicted FRNT50 (Figure 4.9B). The percentages of sera that caused inhibition of 50% or more of DENV3 among SLE and dengue-positive sera were 52% and 70%, respectively, while none of the dengue-negative sera showed neutralization effect at the serum dilution of 1:320 (Figure 4.9C). The percentage of FRNT50 sera of each sample group was lower than 50% at the highest dilution of serum, 1:1280 (Figure 4.9D). Results showed that SLE patients' sera neutralized DENV3,

observed from the significantly higher percentage of virus inhibition of SLE-sera compared to the healthy donors' sera at the dilution of 1:80, 1:320 and 1:1280. SLE patients' sera neutralized DENV3 ($P<0.01$) better than that observed against DENV2 ($P<0.05$), albeit not as profound as the neutralization effect against DENV3 ($P<0.001$). Similar with the neutralization of DENV1 and DENV2, the inhibition of DENV3 by SLE-sera was also in a dose-dependent manner. Overall, the results presented conclude that SLE patients' sera neutralized DENV1, DENV2 and DENV3, with DENV1 being the most vulnerable, followed by DENV3 and DENV2.

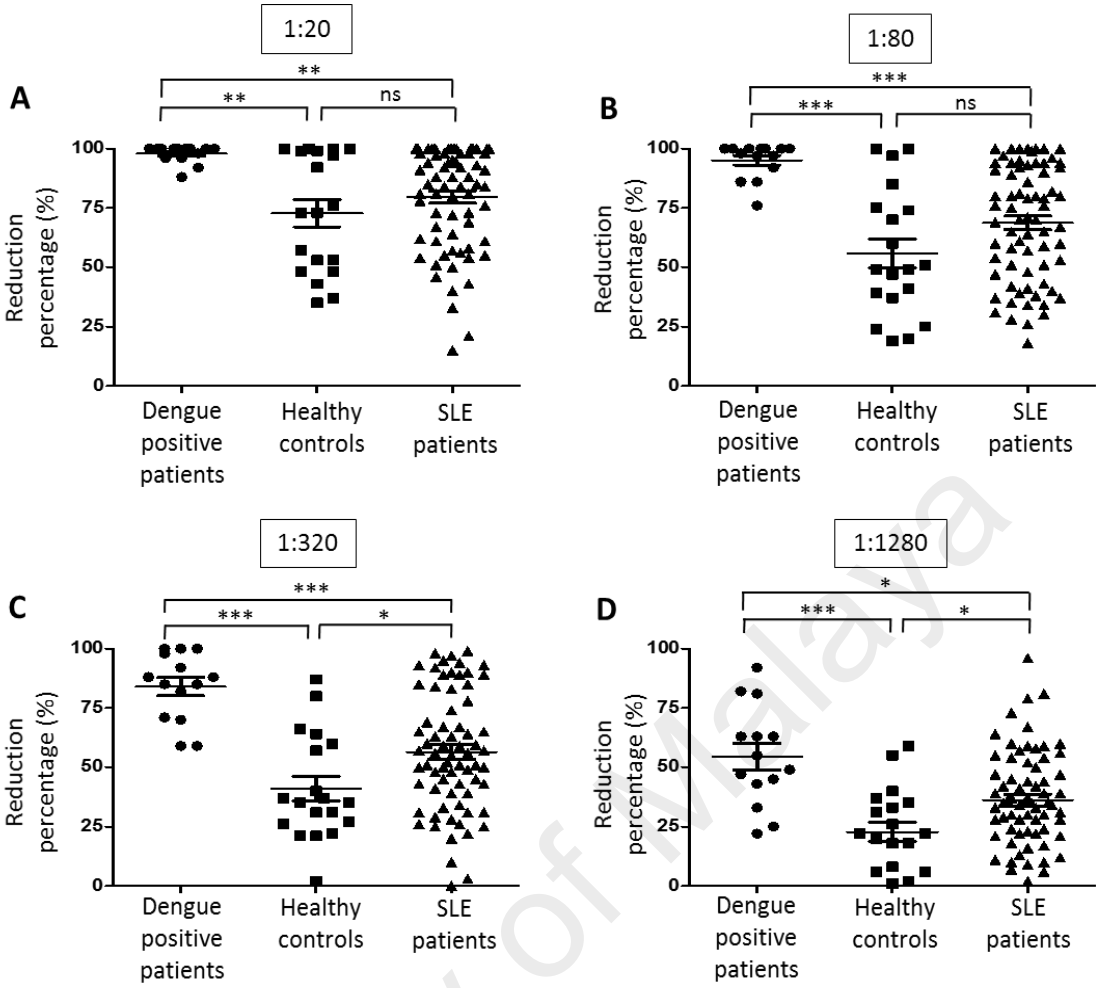


Figure 4.8: SLE-serum neutralizing activity against DENV2. Heat-inactivated dengue positive patients (no known SLE and dengue IgG positive) (n=14), healthy controls (no known SLE and dengue IgG negative) (n=19) and SLE patients' (dengue IgG and IgM negative) (n=70) sera were diluted into 1/20 (**A**), 1/80 (**B**), 1/320 (**C**), 1/1280 (**D**) and incubated with DENV1 for 1 h. The incubated solution was then added to Vero cells and the virus titers were determined using focus forming assay. Values represent the mean ± SEM percentage of DENV2 reduction compared between dengue-positive sera, dengue-negative sera and SLE patients' sera. Significant differences between the groups are shown as ns: not significant, *P<0.05, **P<0.01, ***P<0.001 by Kruskal-Wallis test alongside Dunn's test with 95% confidence intervals.

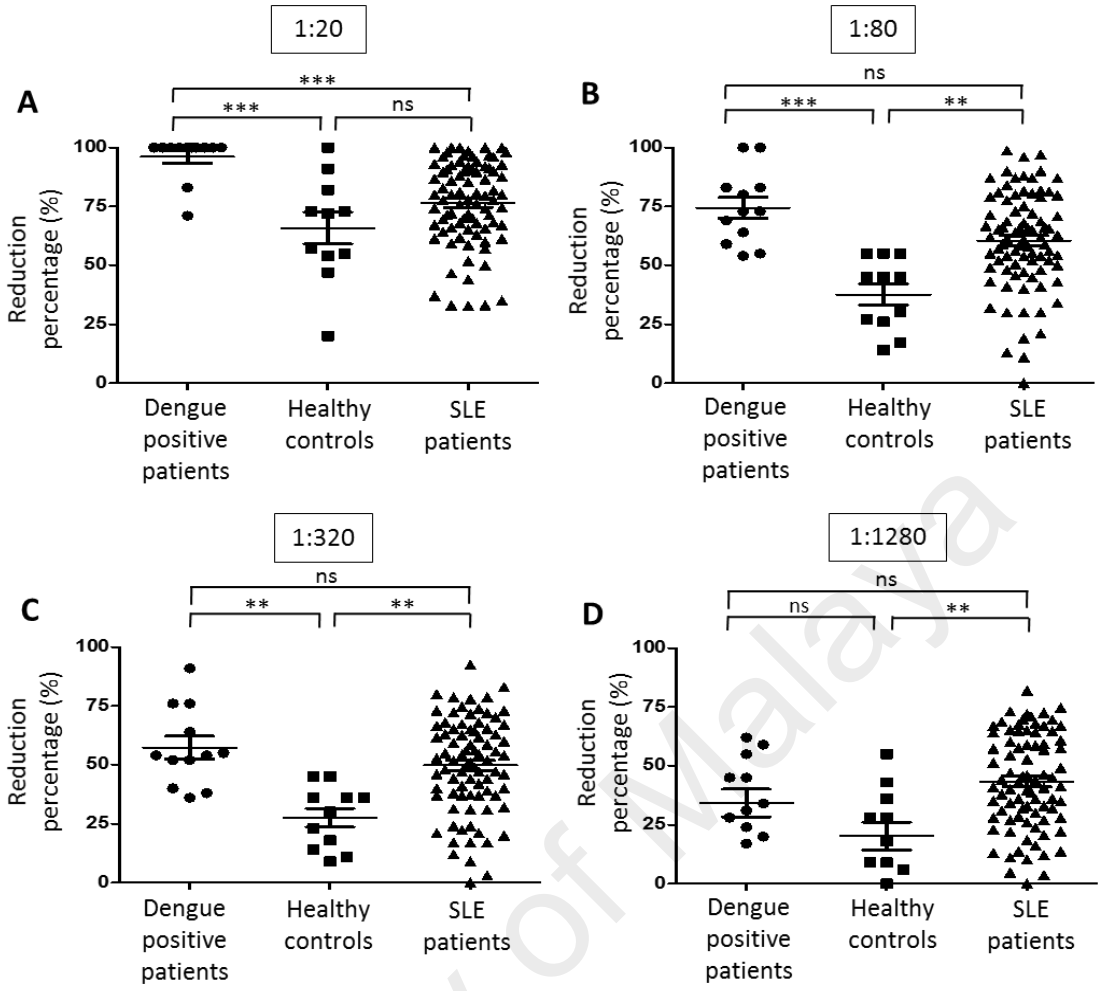


Figure 4.9: SLE-serum neutralizing activity against DENV3. Heat-inactivated dengue positive patients (no known SLE and dengue IgG positive) (n=12), healthy controls (no known SLE and dengue IgG negative) (n=11) and SLE patients' (dengue IgG and IgM negative) (n=82) sera were diluted into 1/20 (**A**), 1/80 (**B**), 1/320 (**C**), 1/1280 (**D**) and incubated with DENV1 for 1 h. The incubated solution was then added to Vero cells and the virus titers were determined using focus forming assay. Values represent the mean ± SEM percentage of DENV3 reduction compared between dengue-positive sera, dengue-negative sera and SLE patients' sera. Significant differences between the groups are shown as ns: not significant, *P<0.05, **P<0.01, ***P<0.001 by Kruskal-Wallis test alongside Dunn's test with 95% confidence intervals.

We then examined the potential role of IgG presence in the SLE-sera. SLE patients' sera were subjected to IgG-depletion procedure, followed by FRNT assay to determine the neutralization percentage of the serum against DENV1 and DENV2. The depletion of IgG was performed using ProteoExtract albumin/IgG removal kit. Among all of the SLE patients' samples used in this study, only 6 of them were randomly selected for IgG depletion test, due to the limited samples. After the depletion of IgG, the dengue IgG ELISA titer of each serum was measured and the average reduction was 60% (Figure 4.10G). Depletion of IgG in the serum of patient 1 showed 34% reduction on the neutralization percentage of DENV1 and 46% reduction on the inhibition percentage of DENV2 at the serum dilution of 1:320 (Figure 4.10A). The IgG-depleted serum of patient 2 showed 30% and 53% decreased of neutralization percentage against DENV1 and DENV2, respectively, whereas patient 3 exhibited 24% and 81% reduction of the inhibition percentage against DENV1 and DENV2, respectively, at the serum dilution of 1:320 (Figure 4.10B, 4.10C). The decrease of the neutralization percentage against DENV1 and DENV2 was also observed in IgG-depleted SLE patients 4 (71% [DENV1] & 86% [DENV2]) and SLE patient 5 (69% [DENV1], 67% [DENV2]) at the serum dilution of 1:320 (Figure 4.10D, 4.10E). IgG-depleted serum of SLE patient 6 at the serum dilution of 1:320 also displayed a reduction of neutralization percentage to 31% against DENV1 and 53% against DENV2 (Figure 4.10F). The reduction of the neutralization percentage of SLE patients' sera after the IgG depletion suggested the importance of IgG in the neutralization ability of SLE patients' sera against DENV.

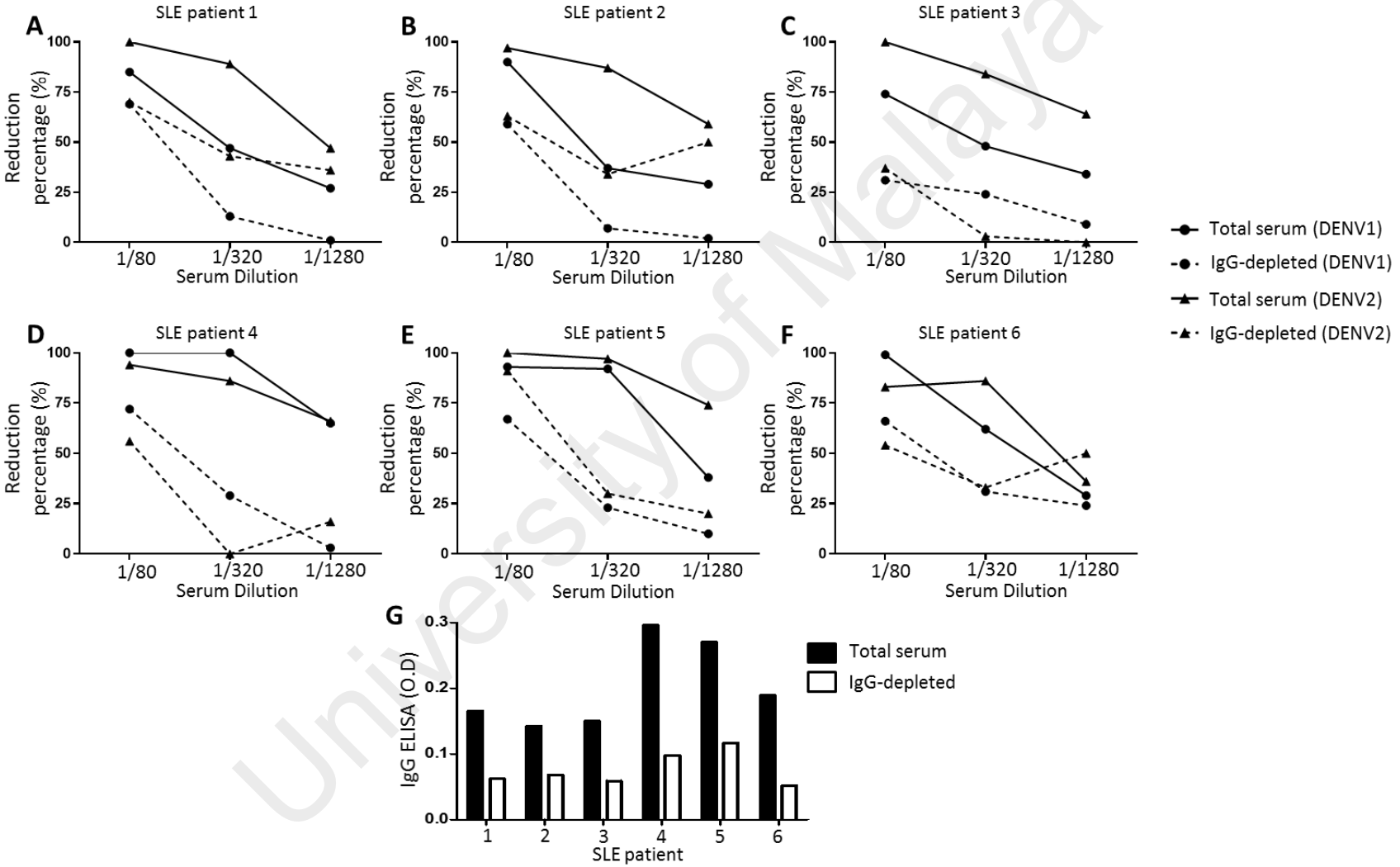


Figure 4.10: Neutralizing capacity of SLE-patients' sera following IgG depletion. Total and IgG-depleted sera of SLE patient 1 (**A**), 2 (**B**), 3 (**C**), 4 (**D**), 5 (**E**), and 6 (**F**) were heat-inactivated and diluted into 1/80, 1/320, and 1/1280. Next, sera were incubated with DENV1 and DENV2 virus for 1 h. The incubated solution was then added to Vero cells and the virus titers were determined using focus forming assay. Dengue IgG titer depletion was confirmed using dengue IgG capturing ELISA (**G**).

Given the involvement of IgG in neutralization of viruses (Palladino *et al.*, 1995; Parr *et al.*, 1997), we investigated the relationship between neutralization percentage of SLE patients' sera against DENV and the level of dengue IgG titer. All of the SLE samples used in this study were dengue-negative sera, determined using ELISA assay with the cut-off value of 0.390 optical density (O.D) absorbance, where serum with <0.390 O.D. absorbance was deemed as dengue-negative, while those with >0.390 O.D. absorbance was regarded as dengue-positive. Despite confirmed as dengue-negative, SLE patients' sera still showed a variant reading of O.D. absorbance (dengue IgG ELISA titer) ranging from 0.00 to 0.389. Hence, we compared the level of IgG ELISA titer of serum to neutralization percentage of DENV1, DENV2 and DENV3. Neutralization percentage of DENV1 by SLE patients' sera showed significant correlation with the level of dengue IgG ELISA titer at both serum dilution of 1/80 and 1/320 (P=0.0015, P=0.0006) (Figure 4.11A). However, in the case of SLE-sera inhibition against DENV2, the neutralization percentage exhibited non-significant correlation with the level of dengue IgG ELISA titer at 1:80 and 1:320 serum dilution (P=0.1518, P=0.4990) (Figure 4.11B). The correlation of neutralization percentage against DENV3 and IgG ELISA titer was only significant at the serum dilution of 1:80 (P=0.001), but not at the serum dilution of 1:320 (P=0.7647) (Figure 4.11C). Oddly, 11% of SLE patients' sera with low level of dengue IgG ELISA titer showed high neutralization capacity against DENV1 (Figure 4.11A, black box). The high neutralization by SLE patients' sera with low level of dengue IgG ELISA titer was also observed among 24% of SLE patients against DENV2 (Figure 4.11B, black box) and 27% of SLE patients against DENV3 (Figure 4.11C, black box). These findings suggested that the neutralizing capacity of the SLE patients' sera against DENV correlated with the presence of dengue virus specific IgG. However, the inherent neutralizing ability of sera with low dengue-specific IgG ELISA titer could possibly cause the statistically insignificant correlation demonstrated between dengue-specific IgG titer and the

neutralizing capacity of SLE-sera against DENV2 and DENV3. Nevertheless, the neutralization effects of the low dengue-specific IgG ELISA titer sera were not observed among the dengue-positive and healthy donors' sera, which displayed significant positive correlation between DENV1 (Figure 4.12A), DENV2 (Figure 4.12B) and DENV3 (Figure 4.12C) reduction percentage and the dengue IgG ELISA titer. Hence, these suggest the possibility that the neutralization effects of SLE patients' sera, may not solely caused by the IgG.

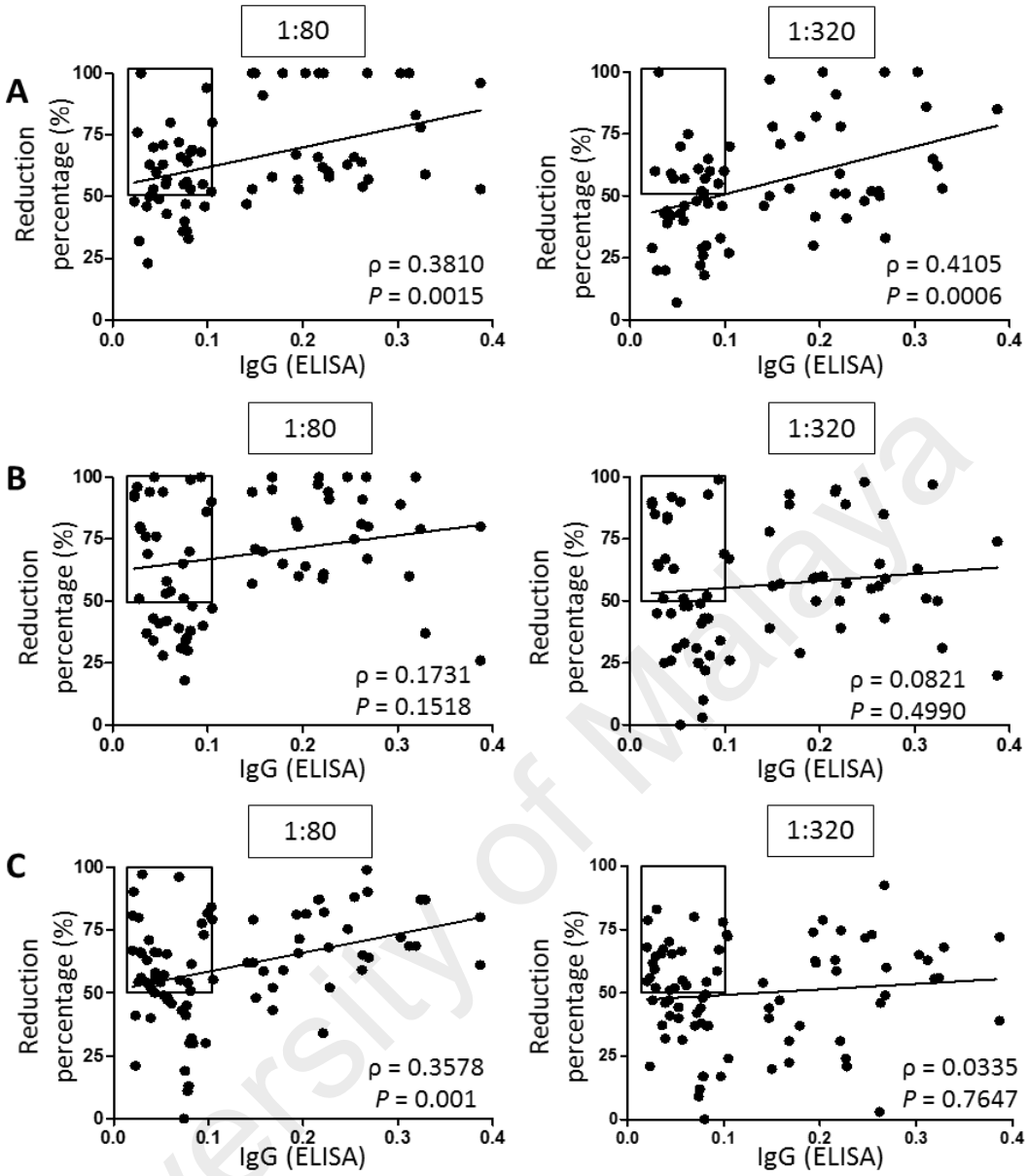


Figure 4.11: Association between dengue neutralization percentage and dengue IgG ELISA titer among SLE patients. SLE patients' sera diluted into 1/80 and 1/320 were incubated with DENV1 (SLE n=67) (**A**), DENV2 (SLE n=70) (**B**) and DENV3 (SLE n=82) (**C**) for 1 h. The incubated solution was then added to Vero cells and the virus titers were determined using focus forming assay. Dengue IgG in the serum was determined using dengue IgG capturing ELISA. Association between DENV neutralization percentage and dengue IgG ELISA titer (O.D. of serum samples) of SLE patients' sera was determined using Spearman test and correlations were indicated by Spearman ρ value and the *P* value.

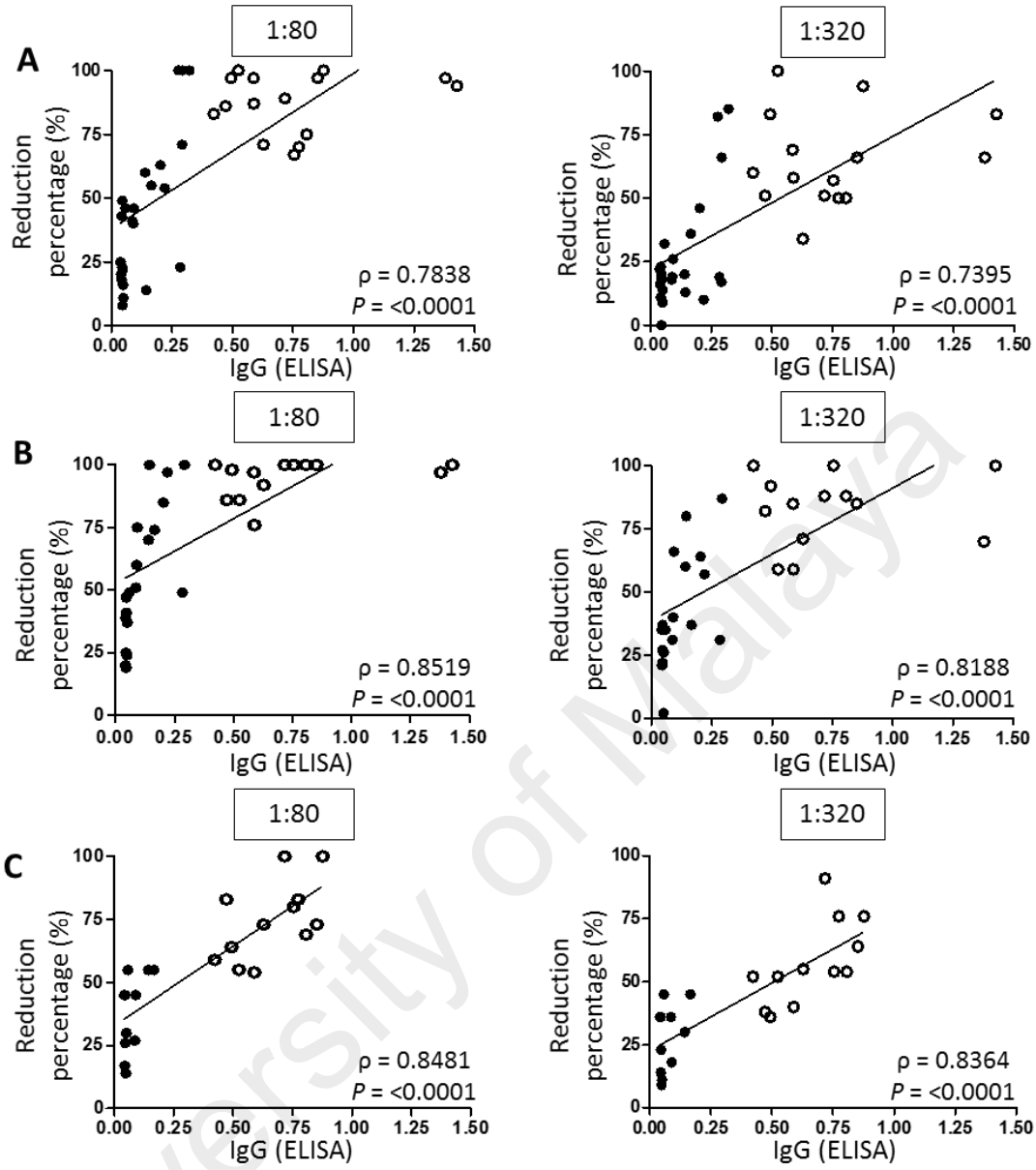


Figure 4.12: Relationship between dengue neutralization percentage and dengue IgG ELISA titer among dengue-positive and healthy sera. Dengue-positive and healthy sera were diluted into 1/80 and 1/320 and incubated with DENV1 (dengue-positive=15, healthy=24) (**A**), DENV2 (dengue-positive=13, healthy=19) (**B**), and DENV3 (dengue-positive=12, healthy=11) (**C**) for 1 h. The incubated solution was then added to Vero cells and the virus titers were determined using focus forming assay. Dengue IgG was determined using dengue IgG capturing ELISA. The black dots represent data points from healthy sera and white dots represent dengue-positive sera. Association between DENV neutralization percentage and dengue IgG ELISA titer (O.D. of serum samples) of dengue-positive and healthy sera was determined using Spearman test and correlations were indicated by Spearman ρ value and the *P* value.

The specificity of neutralizing capability against DENV by dengue serology negative SLE patients' sera was examined by performing similar FRNT assay against another *Flavivirus* endemic in Malaysia (Wong *et al.*, 2008), *Japanese Encephalitis* virus (JEV). The sera of SLE, dengue-positive and healthy participants were diluted to 1:20, 1:80, 1:320 and 1:1280. The diluted serum was then submitted to FRNT assay against JEV and the neutralization percentage was observed. The neutralization percentages of SLE, dengue-positive and healthy donors that inhibited 50% or more JEV at the serum dilution of 1:20 were 75%, 100% and 88%, respectively (Figure 4.13A). At higher serum dilution, 1:80, FRNT50 sera among SLE and dengue-negative patients were 22% and 25%, respectively, whereas dengue-positive patients retained high neutralization percentage of FRNT50 sera at 80% (Figure 4.13B). At serum dilution of 1:320, 3% of SLE patient's and 20% of dengue-positive sera showed 50% or more reduction of JEV, while none of dengue-negative sera showed 50% inhibition against JEV (Figure 4.13C). The percentage of FRNT50 sera were lower than 3% among dengue-positive sera and none among both SLE and dengue-negative sera at the serum dilution of 1:1280 (Figure 4.13D). The neutralization effects of SLE patients' sera showed no significant differences in comparison to the healthy donor's sera at all dilutions, suggesting the inability of SLE patients' sera to neutralize JEV. Low neutralization capacity by SLE patients' sera was in contrast with the data depicted that most of the SLE samples were JEV-positive (above ELISA cut-off value: >1.1 O.D. absorbance) using anti-JEV ELISA (IgG) assay (Figure 4.14A). The association between the neutralization percentage of SLE, dengue-positive, healthy donors against JEV and the level of JEV IgG ELISA was determined (Figure 4.14), and we observed no correlation between them.

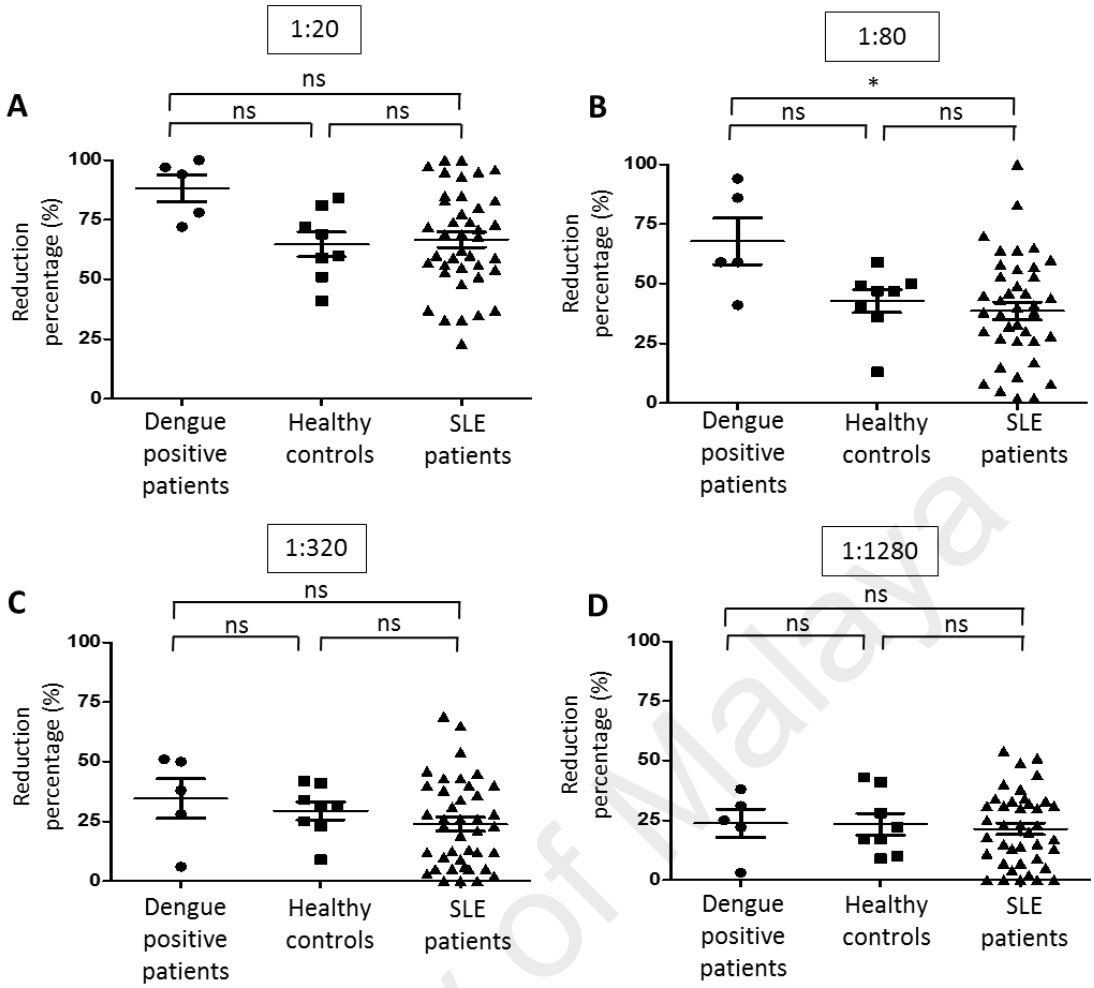


Figure 4.13: SLE serum neutralization activity against JEV. Heat-inactivated dengue-positive (no known SLE and dengue IgG positive) (n=5), healthy controls (no known SLE and dengue IgG negative) (n=8) and SLE patients' (dengue IgG and IgM negative) (n=40) sera were diluted into 1/20 (**A**), 1/80 (**B**), 1/320 (**C**), 1/1280 (**D**) and incubated with JEV for 1 h. The incubated solution was then added to Vero cells and the virus titers were determined using focus forming assay. Values represent the mean ± SEM percentage of JEV reduction compared between dengue-positive sera, dengue-negative sera and SLE patients' sera. Significant differences between the groups are shown as ns: not significant, *P<0.05, **P<0.01, ***P<0.001 by Kruskal-Wallis test alongside Dunn's test with 95% confidence intervals.

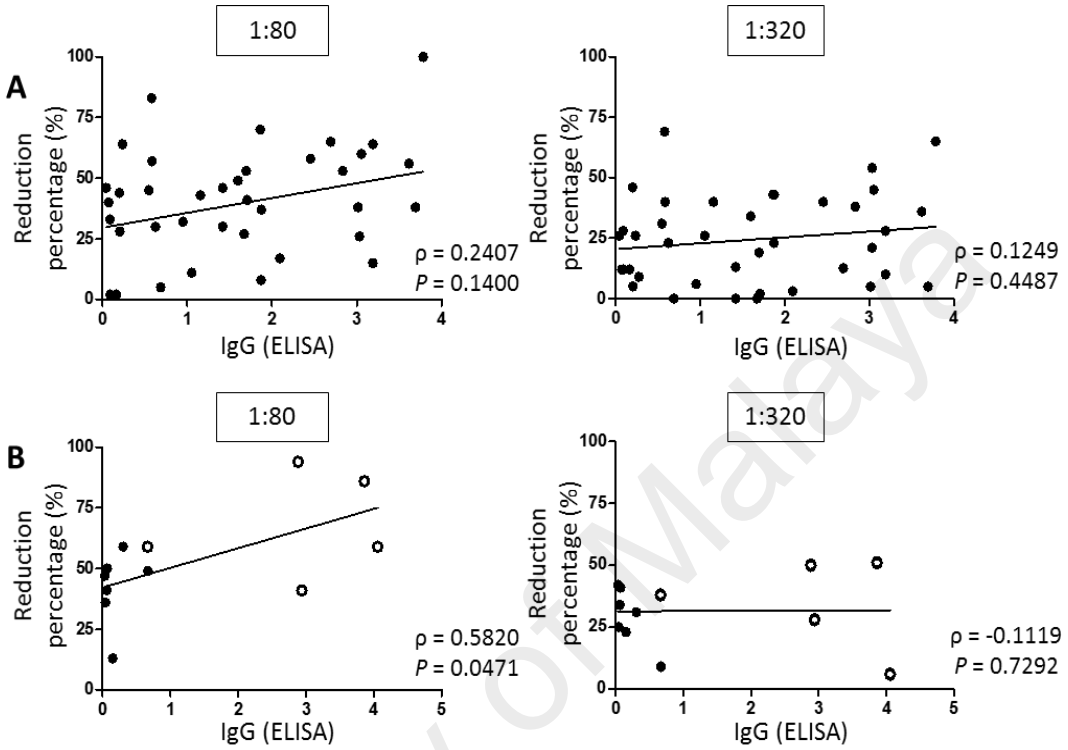


Figure 4.14: Association between neutralization percentage against JEV and JEV-specific IgG ELISA titer. SLE patients' (n=40) (**A**), dengue-positive patients' (n=5) and healthy donor's sera (n=8) (Black dots represent data points of healthy sera and the white dots represent dengue-positive sera) (**B**) were diluted into 1/80 and 1/320 dilution. The diluted sera were incubated with JEV for 1 h. The incubated solution was then added to Vero cells and the virus titers were determined using focus assay. JEV IgG titer was determined using anti-JEV ELISA (IgG). Association between JEV neutralization percentage and anti-JEV IgG ELISA titer (O.D. of serum samples) of SLE, dengue-positive and healthy sera was determined using Spearman test and correlations were indicated by Spearman ρ value and the *P* value.

4.3. The role of HMGB1 in the host antiviral response against DENV

To detect the role of HMGB1 in the antiviral response, we determined the effect of HMGB1-depletion against DENV infection. HMGB1 of Huh7 cells were knockdown using shRNA method and the down-regulation of HMGB1 was determined by the Western blot analysis (Figure 4.15C). Wild type (WT) and HMGB1-knockdown (shHMGB1) cells were infected with DENV at an MOI of 1 and 10. After 24 hours, the cells were harvested and the virus replication was determined by focus forming assay on Vero cells, qRT-PCR and Western blot analysis. Virus titer showed 90% increase of DENV in knockdown cells in comparison to the WT (Figure 4.15A). The level of mRNA NS1 was significantly amplified in knockdown cells compared to the WT (Figure 4.15B). Similarly, Western blot analysis revealed that the protein production of NS3, which used to indicate DENV replication, was higher in HMGB1-knockdown cells as compared to the WT cells (Figure 4.15C). These results suggest that without HMGB1, the antiviral response against DENV was inefficient, suggesting that HMGB1 may play a role in the host antiviral response against DENV replication.

Next, we investigated the effect of HMGB1-depletion on one of the host defence mechanism, type-1 interferon (IFN) response against DENV. WT and HMGB1-knockdown cells were infected with DENV at an MOI of 1 and 10, and subsequently harvested after 24 hours. The detection of IFN-β mRNA level and the Stat-1 protein production were performed using qRT-PCR and Western blot analysis, respectively. The increase of IFN-β mRNA level was significant in HMGB1-knockdown cells in comparison to WT at both 1 and 10 MOI of DENV infection (Figure 4.16A). Western blot analysis showed that HMGB1-knockdown cells increased the production of upstream type-1 IFN response protein, Stat-1, after DENV infection (1 MOI & 10 MOI) (Figure 4.16B). WT and HMGB1-knockdown cells were also stimulated with 50 μM IFN-β for 16 hours and Stat-1 protein production was determined using Western blot analysis. Stat-

1 protein production was also higher in the IFN-β-induced cells compared to the non-induced cells (Figure 4.16C). These data indicate that the effects of HMGB1-knockdown on the type-1 IFN response were in contrast with the reduced antiviral effects observed previously.

We then focused on the downstream of the type-1 IFN responses, which were interferon-stimulated genes (ISGs), including MxA (myxovirus resistance gene A), ISG56 and ISG15. The effects of HMGB1-knockdown on the level of ISGs during DENV infection were examined. WT and HMGB1-depleted cells were infected with DENV at an MOI of 1. The cells were harvested after 24 hours and the mRNA levels of MxA and ISG56 were monitored by qRT-PCR. HMGB1-knockdown cells displayed 1 log reduction of both MxA and ISG56 in comparison to the WT cells (Figure 4.17A, 4.17B). WT and HMGB1-knockdown cells also showed lower levels of ISG15 protein production in IFN-β-stimulated cells compared to the non-stimulated cells (Figure 4.17C). Results show that the cells with deprived HMGB1 expression lead to the reduced protein production of ISGs, implying that HMGB1 is required in the innate immune response against DENV, particularly on the downstream type-1 IFN response.

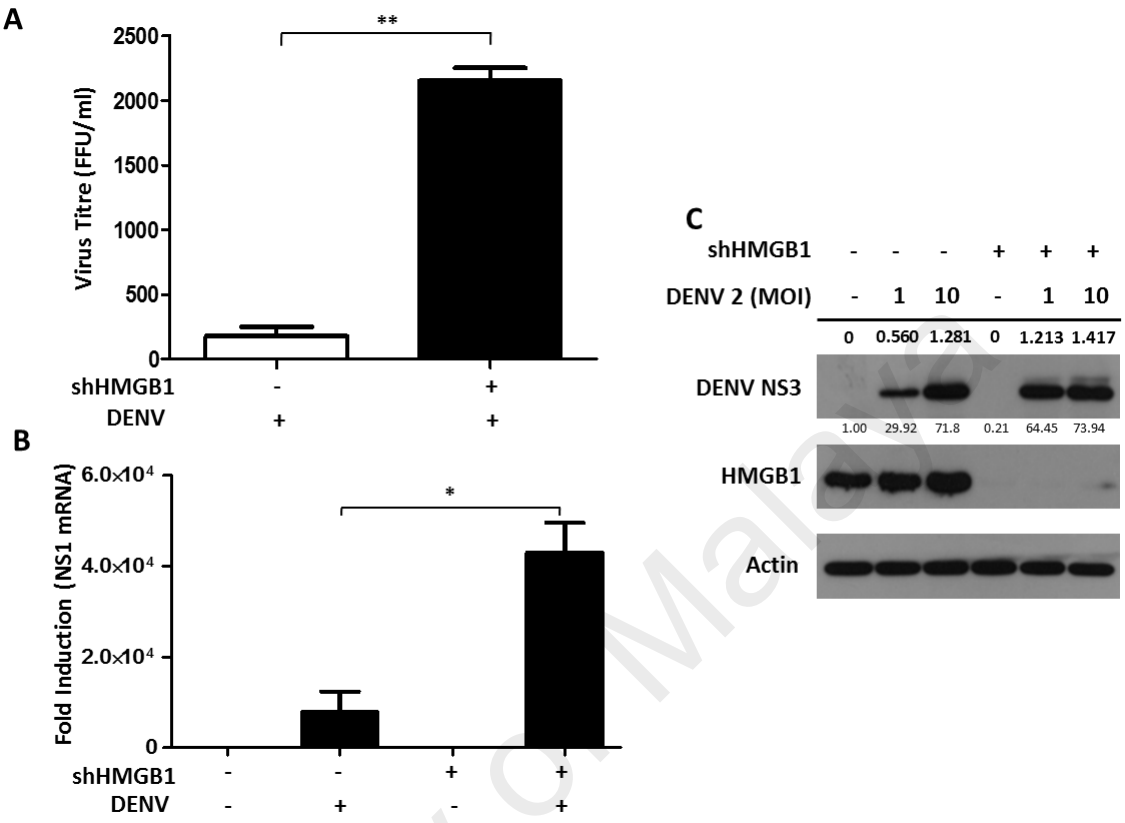


Figure 4.15: HMGB1 knockdown enhances DENV replication in Huh7 cells. Huh7 WT and shHMGB1 were mock-infected or infected with DENV at an MOI of 1. Cells and supernatant were harvested at 24 h post infection (p.i.). (**A**) Cell supernatants were examined using the focus forming assay for determination of virus titers. (**B**) The mRNA NS1 levels were measured by quantitative real-time RT-PCR and normalized to β-actin mRNA. Results are expressed as fold induction compared to control uninfected cells. Statistically significant differences between the groups are as indicated: *P<0.05, **P<0.01. (**C**) Cell cultures were analysed by Western blot for DENV NS3 detection. The band intensity of DENV NS3 and β-actin ratio was determined by the Image J analysis.

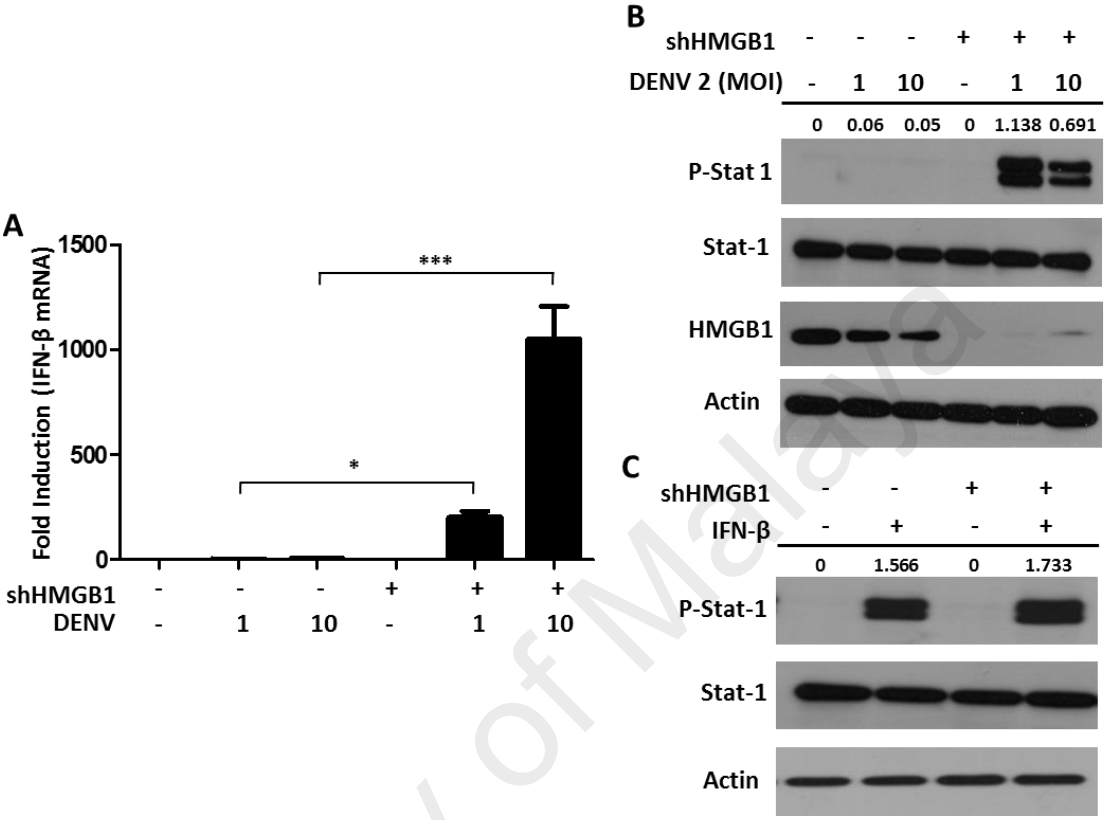


Figure 4.16: HMGB1 knockdown enhances production of IFN-related genes. (**A**) Huh7 WT and shHMGB1 cells were mock-infected or infected with DENV at an MOI of 1 and 10. Cells were harvested at 24 h p.i. The IFN-β mRNA was measured using quantitative real-time RT-PCR, normalized to β-actin mRNA and expressed as fold induction compared to control uninfected cells. Statistically significant variances between the groups are as indicated: *P<0.05, ***P<0.001. (**B**) Levels of Stat 1 and P-Stat-1 were detected using Western blot analysis. (**C**) Huh7 WT and shHMGB1 cells were stimulated with 50 μM IFN-β for 16 h and the levels of Stat-1 and P-Stat-1 were determined using Western blot analysis. The band intensity of Stat-1 and β-actin ratio was determined by the Image J analysis.

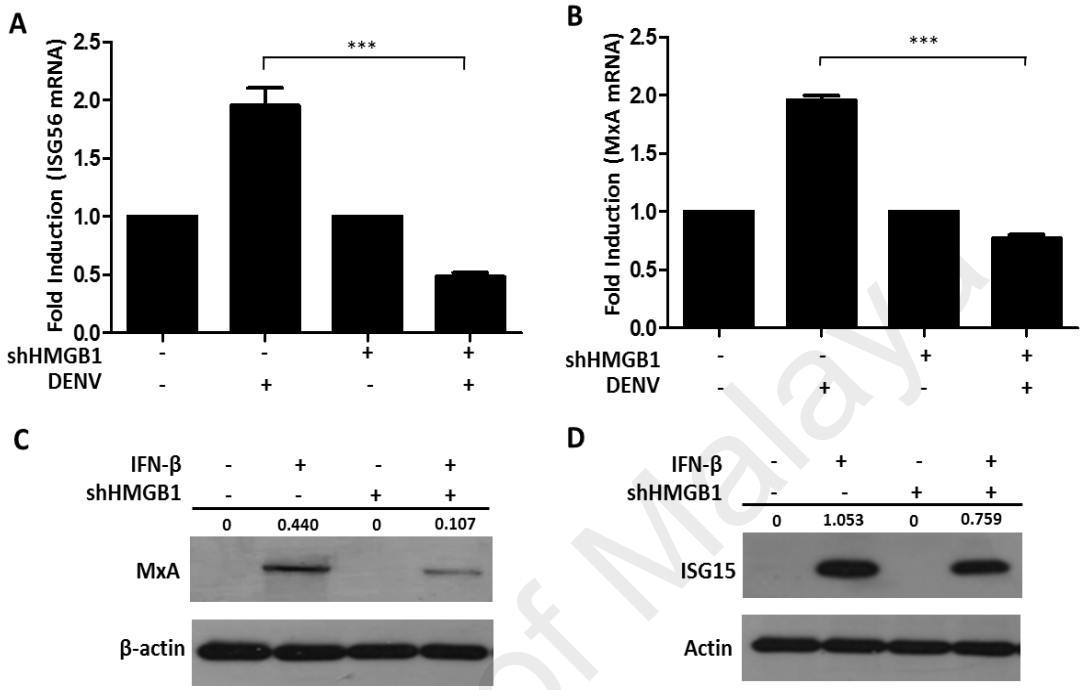


Figure 4.17: HMGB1 knockdown reduces production of ISGs. (**A-B**) Huh7 WT and shHMGB1 cells were mock-infected or infected with DENV at an MOI of 1 and harvested at 24 h p.i. The levels of MxA (**A**) and ISG56 (**B**) mRNA were evaluated using quantitative real-time RT-PCR and normalized to β-actin mRNA level. Statistically significant differences between the groups are as indicated: ***P<0.001. (**C-D**) Huh7 WT and shHMGB1 cells were stimulated with 50 µM IFN-β for 16 h and the levels of ISG15 (**C**) and MxA (**D**) were determined using Western blot analysis. The band intensity of ISG15/MxA and β-actin ratio was determined by the Image J analysis.

In order to understand the role of HMGB1 in the antiviral response against DENV, the characteristic of HMGB1 during DENV infection was studied. The Huh7 cells were mock-infected and infected with DENV at an MOI of 10. The cells were then harvested for the detection of HMGB1 using immunofluorescence assay (IFA), while supernatant was collected for HMGB1 detection by Western blot and ELISA assay. IFA result showed that HMGB1 was translocated out of the nucleus to the cytoplasm (red colour, white arrow) (Figure 4.18A). Western blot and ELISA assay revealed that HMGB1 level in the supernatant was higher in DENV-infected cells than the mock-infected cells, confirming the translocation process of HMGB1 out of the nucleus to the cytoplasm and eventually to the extracellular environment during DENV infection (Figure 4.18B, 4.18C).

To further explore the role of HMGB1 in the host antiviral effect against DENV, the effects of high levels of HMGB1 in the cells against DENV replication was deliberated. However, we first confirmed the high level of HMGB1 in the Huh7 cells resulted from resveratrol (RESV) treatment. RESV acted upon sirtuin1 (Sirt1) protein, a deacetylase that inhibits the translocation of HMGB1 from the nucleus to the cytoplasm, which then leads to high level of HMGB1 in the nucleus. In order to verify the accumulation of HMGB1 in the nucleus by RESV treatment during DENV infection, cytoplasmic and nuclear proteins were harvested from the mock- and RESV-treated cells, both mock-infected and infected with DENV. HMGB1 in the cytoplasm and nucleus were detected using Western blot analysis. HMGB1 was higher in the nucleus (Figure 4.19A) and lower in the cytoplasm (Figure 4.19B) of the RESV-treated cells in comparison to the untreated cells or DENV infection alone. Compatibly, HMGB1 was also lower in the supernatant of RESV-treated cells than the controls (Figure 4.19C). Due to the inhibition trait of Sirt1 against the translocation of HMGB1, the knockdown of the gene (shSirt1) was performed, to induced rapid translocation of HMGB1. The rapid HMGB1 translocation by shSirt1 was confirmed by our Western blot analysis, where HMGB1 was lower in the nucleus of

mock- and DENV-infected shSirt1 cells (Figure 4.19A) than the controls (non-treated, mock- and DENV-infected WT cells). However, no significant difference of the HMGB1 level in the cytoplasm (Figure 4.19B) and supernatant (Figure 4.19C) observed between mock- and DENV-infected shSirt1 cells and the controls (non-treated, mock- and DENV-infected WT cells). Additional confirmation using IFA determined the intracellular location of HMGB1 (red colour). IFA illustrated higher intensity of HMGB1 in the RESV-treated cells, confirming the accumulation of HMGB1 in the nucleus (Figure 4.19D, panel 4 compared to panel 2). Low intensity of HMGB1 in the shSirt1 knockdown cells was observed, resulted from the rapid translocation of HMGB1 out of the nucleus or cells (Figure 4.19D). The RESV-treated shSirt1 cells showed intermediate intensity of HMGB1 in the nucleus (Figure 4.19D). These findings confirm the impact of RESV on the translocation of HMGB1, where the Sirt1-mediated inhibition of HMGB1 translocation leads to an increase level of HMGB1 in the nucleus.

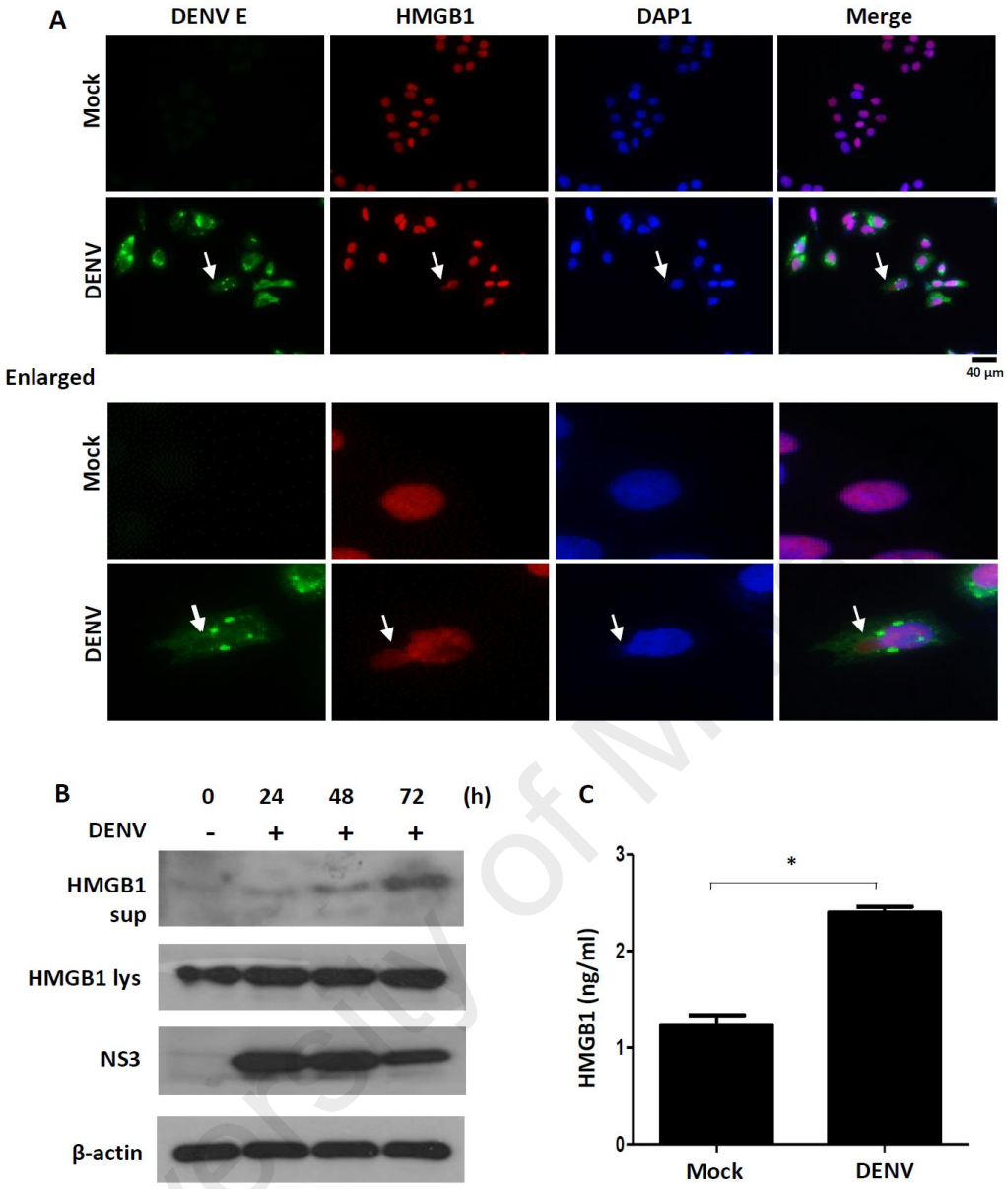


Figure 4.18: DENV infection induces translocation of HMGB1 from the nucleus to the extracellular milieu. (**A**) Huh7 cells were infected with DENV at an MOI of 10. After 24 h, cells were fixed for immunofluorescence analysis (IFA). For detection of HMGB1, cells were stained with rabbit anti-HMGB1 and donkey anti-rabbit-IgG Alexa Fluor 594 (red). For detection of DENV E protein, cells were probed with mouse anti-E protein antibody and goat anti-mouse-IgG Alexa Fluor 488 (green). The cell nuclei were stained with DAPI (blue). Arrows indicate cytosolic HMGB1. (**B**) Cell lysates and the supernatants were collected at 24 h p.i. and analyzed by Western blot for DENV NS3 and HMGB1 detection. (**C**) HMGB1 levels were analyzed by ELISA. Statistically significant differences between the groups are indicated: *P<0.05.

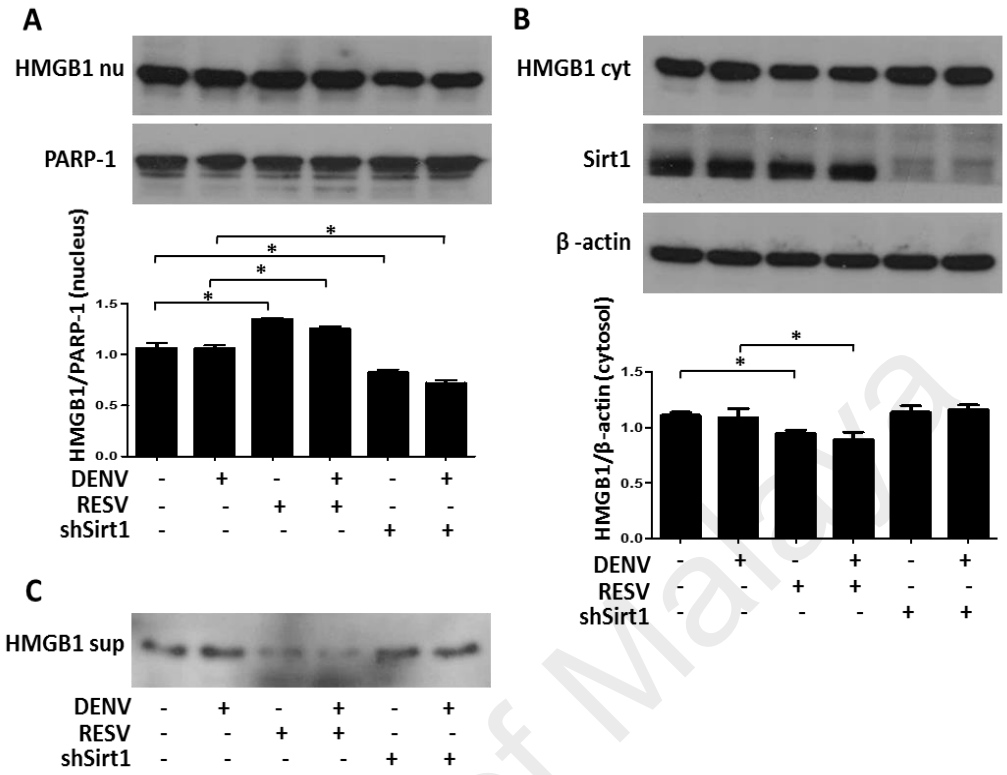


Figure 4.19: RESV increases HMGB1 in the nucleus by Sirt1-mediated translocation inhibition. Mock-treated, 80 µM RESV-treated and Sirt1-knockdown (shSirt1) Huh7 cells were mock-infected or infected with DENV at an MOI of 1 and the cells were harvested at 24 h p.i. The nuclear (**A**) and cytosolic (**B**) proteins were determined using Western blot analysis for detection of HMGB1. Statistically significant differences between the groups are as indicated: *P<0.05. (**C**) The supernatants of the cell cultures were harvested and the levels of HMGB1 were detected using Western blot. (**D**) Mock-treated, 80 µM RESV-treated and shSirt1 cells with different groups of treatment (as marked) were fixed at 24 h p.i. for immunofluorescence analysis (IFA). Staining of HMGB1 was carried out using anti-HMGB1 and donkey anti-rabbit-IgG Alexa Fluor 594 (red). DENV E detection was performed using mouse anti-E protein antibody and goat anti-mouse-IgG Alexa Fluor 488 (green). DAPI (blue) was used to probe cell nuclei.

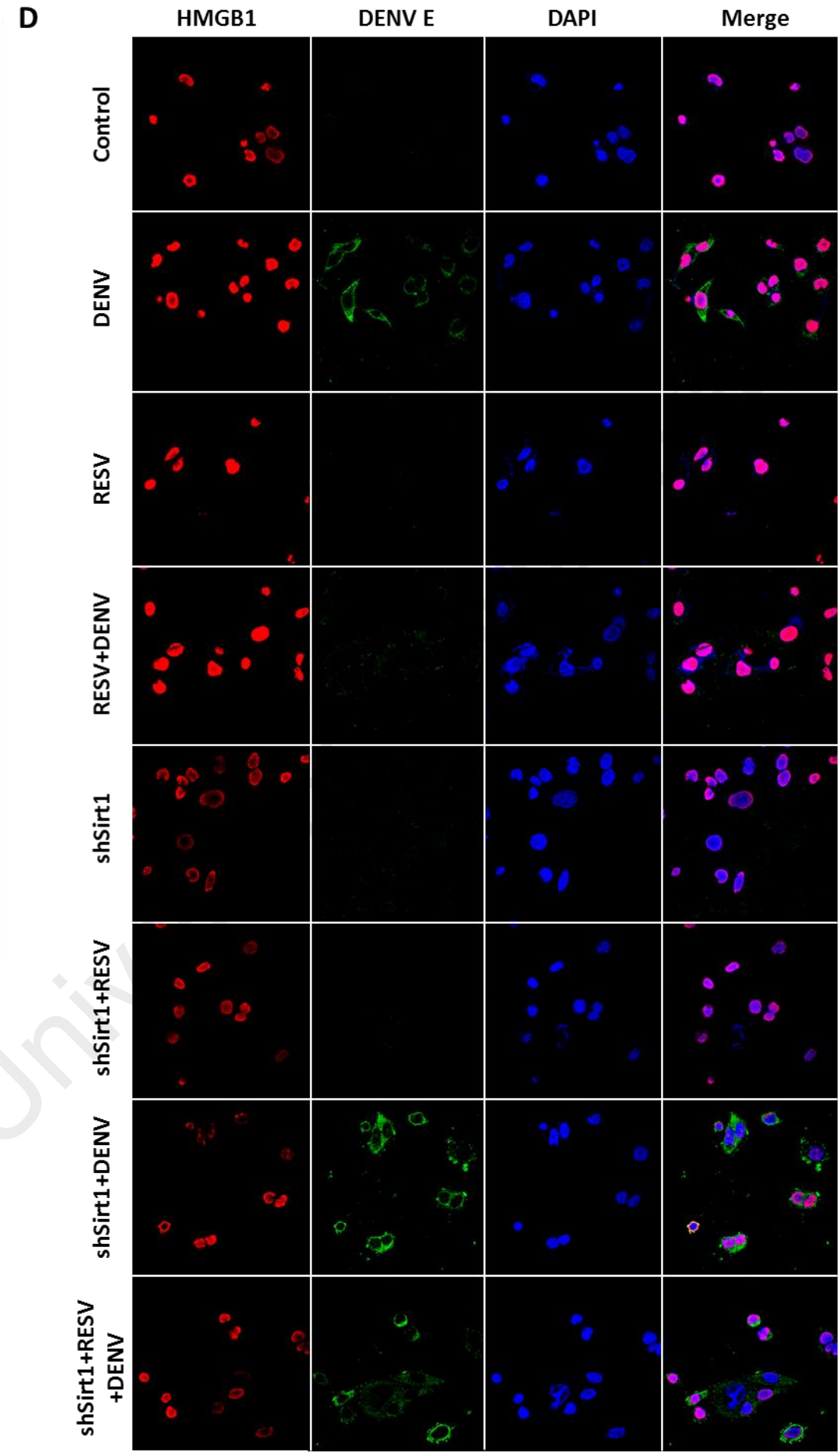


Figure 4.19, continued

We determined the effects of nuclear HMGB1 accumulation by RESV treatment on DENV infection. Huh7 cells were treated with 50 µM, 80 µM and 100 µM of RESV, which afterwards infected with DENV at an MOI of 1. After 24 hours, the cells and supernatant were harvested and the virus titer was determined using plaque assay on the Vero cells. Virus titer was significantly reduced in several concentrations of RESV-treated cells in comparison to the mock-treated cells and the inhibition was in a dose-dependent manner (Figure 4.20A). Western blot analysis showed the reduction of DENV NS3 protein production in 80 µM RESV-treated cells in comparison to the non-treated cells, which confirmed the decrease of DENV replication in the cells subjected to RESV (Figure 4.20B). The cytotoxicity level of RESV treatment in Huh7 cells was also examined by monitoring lactate dehydrogenase (LDH) release. Results showed no significant difference in LDH release in 50 µM, 80 µM and 100 µM RESV-treated cells in comparison to the non-treated mock-infected cells (Figure 4.21). A slight increase of LDH was observed in the RESV-treated cells with DENV infection at an MOI of 1. However, the impact was minor and apparently much lower than the non-treated cells infected with DENV (Figure 4.21). Taken together, these results suggest that RESV treatment, which causes the nuclear HMGB1 accumulation in the cells, leads to an efficient antiviral response against DENV and the effect was not due to the cytotoxic effect of the phytoalexin.

The impact of high levels of HMGB1 in the nucleus by RESV treatment on the type-1 interferon response during DENV infection was also investigated. RESV-treated and non-treated cells were infected with DENV infection at an MOI of 1, which subsequently harvested after 24 hours. The cells were then analyzed using qRT-PCR for the detection of IFN-β, MxA and ISG56. Compared with the non-treated cells, RESV-treated cells showed significantly higher level of IFN-β mRNA during DENV infection (Figure 4.22A). Coherent with the IFN-β, MxA and ISG56 mRNA levels in the RESV-treated

cells were also higher than the non-treated cells during DENV infection (Figure 4.22B, 4.22C). Protein production of MxA in RESV- and non-treated cells was also determined in the IFN-β-induced cells and the result was consistent with the previous DENV infection condition, where the MxA mRNA level was higher in the RESV-treated cells than non-treated cells (Figure 4.22D). These data suggest that the increased amount of HMGB1 in the nucleus by RESV treatment during DENV infection leads to a higher induction of IFN and ISGs, which might be one of the factors to an efficient antiviral activity against DENV.

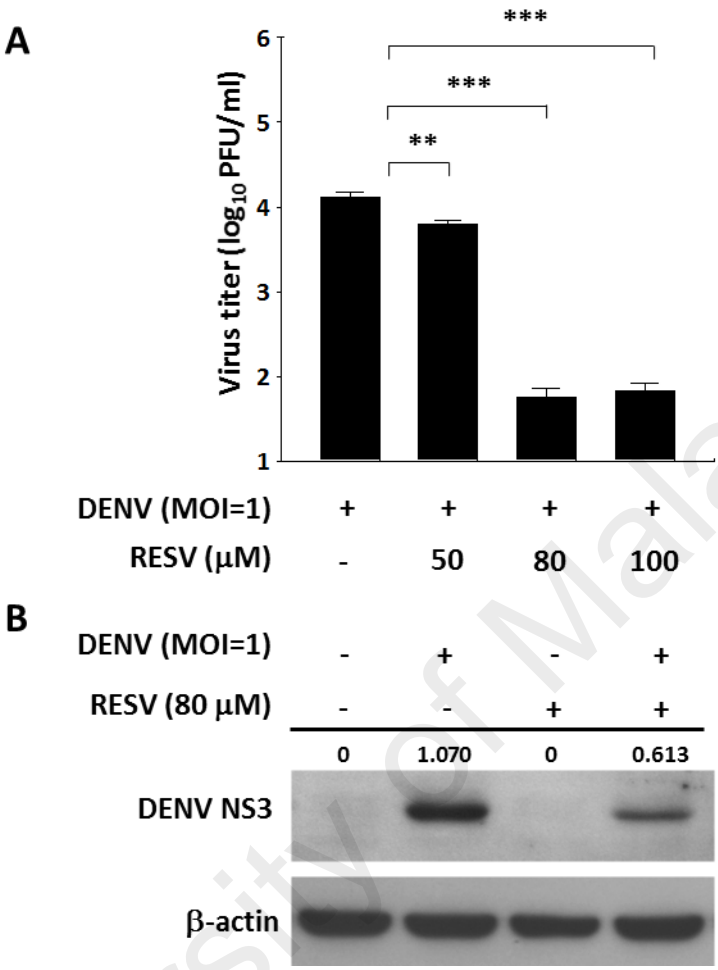


Figure 4.20: RESV treatment reduces virus replication in DENV-infected cells. (**A**) Huh7 cells were mock-treated or treated with different concentrations of RESV (50, 80 or 100 μM) post-infection (p.i.) of DENV at an MOI of 1 and incubated for 24 h. Supernatants of the cell culture were harvested and the virus titers were analysed using plaque assay. Statistically significant differences between the groups are as indicated: **P<0.01, ***P<0.001. (**B**) Huh7 cells were mock-treated or treated with 80 μM of RESV after DENV infection and harvested at 24 h p.i. DENV NS3 was detected using Western blot analysis. The band intensity of DENV NS3 and β-actin ratio was determined by the Image J analysis.

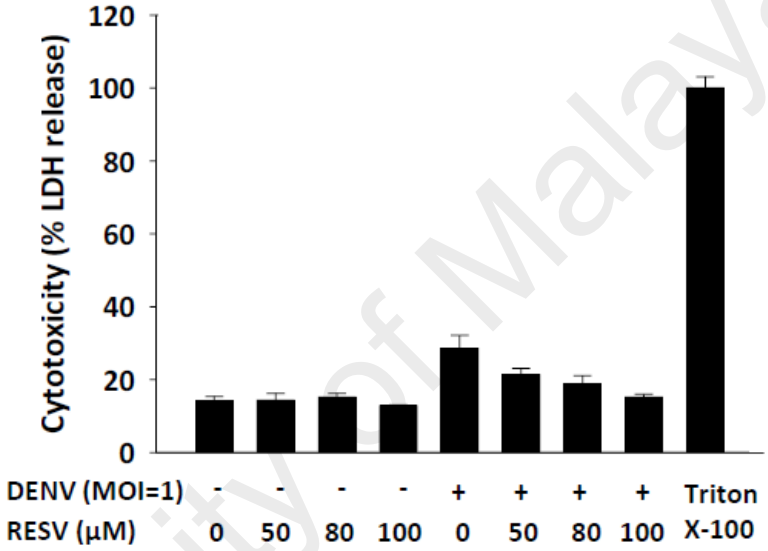


Figure 4.21: RESV treatment does not cause cytotoxicity in Huh7 cells. Huh7 cells were mock-treated or treated with 50, 80 or 100 μM of RESV after DENV infection at an MOI of 1. After 24 h, cell culture supernatants were collected and detected for the release of lactate dehydrogenase (LDH). Triton X-100 served as a positive control.

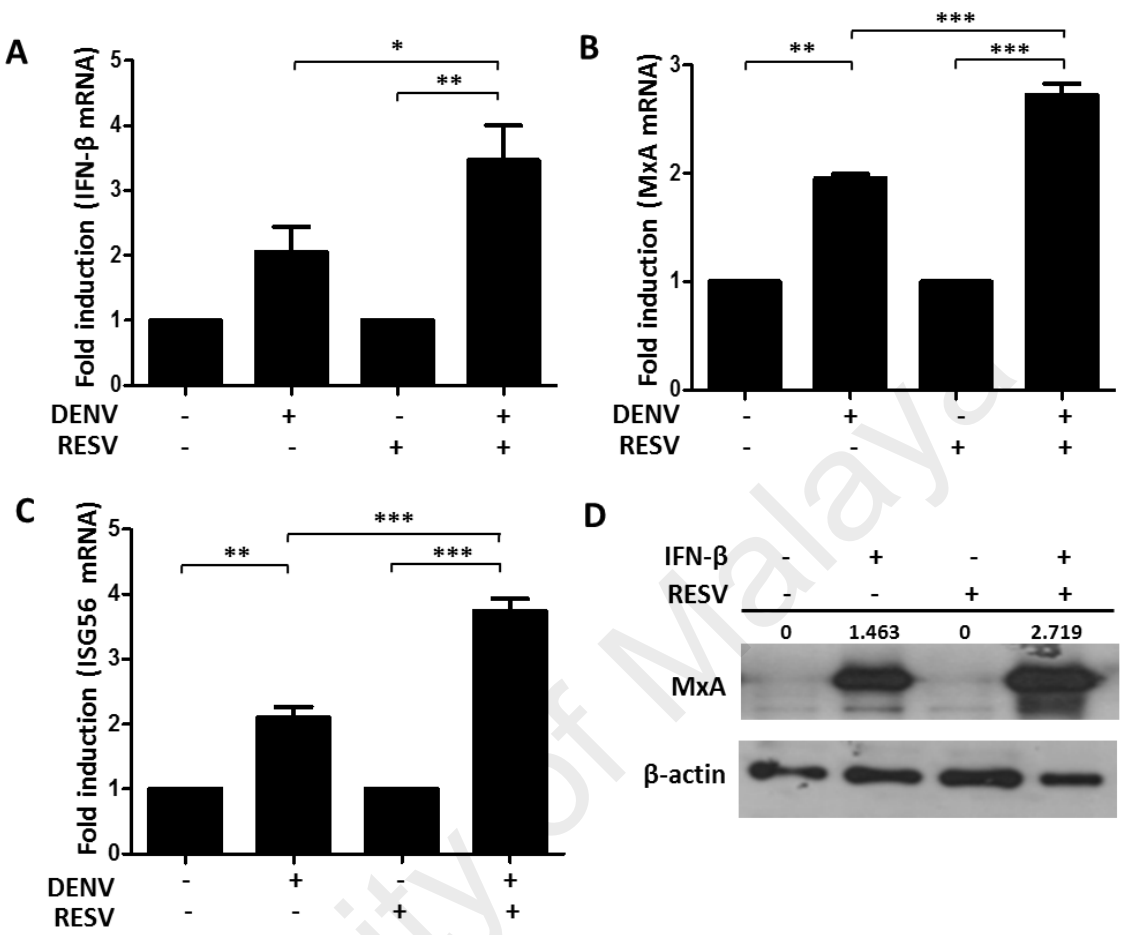


Figure 4.22: RESV increases IFN-β and ISG production. (**A-C**) Huh7 cells were mock-treated and treated with 80 µM RESV post-infection of DENV at an MOI of 1 and incubated for 24 h. Cells were harvested and the levels of IFN-β (**A**), MxA (**B**) and ISG56 (**C**) mRNA were determined using quantitative real-time PCR, normalized to β-actin mRNA. Statistically significant differences between the groups are as indicated: *P<0.05, **P<0.01, ***P<0.001. (**D**) Huh7 cells were mock-treated or treated with 80 µM RESV during stimulation of 50 µM IFN-β for 16 h and the level of MxA was determined using Western blot analysis. The band intensity of MxA and β-actin ratio was determined by the Image J analysis.

We identified the effect of rapid translocation of HMGB1 out of the nucleus in Sirt1-depleted cells on DENV infection. WT and Sirt1-depleted cells were infected with DENV at an MOI of 1. HMGB1-depleted cells, which HMGB1 level was originally low in the nucleus, were also infected with DENV and used for the comparison. The cells were harvested after 24 hours of incubation and analysed using Western blot and focus forming assay. Sirt1-knockdown cells displayed higher DENV NS3 protein production compared to the WT with DENV infection (Figure 4.23A). However, the expression of DENV NS3 in shSirt1 cells was lower in comparison to the shHMGB1 cells (Figure 4.23B). This pattern was also shown on the virus titer result, where shHMGB1 cells exhibited highest virus count, followed by shSirt1 and WT cells. Results show that less amount of nuclear HMGB1 leads to less DENV inhibition, suggesting that the level of HMGB1 in the nucleus is crucial in the antiviral response against DENV.

Given the involvement of ISGs in the antiviral response against DENV as shown earlier, we further determined the effect of Sirt1 knockdown (rapid translocation process of HMGB1 out of the nucleus) on MxA and ISG56 induction during DENV infection. WT and shSirt1 cells were infected with DENV at an MOI of 1 and subsequently harvested after 24 hours of incubation. The qRT-PCR data showed that mRNA levels of MxA and ISG56 were significantly reduced in shSirt1 cells in comparison to WT (Figure 4.24A, 4.24B). IFN-β-stimulated cells also showed lower amount of MxA protein production than the non-stimulated cells (Figure 4.24C). The decreased MxA and ISG56 mRNA expression in shSirt1 and shHMGB1 (Figure 4.17) (less nuclear HMGB1), complementing the high expression of ISGs in RESV treatment (Figure 4.22) (high nuclear HMGB1), dictates that the level of HMGB1 in the nucleus plays an important role in an efficient antiviral response via ISGs induction against DENV.

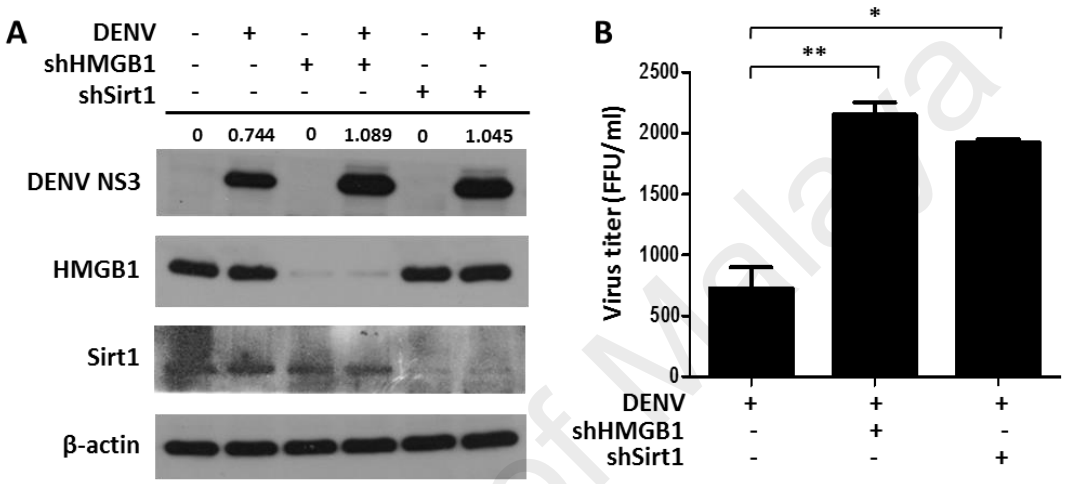


Figure 4.23: Depletion of Sirt1 reduces the efficiency of antiviral response. Huh7 WT, shHMGB1 and shSirt1 cells were mock-infected or infected with DENV at an MOI of 1 for 24 h. (**A**) Cells were harvested and the levels of DENV NS3, HMGB1 and Sirt1 were detected by Western blot analysis. The band intensity of DENV NS3 and β-actin ratio was determined by the Image J analysis. (**B**) Supernatants were collected and virus titers were determined using focus assay. Statistically significant differences between the groups are as indicated: *P<0.05, **P<0.01.

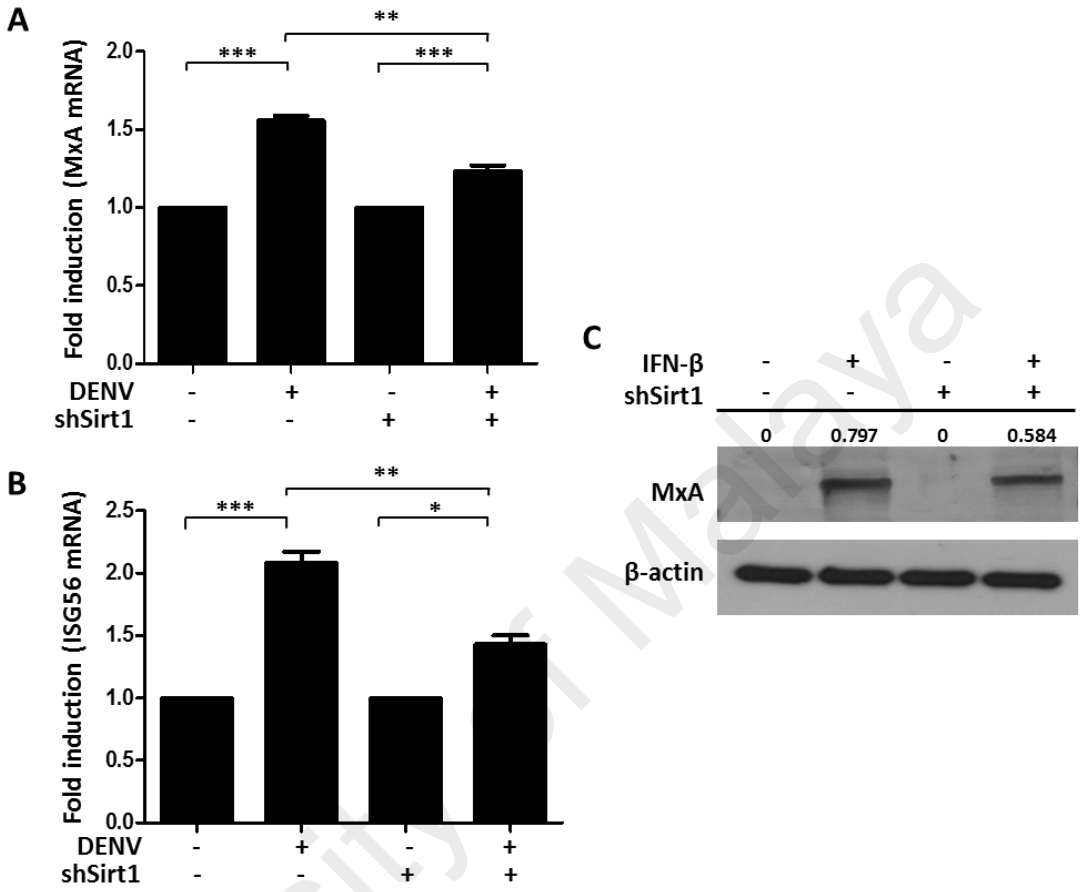


Figure 4.24: Sirt1 is required to trigger ISG expression. (**A-B**) Huh7 WT and shSirt1 cells were mock-infected and infected with DENV at an MOI of 1 for 24 h. Cells were harvested and the levels of MxA (**A**) and ISG56 (**B**) mRNA were quantified using quantitative real-time PCR, normalized to the mRNA level of β-actin. (**C**) Huh7 WT and shSirt1 were stimulated with 50 µM IFN-β for 16 h. Cells were harvested and the level of MxA was determined using Western blot analysis. The band intensity of MxA and β-actin ratio was determined by the Image J analysis.

Next, we clarified whether the mechanism of antiviral activity of RESV depends on the level of HMGB1 in the nucleus. WT, HMGB1-depleted and shSirt1 cells were treated and mock-treated with RESV. Subsequently, the cells were infected with DENV at an MOI of 1 and harvested after 24 hours of incubation. Western blot analysis showed no difference in the DENV NS3 protein production in the shHMGB1 between the RESV-treated and the non-treated cells, whereas WT cells, as reported earlier, showed decreased DENV NS3 with RESV treatment (Figure 4.25A). Sirt1-depleted cells showed slightly less DENV NS3 protein production in RESV-treated cells in comparison to the non-treated cells, although the reduction was not as strong as displayed in the WT cells (Figure 4.25B). This concludes that the antiviral activity of RESV was via its role in regulation of nuclear HMGB1.

The importance of HMGB1 in the induction of ISGs has been shown in this study. However, the exact role of HMGB1 on ISG production was still unknown. Thus, we proceed in investigating whether HMGB1 acted as the transcriptional regulator of one of the ISGs, MxA. We performed *in vivo* DNA binding assay using chromatin immunoprecipitation (ChIP assay). Cross-linked of WT and shSirt1 Huh7 cells were sonicated and the DNA fragments produced were immunoprecipitated with specific antibodies recognizing HMGB1 (α-HMGB1) or rabbit IgG as a control. Precipitated DNA (DNA bound to α-HMGB1) resulted from the reversal of crosslinking was then amplified by MxA-related primer using PCR. The result showed that HMGB1 bound to the region between -842 and -368 from the start codon of MxA after DENV infection (Figure 4.26A). The binding of DNA-protein complexes was also decreased in Sirt1-depleted cells (Figure 4.26A). Moreover, HMGB1 binding to the promoter of MxA only occurred after DENV infection, not in the non-infected cells (Figure 4.26B). Overall, results suggest that DENV infection triggers the binding of HMGB1 to the promoter of

MxA and the decreased of DNA-protein complexes in shSirt1 cells supports the evidence of the binding.

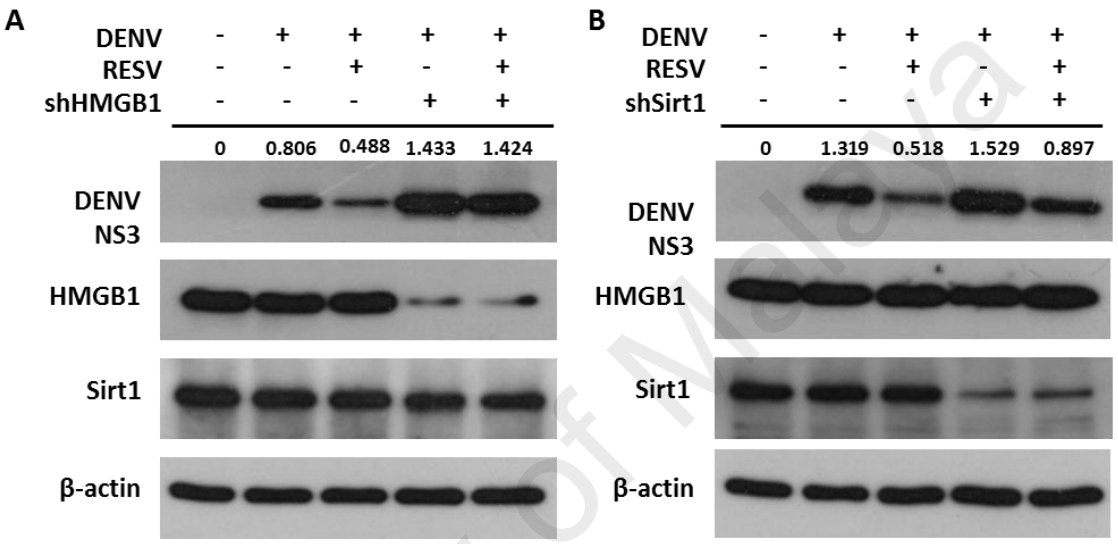


Figure 4.25: Antiviral activity of RESV relies on the presence of HMGB1 and Sirt1. Huh7 WT, shHMGB1 (**A**) and shSirt1 (**B**) cells were mock-treated or treated with 80 µM RESV after DENV infection at an MOI of 1. After 24 h p.i., the cells were harvested and the levels of DENV NS3, HMGB1 and Sirt1 were analyzed using Western blot analysis. The band intensity of DENV NS3 and β-actin ratio was determined by the Image J analysis.

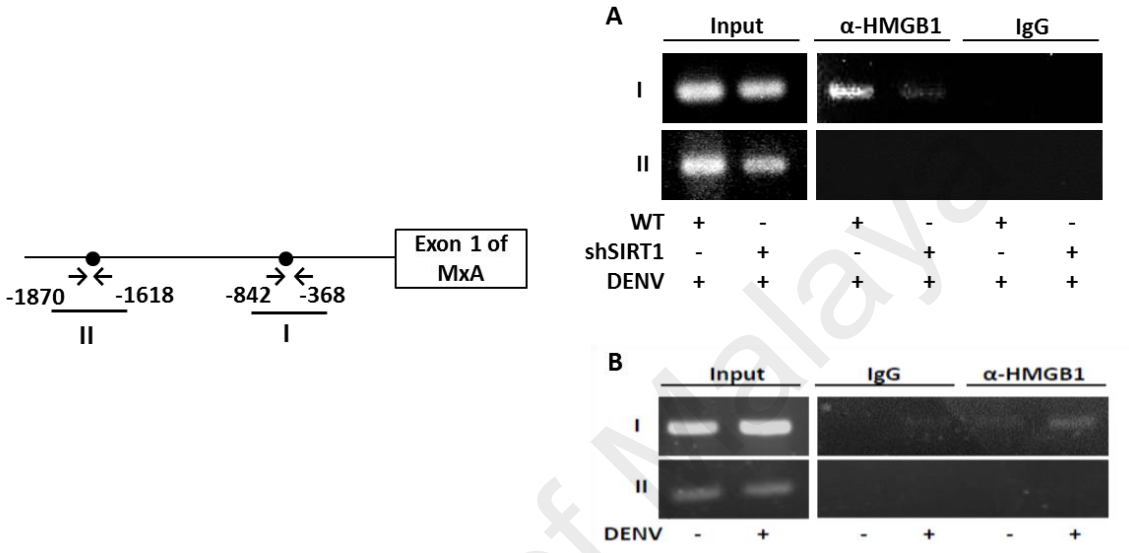


Figure 4.26: HMGB1 binds to the promoter region of MxA. MxA promoter fragment surrounding the proximal HMGB1 binding site (I: from -842 to -368) or region distant from HMGB1 binding site (negative control) (II: from -1870 to -1618) were amplified using designated primers. The ChIP assay was performed on DENV-infected WT or DENV-infected shSirt1 cells at an MOI of 1, where the DNA fragments were immunoprecipitated with α-HMGB1 and IgG antibodies (control) for 12 h. Primers I and II were then used to amplify the MxA promoter fragment (Table 3.3) (**A**). ChIP assay was performed on non-infected or DENV-infected Huh7 WT cells at an MOI of 1 by immunoprecipitation of DNA fragments with α-HMGB1 and IgG antibodies (control) for 12 h (**B**). Primers I and II were used to amplify the MxA promoter fragment (Table 3.3).

CHAPTER 5: DISCUSSION

The relationship between dengue and autoimmunity has been suggested recently, due to the autoantibodies reaction observed in the mechanisms of severe dengue development, demonstrated by the cross-reactivity between the host immune response with its own tissues/cells such as platelets, endothelial cells and coagulatory molecules (Chuang *et al.*, 2014; Chungue *et al.*, 1994; Lin *et al.*, 2003; Lin *et al.*, 2006; Markoff *et al.*, 1991; Wan *et al.*, 2013). In this study, we investigated the association of dengue and autoimmunity through the assessment of genetic variants and its possible contribution underlying severe, non-severe and SLE using the next-generation sequencing method, Ion Torrent. Subsequently, the genetic variant distribution among sample groups were statistically compared, and the association between genetic polymorphism and the protectivity or susceptibility against severe dengue was verified. We also determined the neutralizing capability of autoimmune patients' sera, specifically SLE patients against DENV. This study was to validate the protective effects against dengue in SLE patients from an informal observation of a very rare incident of symptomatic or severe dengue among SLE patients. In addition, we also examined the relationship between HMGB1, a protein linked to multiple autoimmune diseases, including SLE (Lu *et al.*, 2015a; Pisetsky, 2014), psoriasis (Chen *et al.*, 2013), rheumatoid arthritis (Wang *et al.*, 2015), Sjogren's syndrome (Dupire *et al.*, 2012) and multiple sclerosis (Malhotra *et al.*, 2015), and dengue via *in-vitro* observation of the role of HMGB1 in the antiviral responses against DENV.

The association of host genetic variants and the pathogenesis of dengue have been studied previously using genome sequencing (Khor *et al.*, 2011; Loke *et al.*, 2001; Nguyen *et al.*, 2011; Sakuntabhai *et al.*, 2005; Sam *et al.*, 2015; Whitehorn *et al.*, 2013). A genome-wide association study (GWAS) using the Illumina sequencing method showed the association of single nucleotide polymorphism (SNP) at MICB and PLCE1 with the susceptibility to DSS (Khor *et al.*, 2011). In addition, a promoter variant of CD209, DCSIGN1-336,

detected using direct sequencing of PCR-amplified genomic DNA, displayed association with the risk of severe dengue (Sakuntabhai *et al.*, 2005). Previous study on the genotyping of HLA gene among dengue haemorrhagic fever (DHF) patients using PCR-sequence-specific primer (PCR-SSP) revealed the association of variants in the HLA class 1 and the risk factor of severe dengue (Loke *et al.*, 2001). Gene polymorphism of TNF-α was also associated with both protection and susceptibility to severe dengue (Sam *et al.*, 2015). One of the next-generation sequencing method, Ion Torrent was used in this study for the detection of variants in autoimmune- and dengue-related genes among non-severe dengue, severe dengue and SLE patients. Ion Torrent, a semi-conductor sequencing technique which operated via detection of hydrogen released, a by-product of nucleotide binding, did not require optical detection and fluorescence system, resulted in rapid, economical and compact technology for sequencing (Mardis, 2013; van Dijk *et al.*, 2014). This technology allowed up to 400 bp read and the preparation takes up less than 6 hours for approximately 8 samples concurrently. The sequencing quality was more stable with higher map sites and better GC depth detection using Ion Torrent in comparison to other next-generation sequencing (Liu *et al.*, 2012). Using Ion Torrent Personal Genome Machine (PGM) sequencing platform, we inspected the genomic sequences of the target genes (Table 3.1) in the non-severe dengue, severe dengue and SLE patients, and the polymorphisms were then dictated by the comparison with Human Genome Reference Sequence, hg19. The frequency of the variants of each sample group was then analysed and those that showed significant distribution among all groups were further studied. We obtained four significant variants: novel variant at chr3: 49679737, rs76018112, rs1557370 and rs945635.

Our study showed a significant association between a novel variant at chr3:49679737 in the exonic region of BSN gene, and patients' susceptibility to severe dengue (Table 4.3). BSN, a presynaptic scaffold protein, comprised of ribbons that channel calcium (Ca^{2+})

released, also played a role in promoting vesicle replenishment (Frank *et al.*, 2010). Low Ca^{2+} has been detected in DENV-infected patients and the effect was linked to the hypocalcemia in severe dengue (Constantine *et al.*, 2014; Dahanayaka *et al.*, 2017). Furthermore, Ca^{2+} is important in the platelet aggregation, proposing the importance of Ca^{2+} in the development of dengue treatment (Authi, 2007; Blaustein *et al.*, 1991). Hence, decreased or altered BSN gene expression might affect the protein structure and leads to predisposition to severe dengue, perhaps by reducing platelet accumulation. Correspondingly, *in-silico* analysis revealed the possible obstruction of BSN gene by the variant effect on the splicing event of the gene (Figure 4.1). Moreover, another *in-silico* analysis, PROMO predicted that this variant diminished three transcription factor binding sites, including RAR-beta:RXR-alpha, Pax-5 and p53 and added a transcription factor binding site for AP-2 alpha (Figure 4.4). Retinoid acid inhibits reactivation of latent Epstein-Barr virus (EBV) infection by the abolition of a viral protein, Z (Sista *et al.*, 1995; Sista *et al.*, 1993). Pax5 also showed important role in the regulation of EBV, where depletion of the gene caused higher viral genome copies and BZLF1 protein expression (Arvey *et al.*, 2012), a protein that converts latent infection to lytic phase (Wen *et al.*, 2007). Transcription factor p53 displayed dengue inhibiting effect by type-1 interferon activation (Fischer *et al.*, 2013; Hu *et al.*, 2017). The reduction of HIV was also observed with the overexpression of p53, suggesting a possible therapeutic target for AIDS (Mukerjee *et al.*, 2010). The abrogation of a RAR-beta:RXR-alpha, Pax-5, p53 clarified the vulnerability of patients with this variant towards severe dengue. AP2-alpha promotes the expression of oncogene, LMP1 expression in EBV latent infection (Murata *et al.*, 2016). Hepatitis B regulates oncogene *Raf1* expression via activation of AP-2 alpha transcription factor (Lu *et al.*, 2015b; Qu *et al.*, 2013). The activation of oncogenes by AP-2 alpha transcriptional factor may contribute to the inflammatory manifestation observed in severe dengue (Aggarwal *et al.*, 2006). Disruption and the addition of these

transcriptional binding sites, together with the possible disruption of Ca^{2+}-mediated immune response of the gene, supported the association of novel variant at chr3:49679737 and the susceptibility to severe dengue.

Our data showed that rs76018112 variant in exonic region of ABCF1 gene was significantly associated with protection against development of severe dengue (Table 4.3). Variant rs76018112 in ABCF1 gene is a very rare polymorphism and the information regarding to this mutation is not available. Earlier study showed that rare copy number variant (CNV) in ABCF1 gene was detected in DENV-infected patients (Hoh *et al.*, 2014), though no significant correlation between ABCF1 and dengue can be postulated. ABCF1 gene was associated with the activation of antiviral response, observed from the significantly reduced innate immune reaction against retroviral infection in ABCF1 depleted (*Abcf1*) mouse embryonic fibroblasts cells (MEFs) (Lee *et al.*, 2013). Depletion of ABCF1 affects the induction of CXCL10, an essential protein in the defence against primary dengue infection (Hsieh *et al.*, 2006). Higher copy number of ABCF1, however, contributed to higher risk of gout, possibly by stimulating inflammatory process of the disease (Dong *et al.*, 2017). Splicing events of the ABCF1 were not affected by rs76018112 variant, suggesting the possibility of non-disruption of the gene (data not shown). The high frequency of this polymorphism among SLE patients (Table 4.3) suggested that the variant may lead to the overexpression of ABCF1-mediated immune response. The prediction of the transcription factor binding site around the rs76018112 variant site showed the addition of nuclear factor of activated T-cells (NF-AT2) binding site when compared to the WT sequence (Figure 4.5). NFAT transcription factor was recently reported to downregulate the expression of one of HIV-1 proteins, long terminal repeat (LTR) (Macian *et al.*, 1999), which is important in the transcription of the virus (Jacque *et al.*, 1996). The synergies reaction of NF-AT2 with Jun proto-oncogene (cJun) protein induced IL-2 (Nguyen *et al.*, 2010), a protein released early during dengue

infection (Chaturvedi *et al.*, 1999). Moreover, the frequency of IL-2 producing T-cells were higher among school children with subclinical (asymptomatic) secondary dengue infection in comparison to those that developed symptomatic secondary dengue infection (Hatch *et al.*, 2011), suggesting the protective effect of IL-2 against dengue. The postulated overexpression of ABCF1-mediated immune response and early defence against DENV through transcriptional factor NF-AT2 by rs76018112 variant may contribute in the protective attribution of rs76018112 polymorphism against the development of severe dengue.

From the analysis of our sequencing result, SNP rs1557370 in the 3-prime untranslated region (3'UTR) of Mx1 gene was significantly correlated with protective effects against severe dengue. Mx1 is one of the ISGs and plays an important role in the innate immune response against virus infection (Pillai *et al.*, 2016), particularly Orthomyxoviruses, by accumulating in the nucleus and blocking the virus replication at the early stage of the infection (Haller *et al.*, 1995; Turan *et al.*, 2004; Zurcher *et al.*, 1992). DENV2 replication was significantly reduced by IFN-λ via increased level of Mx1 (Palma-Ocampo *et al.*, 2015). Mx1 also positively regulates type 1 IFN in influenza virus infection (Schattgen *et al.*, 2016). Study on SNP rs1557370 showed insignificant association of the polymorphism with chronic HBV infection (He *et al.*, 2015), yet there is no other research studying the effect of this variant against other virus infections. *In silico* prediction on the splicing factor binding sites showed that SNP rs1557370 may affect the normal splicing events of the gene (Figure 4.2), thus presumed to cause disruption. However, previous studies on multiple variants in Mx protein revealed that the antiviral effect of the gene will only be inhibited by the alteration of amino acids in the GED domain (Lee *et al.*, 2002), suggesting the possibility of Mx1 intact expression in SNP rs1557370. Nevertheless, due to the location of SNP rs1557370 in the 3'UTR region of Mx1, the variant probably altered the gene expression (Ye *et al.*, 2017). The high frequency of SNP

rs1557370 among SLE patients inferred the possibility that the polymorphism caused Mx1 overexpression, a phenomenon that typically occurred in SLE patients (Feng *et al.*, 2006). The protective characteristic of SNP rs1557370 against severe dengue might be acquired from the early and efficient antiviral responses by Mx1 overexpression, postulated from the effects of the polymorphism.

This study also showed that SNP rs945635 in FCRL3 genes was significantly correlated with protective effects against severe dengue (Table 4.4). FCRL3 is a transmembrane protein that exhibits high homology with the Fc receptor (Davis *et al.*, 2001). Nonetheless, the actual function of FCRL3 is still indefinite. Variants in FCRL3 gene, including rs945635 have been associated with the susceptibility against autoimmune diseases, including rheumatoid arthritis, autoimmune thyroid disease (Kochi *et al.*, 2005), SLE (Mao *et al.*, 2010) and multiple sclerosis (Yuan *et al.*, 2016), proposing the importance of FCRL3 in regulating host immune response. Indeed, the presence of ITAM and ITIM in the cytosolic domain of FCRL3 suggests the inhibitory effect of the gene on B-cell receptor, hence confirming the regulatory function of FCRL3 in host immune response (Kochi *et al.*, 2009). Our study showed that SNP rs945635 altered the splicing events of FCRL3, signifying the disruption of gene expression (Figure 4.3). Moreover, the mutation located at 5 prime untranslated region (5'UTR) (Table 4.5) may alter the regulation of gene translation (Hamilton *et al.*, 2006), hence, depleted or reduced the regulatory function of FCRL3. This theory coordinates with our finding that showed a higher frequency of SNP rs945635 among SLE patients in comparison to the severe dengue (Table 4.4). Ineffective FCRL3 immune regulation by the polymorphism may cause abnormal increased of host immune response, a typical occurrence in autoimmune diseases, and possibly the factor that contributes in the protective aspects of the variant. Prediction of transcriptional factor binding sites revealed that SNP rs945635 added transcription factor E2F-1 binding site compared to the WT (Figure 4.6). Transcription

factor E2F-1 is a cell proliferation regulator that is involved in both cell growth and apoptosis (Dimova *et al.*, 2005; Wu *et al.*, 2009). E2F-1 reduced the viral replication of HIV-1 in transfected cells by suppression of viral protein, Tat (Kundu *et al.*, 1995). In addition, E2F-1 inhibits the binding of NF-κβ, a critical process in the transcription of HIV, thus resulted in the repressed virus replication (Kundu *et al.*, 1997). The enhanced antiviral response by the disorderly increased immune response and antiviral reaction by the transcription factor E2F-1 by SNP rs945635 may confer the protective effect against severe dengue.

All of the significant variants found in this study required further investigation, especially on the effects of the polymorphism on both gene and protein expression. Functional studies, including knockdown or knockout gene method at both *in vitro* and *in vivo* environment may provide more details and accurate outcomes of the variants against dengue. In addition to the discovery of potential biomarkers associated with protection and predisposition to severe dengue, this study also exposed the unexpected protective correlation of overactive or abnormal immune response against severe dengue. This finding is rather conflicting with the previous studies that emphasized on the similarity between severe dengue manifestation and autoimmune features, especially on the ability of host immune response to cross-react with its own cells and tissue (Lin *et al.*, 2006; Wan *et al.*, 2013). Host antibodies that supposedly be targeting DENV NS1, prM and E protein cross-react with platelets, endothelial cells and coagulation factors (Chuang *et al.*, 2014; Lin *et al.*, 2003), hence, increasing the risk of bleeding and platelet function failures (Chen *et al.*, 2009), two of the main hallmarks of severe dengue (Michels *et al.*, 2014). The preference of the host to produce autoantibodies was partially due to the defects of B-cells tolerance caused by certain genetic variants (Bentham *et al.*, 2015; Dam *et al.*, 2016), a typical occurrence in most autoimmune patients. Although not knowing the exact factors contributing to the mimicry effect between host antibodies and DENV protein, we

were initially drawn to expect some similarities in the genetic polymorphism between autoimmune patients with severe dengue patients. In contrary, our study showed that SLE patients shared similar protective variants against severe dengue with the non-severe dengue patients, thus suggesting that SLE patients possess certain protective factors against severe dengue.

Analysis on the possible effects of the protective variants (associated with protection against severe dengue) on the function of the genes, demonstrated the importance of effective innate immune response and early inhibition of virus replication in preventing acute dengue manifestation. Earlier studies showed that SLE patients displayed protective effects against HIV infection (Sekigawa *et al.*, 2002b) through the antiviral effect of IL-6 (Center *et al.*, 1996b) and the high levels of autoantigens such as anti-ribonucleoprotein (anti-RNP) and antibody-dependent cellular cytotoxicity (ADCC) that shared homologous region with the retroviral *gag* and/or *env* proteins (Perl, 2001; Sun *et al.*, 2011). In addition, an SLE patient with HIV infection produces broadly neutralizing antibodies (bNAbs), an autoreactive antibody that broadly neutralizes HIV-1, more readily than those without the autoimmune disease (Bonsignori *et al.*, 2014). The association of virus replication or viral load and the development of severe dengue has been suggested before, particularly in the progress of plasma leakage (Ben-Shachar *et al.*, 2016; Vaughn *et al.*, 2000). Furthermore, severe dengue also related to the rate of virus clearance (Tricou *et al.*, 2011; Wang *et al.*, 2006), suggesting the importance of the antiviral effects of host immune response in combating DENV and subsequently stunted acute manifestation. Delayed virus clearance leads to prolonged activation of the cross-reactive $CD8^+$ T cells, resulted in persistent and high stimulation of pro-inflammatory cytokines, including TNF-α, IL-6 and other molecules, which may contribute to vascular permeability (Wang *et al.*, 2006). Taken together, virus replication and virus load that associate with the antiviral reactions and virus clearance rate might be among the

important factors in the pathogenesis of severe dengue. Observation from our gene sequencing study suggests that individual with efficient activation of early antiviral responses and rapid clearance of DENV might have higher possibilities to avoid severe dengue manifestation.

The protective characteristics of autoimmunity against severe dengue suggested in our study correlates with the rare to almost none cases of severe dengue among the autoimmune disease patients. There were a few studies reporting the development of autoimmunity after DENV infection (de Souza *et al.*, 2010; Morel *et al.*, 2014; Rajadhyaksha *et al.*, 2012; Sane *et al.*, 2016; Talib *et al.*, 2013), however, cases where the development of severe dengue among already an autoimmune disease patient was not found in the literature. There was only one case of dengue in SLE patient, but the patient, however, was discharged as an outpatient due to the mild-symptoms of dengue (Verdolin *et al.*, 2014). This observation and the possible protection of autoimmunity via efficient antiviral response suggested by our genes sequencing study drove us to further investigate the probability of protection against DENV in SLE patients. We determined the neutralization capability of 82 SLE patients' sera against DENV1, DENV2 and DENV3, using foci-reduction neutralization (FRNT) assay. The results showed that SLE patient's sera have the capacity to neutralize DENV1 (Figure 4.7), DENV2 (Figure 4.8) and DENV3 (Figure 4.9) up until the serum dilution of 1/1280 when compared with the neutralizing capacity of the healthy donors, proposing the ability of SLE patients to efficiently inhibit DENV. Unusual high neutralization was observed among healthy donors' sera against DENV especially at the lowest serum dilution (1/20). This incident might be due to the non-specific reaction caused by high concentration of the serum (Terato *et al.*, 2014). Nevertheless, a few healthy donors' sera still showed high neutralization at higher serum dilution 1/80 and 1/320 (Figure 4.7B, 4.7C & Figure 4.8B, 4.8C). Although regarded as negative for dengue serology using dengue IgG and IgM

ELISA assays, these healthy donors probably had encountered other closely-related virus infection, which led to the presence of non-specific recognition towards DENV. On the other hand, we could not ascertain if the healthy volunteers had an underlying undiagnosed autoimmune disease.

The current study revealed the association of the neutralization percentages of SLE-sera against DENV and the dengue IgG level (Figure 4.11). However, some of the SLE-sera displayed the neutralization effects against DENV even with low levels of the dengue IgG ELISA titer, a phenomenon that occurs only in SLE patients (Figure 4.12), suggesting the uncommon incident of neutralization when specific IgG was below the detectable level (Black box, Figure 4.11). We emphasized the importance of IgG using IgG-depleted SLE-sera. IgG-depleted SLE-sera showed significant reduction of the neutralizing capacity against DENV (Figure 4.10A-F), confirming the prominent role of IgG in the neutralization effect of SLE-sera against DENV. This outcome is in accordance with the previous studies of the other viruses, including simian-human immunodeficiency virus (SHIV) (Moldt *et al.*, 2011), West Nile virus (WNV) (Chua *et al.*, 2013), measles and human cytomegalovirus (Corrales-Aguilar *et al.*, 2016), where IgG plays a major role in the antiviral responses. Depletion of IgG in the sera of SLE patients leads to the decrease of O.D absorbance (dengue IgG ELISA) to lower than 0.1 in most sera tested (Figure 4.10G). It is noteworthy that the reduction of the neutralizing effects of IgG-depleted SLE-sera against DENV was not in accordance with the high neutralizing capacity of SLE-sera with similarly low dengue IgG ELISA titer. This irregularity might be due to the decrease of other unidentified protein or low abundance antibody during IgG depletion (Granger *et al.*, 2005). Those depleted proteins during IgG-depletion procedure might play an important role in the neutralizing capacity of SLE patients against DENV. Thus, the possibility of other antibodies involvement in the neutralization effects of SLE-sera against DENV could not be excluded.

There was no association between the dengue-specific IgM ELISA levels with the neutralization percentage of SLE patients against DENV. Unfortunately, we did not examine the level of IgE antibody in this study, which was reported to be upregulated in the sera of dengue fever patients during the convalescent phase (AbuBakar *et al.*, 1997). IgE antibody was also prevalent among SLE patients and found to be associated with the activity of the disease (Charles *et al.*, 2011; Dema *et al.*, 2014; Henault *et al.*, 2016). These earlier findings may raise the possibility that IgE might be involved in the neutralization effects against DENV by SLE-sera. Previous study on the sera of HIV-infected children via maternal transmission showed significant inhibition of HIV in the cell culture due to the cytotoxic effects of the anti-HIV-1 IgE antibody (Pellegrino *et al.*, 2002), hence supporting the role of IgE in the antiviral responses. Furthermore, hyper-IgE condition in the serum of HIV patient conferred protection against HIV replication, postulated by maintaining low viral burden in the patient (Seroogy *et al.*, 1999). In addition to HIV, IgE was also found to be important in the antiviral response against Parvovirus B19 infection (Bluth *et al.*, 2005). Nevertheless, the mechanisms on how IgE confer antiviral responses are still uncertain. The antiviral effects might be mediated by the IgE-induced mast cells activation (Pandey *et al.*, 2004). Mast-cell deficient mice showed a higher titer of virus on the skin inoculation site and longer bleeding time than the WT mice, suggesting the role of mast cells in limiting DENV (Chu *et al.*, 2015). Moreover, mast cells not only induced mediators such as histamine, proteases and inflammatory cytokines, it also instigated antiviral chemokines including CCL3, CCL4 and CCL5, which are important for recruitment of NK cells, T cells and monocytes (Brown *et al.*, 2012; Graham *et al.*, 2015; John *et al.*, 2011). Though it is possible that IgE antibody may be involved in the neutralization effect of SLE-sera against DENV, we were unable to verify the theory due to the limited samples.

The specificity of the neutralization effects of SLE-sera towards DENV was also examined by replicating the experiment on JEV, a virus of the same genus and had also caused an endemic infection in Malaysia (Wong *et al.*, 2008). Our data showed no neutralizing capacity by SLE-sera against JEV (Figure 4.13). This data is comparable to a study demonstrating low cross-neutralizing capacity between DENV and JEV, due to the less antigenic homology between these viruses (Kimurakuroda *et al.*, 1986). This finding may clarify the preference of SLE-sera to recognize antibody- recognition sites on DENV but not JEV.

The protective effects of SLE patients in another virus infection such as HIV were also observed previously. A study investigating 400 HIV-infected SLE patients showed that only 5% of them displayed both autoimmunity and HIV infection simultaneously, suggesting the possible protective mechanism by the earlier established autoimmune disease against HIV manifestation (Chang *et al.*, 1999; Diri *et al.*, 2000b; Fox *et al.*, 1997b). SLE patient was postulated to confer protection against HIV via broadly HIV-1 neutralizing antibodies (bNAbs), where HIV-infected SLE patient was postulated to readily produced bNAbs than those without autoimmune disease, observed from the more controlled virus load in the HIV/SLE patient in comparison to those that were non-SLE (Bonsignori *et al.*, 2014). Moreover, a few other reports also confirmed that bNAbs are one of the promising prospects for HIV prevention (Bruel *et al.*, 2016; Cheeseman *et al.*, 2017; Zhang *et al.*, 2016). Intriguingly, the mapping of DENV E DIII for binding to the bNAbs suggested flexible and broader recognition of two bNAbs, 4E11 and 4E5A, on all serotypes of DENV (Frei *et al.*, 2015). ADCC, typically produced by patients with an immune tolerance defect, including SLE (Kumagai *et al.*, 1981; Miller *et al.*, 1983; Penning *et al.*, 1984), also showed protection against DENV via early inhibition against virus replication (Laoprasopwattana *et al.*, 2007). ADCC that was produced as early as 3 weeks after HIV infection (Pollara *et al.*, 2010) showed an inverse correlation with plasma

virus RNA (Sun *et al.*, 2011) and associated with the decreased mortality in HIV-infected infant (Milligan *et al.*, 2015). ADCC also protects the spreading of the HIV from mother to the infant (Ljunggren *et al.*, 1990). A protein produced abundantly in SLE patients, cytokine IL-16, showed inhibition against HIV (Center *et al.*, 1996a) while anti-RNP displayed 70-99% neutralization capacity against HIV (Douvas *et al.*, 1996). Anti-phospholipid monoclonal antibody isolated from SLE patients released soluble chemokines that inhibit the entry of HIV, hence reduced virus infection (Moody *et al.*, 2010). These evidences of virus inhibition and alleviated manifestation of HIV by SLE patients' efficient antiviral responses and autoreactive antibody production imply the possibility that the rare incident of severe dengue among SLE and their neutralizing capability against DENV may also due to the similar mechanisms.

In summary, this study showed the neutralization effects against DENV by dengue serology negative SLE patients. The neutralizing capacity of SLE-sera depends on the dengue specific IgG antibodies and possibly other isotypes or cross-reacting antibodies. The protection conferred by SLE patients against DENV, which possibly reflects those shown in HIV, promised a better understanding on the potential antibody-therapeutic strategies against DENV. *In-vitro* DENV inhibition of SLE-sera in this study, however, required more refined experiments in the future to further understand the autoimmunity factors conferring protection against severe dengue. Monoclonal antibody isolated from the SLE patient could provide information on the specific binding required for effective DENV neutralization. In addition, research on the B-cells of autoantibodies in SLE patient also helps to determine the antibodies origin that may lead to an efficient neutralization of DENV. The antiviral effects of IgE antibody and autoantibodies-derived SLE, including ADCC, anti-RNP and anti-phospholipids may provide beneficial insight in limiting DENV acquisition. *In vivo* observations on the protection of these SLE-derived antibodies against DENV infection and the development of severe dengue are

also required. Altogether, the protection of autoimmune responses against dengue might be an additional platform for the development of effective therapeutic antibodies or vaccines against DENV.

We then took another approach in understanding the association between autoimmunity and dengue, by investigating the involvement of HMGB1, a protein commonly linked to inflammatory reactions, in the pathogenesis of dengue. HMGB1 is involved in the pathogenesis of SLE disease due to the activation of macrophage inflammatory activity (Li *et al.*, 2015; Lu *et al.*, 2015a; Pisetsky, 2014). Owing to the fact that HMGB1 contributes in the disease activity of SLE with high specificity, it is identified as one of the SLE biomarkers (Schaper *et al.*, 2017) and a therapeutic target for SLE (Pan *et al.*, 2010; Urbonaviciute *et al.*, 2011). Upregulation of HMGB1 in the serum of dengue patients leads to the deduction that HMGB1 also plays a role in the pathogenesis of dengue (Allonso *et al.*, 2013). The activation of host immune responses via HMGB1 occurs once the protein was released out from the nucleus and binds to RAGE, TLR2 or TLR4 receptors (Lotze *et al.*, 2005). Typically, extracellular HMGB1 caused over-activation of the immune response that postulated to be the 'culprit' in the broad-range of infectious, inflammatory and malignant diseases, hence, the label of 'danger molecule' (Hosakote *et al.*, 2016; Štros, 2010; Yang *et al.*, 2013).

In contradictory, our results showed higher DENV replication in HMGB1-depleted cells (Figure 4.15), suggesting the positive role of the protein in the antiviral response against DENV. Previous studies showed that HMGB1 may facilitate or block virus replication (Cassetta *et al.*, 2009; Jung *et al.*, 2011; Moisy *et al.*, 2012; Naghavi *et al.*, 2003; Yu *et al.*, 2016). HMGB1 in the nuclei was found to bind to the nucleoprotein (NP) of influenza virus and promotes virus replication (Moisy *et al.*, 2012). Studies on the effects of HMGB1 on hepatitis C virus replication showed mixed results, where a report displayed that HMGB1 released from virus-infected cells block virus infection (Jung *et al.*, 2011),

whereas another report showed that extracellular HMGB1 interacts with stem-loop 4 in the viral 5'UTR of hepatitis C virus and facilitates its replication (Yu *et al.*, 2016). Extracellular HMGB1 exhibits inhibition against virus replication of R5 and X4 HIV-1 strain (Cassetta *et al.*, 2009), while intracellular HMGB1 blocks HIV-1 replication by suppressing viral LTR-mediated transcription (Naghavi *et al.*, 2003).

In our study, we examined the effects of HMGB1 depletion on the type-1 interferon signalling pathways, one of the early host defence against virus infection (Nasirudeen *et al.*, 2011). It is notable to highlight the increase of IFN-β mRNA level in HMGB1-depleted cells (Figure 4.16A). Moreover, the expression of Stat-1, an upstream protein induced by type 1-IFN responses, also showed an upregulation in the HMGB1-knockdown cells when compared to the wild-type (Figure 4.16B). The increase of virus replication in the HMGB1-knockdown cells did not complement with the high expression of IFN-β and Stat-1, suggesting that the knockdown of HMGB1 negatively altered the antiviral effects of the upstream of type-1 IFN responses. There is also a possibility that the high expression of the non-neutralizing IFN-β and Stat-1 in the HMGB1-depleted cells were triggered by the virus, observed from the non-augmentation of Stat-1 protein expression in IFN-β-induced HMGB1-depleted cells when compared to the wild-type (Figure 4.16C). Nevertheless, additional experiments need to be carried out to further confirm these theories.

We also determined the effects of HMGB1 depletion on the expression of ISGs including MxA, ISG56 and ISG15. Data showed that the expression of these proteins was affected by the depletion of HMGB1 in the cells during DENV infection, suggesting the regulation of ISGs by HMGB1 in the antiviral responses against DENV (Figure 4.17). ISGs could directly block any steps in a virus replication and induce steady and robust antiviral activity by further inducing other ISGs (Schneider *et al.*, 2014; Schoggins *et al.*, 2011). MxA exerts the antiviral responses at the early step of influenza A virus replication by

blocking the synthesis of viral mRNA (Haller *et al.*, 2015). *In vivo* study showed that the mutant mice carrying human MxA were protected from lethal Thogoto virus and avian influenza A infection (Deeg *et al.*, 2013). ISG56, another ISGs protein, suppressed the internal ribosome entry site and inhibits HCV replication (Raychoudhuri *et al.*, 2011). ISGylation substrates produced by ISG15 caused loss of the viral protein function (Skaug *et al.*, 2010).

Our data suggested the importance of the nuclear HMGB1, via inhibition of HMGB1 translocation process using RESV. Our study in verifying the inhibition of HMGB1 translocation out of the nucleus by RESV treatment (Figure 4.19) was comparable with the earlier studies (Xu *et al.*, 2014). DENV replication was reduced in the RESV-treated cells (high level of nuclear HMGB1) (Figure 4.20) and the observation of increased MxA and ISG56 mRNA levels confirmed the influence of nuclear HMGB1-induced antiviral responses on the expression level of the ISGs (Figure 4.21). It is noteworthy to mention the increase of IFN-β in the RESV-treated cells, signifying the regulation of type 1-IFN response by HMGB1 in the nucleus. Thus, we concluded the novel role of HMGB1 in the regulation of type 1-IFN response against DENV through RESV treatment (Zainal *et al.*, 2017). Previous studies showed that RESV possessed the antiviral activity against a wide-range of different viruses (Abba *et al.*, 2015; Berardi *et al.*, 2009; Campagna *et al.*, 2010; Drago *et al.*, 2008). However, the exact mechanism of the virus inhibition is still uncertain. A few mechanisms of RESV-mediated antiviral effects have been proposed, including blocking of NF-κB pathways (Holmes-McNary *et al.*, 2000; Kundu *et al.*, 2006), nucleotide reductase action inhibition (Liu *et al.*, 2014), blocking of EGR-1 (Evers *et al.*, 2004; Wang *et al.*, 2004c), interference of the autophagy pathways (Morais *et al.*, 2009) and sirtuins manipulation (Zhang *et al.*, 2009). Present study showed that the inhibition of HMGB1 migration out of the nucleus via RESV treatment was mediated by Sirt1. This finding is in parallel with an earlier study that suggested the induction of Sirt1

by RESV leads to deacetylation of HMGB1, constricting the migration of HMGB1 out of the nucleus (Xu *et al.*, 2014; Yang *et al.*, 2015). Examination of Sirt1-knockdown cells showed low level of nuclear HMGB1 (Figure 4.19), decreased antiviral effects against DENV (Figure 4.23) and reduced ISGs induction (Figure 4.24), which further validated the antiviral effects of nuclear HMGB1 via its regulation on the expression of ISGs. The increased virus titration in shSirt1, even when there was high level of HMGB1 in the cytoplasm and extracellular milieu (Figure 4.19), implied that there was probably no direct inhibition of HMGB1 against DENV. We also confirmed that the antiviral effects of RESV were dependent on the presence of HMGB1 and Sirt1 (Figure 4.25). Our ChIP assay demonstrated binding of HMGB1 to the promoter region of an ISG, MxA, during DENV infection (Figure 4.26). Moreover, Sirt1-knockdown cells, which associated with the low nuclear HMGB1, showed reduced binding activity between HMGB1 and the promoter of MxA (Figure 4.26A). Thus, we postulated that HMGB1 transcriptionally stimulates the expression of MxA. It should be noted, however, that our data do not verify the direct binding of HMGB1 with DNA, thus the possibility that HMGB1 binds to another protein that also binds DNA, such as transcription factor protein, cannot be excluded. Overall, we concluded that the accumulation of HMGB1 in the nucleus by RESV treatment via induction of Sirt1, promotes the expression of ISGs, such as MxA, hence augmenting the antiviral response (Figure 5.1).

Extensive researches on vaccine production, antiviral development, and vector management have been done to prevent dengue. Yet, fatal cases of dengue are still increasing. Approval of the new DENV vaccine showed a promising progress, however, the weak efficacy of the vaccine remains a concern (Wilder-Smith *et al.*, 2015). The obstacles in the development of a vaccine or therapeutic antiviral against DENV are due to the complex relationship between the eradication of the virus and the hyperactivity of the host immune response. Specifically, the immune response induced for the efficient

elimination of the virus might be excessive and inflicted injuries to the host cells through mimicry. Therefore, strategies in achieving the perfect balance in regulating these two factors, which still unknown, are critically required to limit fatal dengue.

RESV, known as an anti-inflammatory agent, might possess another benefit in addition to its property as an antiviral response against DENV. HMGB1 that translocated out of the nucleus (extracellular HMGB1) binds to RAGE, TLR2 or TLR4, which then activates NF-κB and its signalling pathway and eventually multiples pro-inflammatory genes (Lotze *et al.*, 2005). Previous study suggested that extracellular HMGB1 contributes to vascular endothelial leakage during DENV infection (Ong *et al.*, 2012). Extracellular HMGB1 was also involved in the disease activity of SLE through the activation of macrophage inflammatory responses (Lu *et al.*, 2015a). Therefore, the inhibition of HMGB1 translocation out of the nucleus via RESV treatment may assist in the decreased activation of pro-inflammatory genes in dengue, thus preventing the induction of the vascular leakage and haemorrhage, the hallmarks of severe dengue. This theory is in agreement with an earlier study suggesting that RESV suppresses the NF-κB and JAK/STAT signalling pathways via the inhibition of HMGB1 release (Ma *et al.*, 2015). Moreover, RESV was found to be an effective resuscitation fluid due to its capability to improve the survival and prolongs life of hemorrhagic injured rat (Ayub *et al.*, 2015). We postulated the dual benefits of RESV in dengue via manipulation of HMGB1 translocation pattern in the cell. As an antiviral agent for DENV, RESV increased the nuclear HMGB1, a protein that will then positively regulate the activation of ISGs in the nucleus, hence leads to an efficient antiviral response against DENV. As an anti-inflammatory reagent, RESV reduced the release of HMGB1 as well as the pro-inflammatory reactions activated by extracellular HMGB1, hence preventing the injuries against host cells or tissues by the extreme activation of immune response.

Our study is limited by the fact that we only validated the efficiency of antiviral effects against DENV by HMGB1 using gene depletion cells and RESV treatment in an *in-vitro* environment. We did not, however, observe the prevention or alleviation of vascular leakage during DENV infection via HMGB1 translocation inhibition using RESV treatment. Accordingly, the comparison on the level of HMGB1 protein in the serum of SLE patients, dengue positive and healthy individuals may also provide interesting results on the possibilities of HMGB1 mediating the severity of dengue disease. Furthermore, *in-vivo* studies on both antiviral efficiency and anti-inflammatory effects against dengue by HMGB1 using RESV-treated cells and gene knockdown methods are also required. There were numerous *in vitro* an *in vivo* studies of RESV and its benefits in anti-inflammatory, antiviral responses and cancer, but little information on its effects in human and none of the antiviral responses against DENV (Tomé Carneiro *et al.*, 2013b). The bioavailability of RESV have been deemed low (Goldberg *et al.*, 2003; Nunes *et al.*, 2009; Walle *et al.*, 2004), but a few efforts in improving the bioavailability of the compound have been made (Amri *et al.*, 2012; Santos *et al.*, 2011). Most of the RESV study on humans showed no adverse effects on the hematological profile, hepatic, thyroid and renal complication (Tomé Carneiro *et al.*, 2012a; Tomé Carneiro *et al.*, 2012b; Tomé Carneiro *et al.*, 2013a). Moreover, the *in vitro* study showed that the cell viability of human breast adenocarcinoma (MDA-MB 231), human cervical cancer (HeLa) and Chinese hamster lung fibroblast (V79) cells remains high at 60% even at 400 µM RESV concentration (Anlar *et al.*). Nevertheless, a substantial amount of works, especially in determining the safety of RESV, as well as the antiviral and anti-inflammatory effects of the compound against DENV and its severe manifestation on animal and human are required. Although with limitation, our study introduces a novel role of HMGB1 in the host antiviral responses against DENV and provides an insight in the development of safe antiviral and therapeutic agents for dengue.

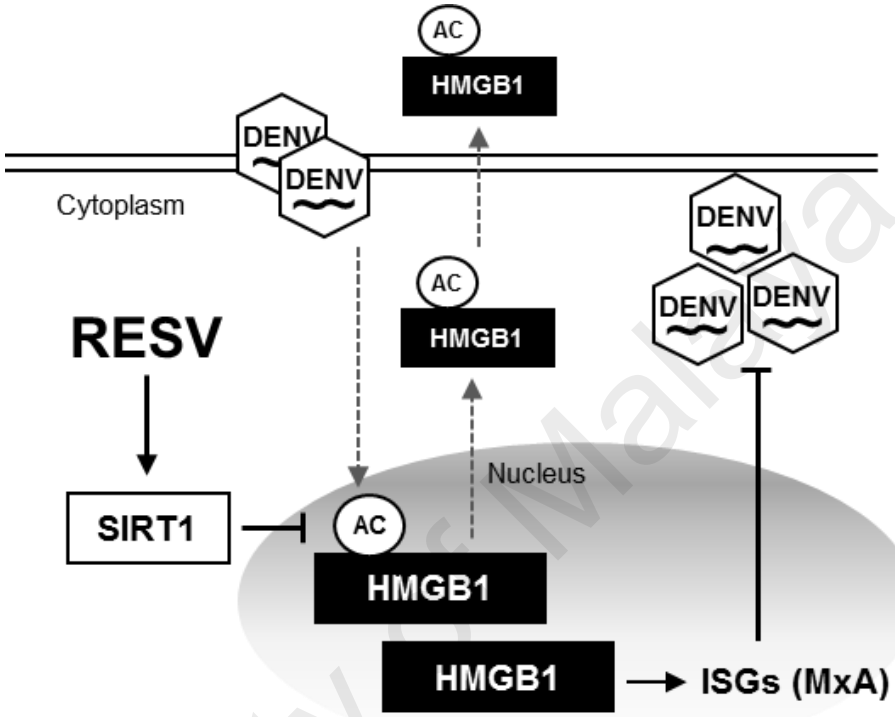


Figure 5.1: Model of HMGB1 and its role in DENV infection. DENV infection induces the acetylation (AC) of HMGB1 in the nucleus, which then leads to the release of HMGB1 into the cytoplasm and extracellular milieu. RESV treatment stimulates production of Sirt1, causing deacetylation of HMGB1. Deacetylation of HMGB1 decreases the translocation process and increases the accumulation of HMGB1 in the nucleus. The higher accumulation of nuclear HMGB1 enhances the production of ISGs, leading to an efficient antiviral response in the cell.

CHAPTER 6: CONCLUSION

Novel variant at chr3:49679737 of BSN gene showed an association with the susceptibility to severe dengue, while polymorphisms rs76018112 (chr6:30558477) of ABCF1 gene, rs1557370 (chr21:42830690) of Mx1 gene and rs945635 (chr1:157670290) of FCRL3 displayed an association with the protective effects against severe dengue. Unexpectedly, SLE patients showed significantly high distribution of all variants linked to protective effects against severe dengue, suggesting the protective association of autoimmune phenotype against acute dengue development. This result correlates with the protective effects of SLE patient's sera against DENV observed in the second experiment of our study. These findings suggest the importance of early antiviral response and efficient virus clearance in preventing the development of severe dengue. This is in agreement with the previous study that showed a relationship between the virus clearance rate and severe dengue (Ben-Shachar *et al.*, 2016; Tricou *et al.*, 2011; Wang *et al.*, 2006). However, we cannot overrule the fact that inflammatory effects of excessive immune molecules may lead to the severity of dengue, as indicated in a few earlier studies (Chaturvedi *et al.*, 2000; Chousterman *et al.*, 2017; Chuang *et al.*, 2014; Pang *et al.*, 2007; Srikiatkhachorn *et al.*, 2017). Taken both factors into account, we postulated that there is a particular time-point threshold that the virus needs to be cleared, in order to prevent the development of severe manifestation of dengue. Correspondingly, previous study showed that delayed virus clearance leads to prolonged stimulation of $CD8^+$ T cells and resulted in high level of cytokines and pro-inflammatory molecules, the main elements in disruption of the vascular barrier (Wang *et al.*, 2006). Hence, those that have the genetic capability to produce early, efficient antiviral responses that leads to rapid virus clearance may be protected, while those without the similar genetic capability might be susceptible to severe dengue. Nevertheless, more studies need to be conducted to comprehend the optimum rate of virus clearance and its underlying mechanisms that may confer

protection against the development of severe dengue. We also disclosed the novel role of HMGB1 in regulating innate immune response against DENV using RESV, a suitable platform for the antiviral development of DENV. In addition, RESV also acts as an anti-inflammatory agent that capable to reduce the extracellular HMGB1 levels, signifying the possible dual beneficial effects of the compound against dengue.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Article in Academic Journals

1) **Zainal, N**., Chang, C. P., Cheng, Y. L., Wu, Y. W., Anderson, R., Wan, S. W., Chen, C. L., Ho, T. S., AbuBakar, S., and Lin, Y. S. (2017). Resveratrol treatment reveals a novel role for HMGB1 in regulation of the type 1 interferon response in dengue virus infection. *Scientific Reports, 7*, 42998.

2) **Zainal, N.**, Tan, K. K., Johari, J., Hussein, H., Wan Musa, W. R., Hassan, J., Lin, Y. S., AbuBakar, S. Sera of patients with systemic lupus erythematosus cross-neutralized dengue viruses. (**manuscript submitted**)

3) Mohd, A., **Zainal, N.**, AbuBakar, S. Antiviral activity of resveratrol against Zika virus replication. (**manuscript to be submitted)**

Abstracts and Proceedings

1. **Zainal, N.**, Chang, C. P., Cheng, Y. L., Wu, Y. W., Anderson, R., Wan, S. W, Chen, C. L., Ho, T. S., Abubakar, S., Lin, Y. S. (2017). Resveratrol treatment reveals a novel role for HMGB1 in regulation of the type 1 interferon response in dengue virus infection. Asia Dengue Conference, Dengue: New Challenges, New Strategies, JW Marriott Kuala Lumpur, Malaysia, 23-24 April 2016.

2. Tan, K. K., **Zainal, N.**, Abd-Jamil, J., Azizan, N. S., Yaacob, C. N., Che Mat Seri, N. A. A., Samsudin, N. I., Mahfodz, N. H., AbuBakar, S. (2017). Emergence of dengue virus type 3 genotype I associated with upsurge of DENV3 cases in Klang Valley, Malaysia. 17th International Congress of Virology, IUMS 2017, Sands Expo & Convention Centre, Singapore, 17-21 July 2017.

3. Tan, K. K., Johari, J., Abd-Jamil, J., Zulkifle, N. I., Sulaiman, S., **Zainal, N.**, Ab.Rahman, H. A., Sam, S. S., Teoh, B. T., AbuBakar, S. (2012). Comparison of Real Time Reverse Transcription-Polymerase Chain Reaction Assays for the Detection of Dengue Virus. Singapore International Conference on Dengue and Emerging Infections, Grand Copthorne Waterfront, Singapore, 21-23 November 2012.

4. Ab-Rahman, H. A., **Zainal, N.**, Jee, P. F., Tan, K. K., Teoh, B. T., Sam, S. S., Danlami, M. B., NorAmdan, A. S., Shu, M. H., AbuBakar, S. (2012). Optimization of Burkholderia pseudomallei Antigenic Protein Expression in

Escherichia coli. 31st Symposium of the Malaysian Society for Microbiology, Promenade Hotel, Kota Kinabalu, Sabah, Malaysia, 13-15 December 2012.