Altered responses of Dengue virus infected cells to TNF-α and induction of GRP78 and HSP70 – *in vitro* studies

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ABSTRACT

Dengue virus (DENV) infection of humans is characterised by immunopathology with elevated levels of many inflammatory mediators. Tumour necrosis factor alpha (TNF-α) plays a significant role in the pathogenesis of DENV infection with elevated levels of TNF-α in the sera of DENV infected patients that parallel the severity of disease and release of TNF-α coincident with the peak of DENV production from infected monocyte-derived-macrophages (MDM) *in vitro*. However, the effect of TNF-α on DENV replication is not fully clarified. In this study we aimed to determine (1) the effect of TNF-α on DENV replication and (2) the changes in host cell protein expression, in response to DENV-infection. Since macrophages are a primary cell target *in vivo* for DENV-infection, this study mainly used primary monocyte-derived-macrophages (MDM) and macrophage-like cell lines (K562, U937) to represent this cell type. Initially methods were developed for specific analysis of DENV replication, including a tagged RT-PCR method for quantitation of DENV positive (+ ve) and negative (- ve) strand RNA.

Next the potential antiviral role of TNF-α in regulating DENV replication in MDM was investigated. While pre-treatment of MDM with TNF-α had a minor inhibitory effect, addition of TNF-α to MDM with established DENV-infection had no effect on DENV replication as measured by DENV RNA levels or virion production. Blocking endogenous TNF-α using TNF-α antibodies or TNF-α siRNA also had no effect on infectious DENV production or RNA synthesis. Together, these results demonstrate that DENV replication in MDM is not affected by TNF-α. Additionally, normal cellular TNF-α signalling, measured by quantitation of TNF-α-induced stimulation of transcription from a nuclear factor-kappa B (NF-kB) responsive reporter plasmid or NF-kB protein nuclear translocation, was blocked in DENV-infected MDM. Thus, DENV replication in MDM is not affected by TNF-α, and infected cells do not respond normally to TNF-α stimulation. It is therefore unlikely that the increased production of TNF-α seen in DENV-infection and correlating with DENV pathology contributes directly to DENV clearance by inducing anti-viral defence mechanisms and reducing DENV replication in MDM. These results also highlight an example of viral subversion of potential anti-viral cellular responses.

Secondly, the host cell response to DENV-infection was analysed, presenting the first proteomic analysis on the cellular response to DENV-infection. The differential proteomes of K562 cells with or without DENV infection were resolved and quantitated with two dimensional differential gel electrophoresis (2D PAGE). One 72 kDa protein, was identified by mass spectrometry to be GRP78 (a member of HSP70 protein family) and was up-regulated 2 to 3 fold in infected cells. Up-regulation of GRP78 in DENV-infected cells was confirmed by immuno-staining and confocal microscopy. GRP78 and HSP70 have previously been identified as a component of the DENV receptor complex and blocking of these proteins has been found to inhibit DENV entry into the cell. By confocal microscopy we found that cytoplasmic GRP78 and HSP70 were also up-regulated in DENV-infected cells. The role of cytoplasmic GRP78 and HSP70 in DENV-infected cells has not been established; however there are precedents in other viral infections that cytoplasmic GRP78 and HSP70 could enhance viral protein production.

Thus, this thesis shows that (1) the high levels of circulating TNF-α seen in DENV-infection does not influence DENV replication (2) the cellular responses to TNF-α are altered in DENV-infected cells and (3) we have identified two protein chaperones and stress response proteins (GRP78 and HSP70) that are up-regulated during DENV-infection. With the advancement in proteomic techniques since initiation of this project future proteomic analysis could further identify other novel host factors that may either regulate DENV-infection or be involved in a host cell response to DENV-infection and help our understanding of DENV pathogenesis at the protein level.

DECLARATION

This work contains no material that has been accepted for the award of any degree or diploma in any university or other tertiary institution and to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

In accordance with the University of Adelaide regulations, I give my consent to this thesis being made available for photocopying and loan if accepted for the award of the degree.

Satiya Wati

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ABBREVIATIONS

2D – 2 dimensional

^{o}C – degrees Celsius

g – (units) gravity force

(+ ve) - positive

(- ve) - negative

approx - approximately

ALT – alanine aminotransferase

AST – aspartate aminotransferase

ATCC – American Type Culture Collection

ATF6 – activating transcription factor 6

BME – Basal media Eagle

bp – base pairs

BSA – bovine serum albumin

BVDV – bovine viral diarrhea virus

C - complement

CAP - capsid

CPE – cytopathic effect

CS – cyclisation sequence

CT – cycle threshold

DENV – dengue virus

DEPC - diethylpyrocarbonate

DF – dengue fever

DHF – dengue haemorrhagic fever

dH_20 – de-ionised water

DMEM – Dulbecco modified Eagle medium

DNA – deoxyribonucleic acid

dNTP – 2'- deoxynucleoside 5'-triphosphate

ds – double stranded

DSS – dengue shock syndrome

DTT - dithiothreitol

E - the envelope glycoprotein

EDTA – ethylene diamine-tetra-acetic acid

ELISA – enzyme linked immunosorbent assay

ER – endoplasmic reticulum

FBS – fetal bovine serum

FITC – fluorescein isothiocyanate

GRP78 – glucose regulated protein 78

HBBS – Hanks balanced salts

HBBS+ - Hanks balanced salts with $CaCl_2$ and $MgSO_4$

HBV – Hepatitis B virus

HCL – hydrochloric acid

HCV – hepatitis C virus

HIV – human immunodeficiency virus

HCMV – human cytomegalovirus

HUVEC – human umbilical vein endothelial cell

hNRBP - human nuclear receptor binding protein

hr – hour (s)

HSP – Heat shock protein

HSV – Herpes Simplex virus

IEF – Isoelectric focussing

IgG – immunoglobulin G

IL - interleukin

INF- Interferon

IRE1 – ER trans-membrane protein kinase/endoribonuclease

JE – Japanese encephalitis virus

kb – kilobase

kDa - kilodaltons

LPS - lipopolysaccharides

LUC - luciferase

M – membrane protein

MDM – monocyte derived macrophages

MEM – minimum essential medium

min – minute (s)

ml - millilitre

mM – millimolar (millimoles per litre)

MOI - multiplicity of infection

mRNA – messenger RNA

MS – mass spectrometry

MW – molecular weight

NF-kB – nuclear factor-kappa B

ng - nanogram

NMR - nuclear magnetic resonance

NO – Nitric oxide

NS– non structural proteins

OD – optical density

oligo - oligonucleotide

O/N - overnight

ORF – open reading frame

PAGE – polyacrylamide gel electrophoresis

PBS – phosphate buffered saline

PBMC – peripheral blood mononuclear cells

PCR – polymerase chain reaction

PERK – PKR-like endoplasmic kinase

pfu – plaque forming units

pg - picograms

PMA – phorbal myristate acetate

pmol - picomoles

PrM- the precursor to the membrane protein

RF – replicative form

RI – replicative intermediate

RNA – ribonucleic acid

RT – reverse transcription

SDS – sodium dodecyl sulphate

sec – second (s)

siRNA - small interfering RNA

SRBC – sheep red blood cells

ss – single stranded

TNF-α - tumor necrosis factor alpha

μg – microgram

μM – micromolar (micromoles per litre)

UPR – unfolded protein response

UTR – untranslated region

MW – molecular weight

v/v – volume per volume

WHO – World Health Organisation

w/v – weight per volume

WNV – West Nile virus

PUBLICATIONS AND PRESENTATIONS ARISING

PUBLICATIONS

2007 **Satiya Wati,** Peng Li, Christopher Burrell, Jillian Carr. **Dengue virus (DV) replication in monocyte derived macrophages is not affected by TNF alpha, and DV infection induces altered responsiveness to TNF alpha stimulation.** *Journal of Virology* 81 (18):10161-10171

CONFERENCE PRESENTATIONS

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The role of exogenous TNF alpha in dengue virus replication

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Thesis Amendments

Abbreviations – pxii should include CT - cycle threshold

Chapter 1 – Introduction
Section 1.2.2 p2 should read Since then epidemic outbreaks have been restricted to North Queensland and the Torres Strait islands and a summary of DENV outbreaks reported in Queensland in the last ten years to date is summarised in Table 1.1.

Section 1.4 p6 should read The *Flaviviridae* family contains three genera: (1) *Flavivirus* (e.g. dengue virus, yellow fever virus, Japanese encephalitis virus (JEV) and tick borne encephalitis virus.

Section 1.4 p6 should read Members of the *Flavivirus genus* are distinguished by presence of a type I cap structure (m7GpppAmp) at the 5' end of the genome, a highly structured 3' untranslated region (UTR) (Brinton and Dispoto, 1988) and by the absence of a 3'-terminal poly (A) tract (Chambers *et al.*, 1990).

Section 1.5 p7 DENV genome comprises of approximately 10,600 nucleotides.

Section 1.5.1 p7 should read.....the DENV poly-protein includes viral serine proteases (NS2B-NS3) and host cellular proteases.

Section 1.6.1.1 p10 should read Heparan sulphate and GRP78 (BiP) .....

Section 1.8.2.4 p26 should include TNF-α also has been implicated in transiently changing permeability of the blood-brain barrier and hence allowing West Nile virus to cross the central nervous system (Wang *et al.*, 2004).

Chapter 2 – Materials and Methods
Section 2.6.7.2 p48 should include DENV anti-mouse monoclonal antibodies were used instead of DENV positive patient sera in K562 and macrophages due to high background issues.

Chapter 6 – General and Discussion
Section 6.1 p86 should read *In vitro,* TNF-α release coincides with the peak of DENV production from infected MDM (Carr *et al.*, 2003) and high levels of TNF-α are released from other cells of the immune system such as B and T cells when exposed to DENV (Lin *et al.*, 2002b; Mangada *et al.*, 2002; Mangada and Rothman, 2005).

References should include

Wang, T., Town, T., Alexopoulou, L., Anderson, J. F., Fikrig, E. and Flavell, R. A. (2004) Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. Nat Med 10(12), 1366-1373

CHAPTER 1

INTRODUCTION

1.1 DENGUE VIRUS (DENV)

Dengue virus (DENV), is a single stranded (ss) RNA virus of the family *Flaviviridae* (Henchal and Putnak, 1990) and is the most common cause of arboviral (arthropod borne) disease in the world. DENV-infection causes a range of syndromes from an undifferentiated fever referred to as dengue fever (DF), to the potentially fatal dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). DENV exists as four antigenically related but distinct serotypes, designated DENV-1, DENV-2, DENV-3, and DENV-4, any of which can cause any form of the disease. Infection with one serotype provides life-long immunity to the homologous serotype but does not provide immunity to the heterologous serotypes. The more severe forms of the disease, DHF/DSS, are predominantly, but not exclusively, associated (Scott *et al.*, 1976; Morens *et al.*, 1987; Martinez *et al.*, 1993) with secondary infection of a different serotype to that type which caused the primary infection (Burke *et al.*, 1988; Vaughn *et al.*, 2000).

1.2 EPIDEMIOLOGY

In the past 25 years there has been a huge global increase in epidemic dengue, including more frequent and larger epidemics associated with severe forms of the disease (Mackenzie *et al.*, 2004). This global pandemic has been attributed to major demographic and societal changes resulting in both virus and vector being distributed worldwide (Mackenzie *et al.*, 2004). Unprecedented population growth, increased movement of virus through modern transportation, global climate warming and lack of effective mosquito control, have all contributed to the marked increase in epidemic activity.

1.2.1 Worldwide distribution of DENV and threat to public health

Humans and mosquitoes are the principal hosts of DENV. The virus is only known to cause illness in humans and is transmitted by bites from *Aedes sp* mosquitoes. The most common vector is the domestic mosquito *Aedes aegypti* that is closely associated with human habitation and is found in tropical and sub-tropical regions (35^{o}N – 35^{o}S, latitudes) (Gibbons and Vaughn, 2002) (Fig 1.1). Due to increased incidence and geographical distribution of

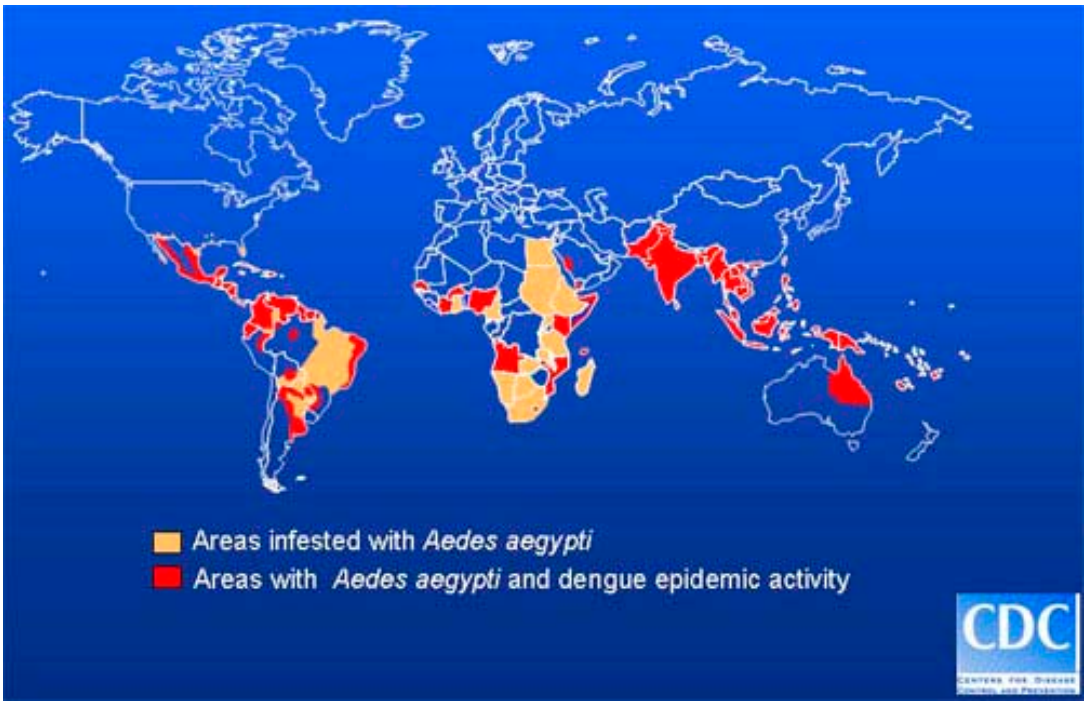


Fig 1.1. World distribution of the predominant dengue mosquito vector (*Aedes aegypti*) and areas with dengue epidemic acitivity (www.cdc.gov).

DENV-infections in the last 50 years, DENV is being increasingly recognised as one of the world's major infectious diseases that represent a serious threat to public health (Mairuhu *et al.*, 2004). WHO reports an estimated 50-100 million cases of DF occur every year, with 500,000 patients developing the more severe disease, namely DHF, leading to hospitalisation and resulting in around 24,000 deaths. DENV-infection is a global problem which is also relevant to Australia (as discussed in section 1.2.2) and as there is no vaccine or current medical means to prevent infection, vector control and personal protection are the main control measures available. Preventing man-vector contact is a difficult task, and the predictions of potential climate changes, which are likely to increase the distribution of these mosquitoes (Hales *et al.*, 2002), suggest that an even larger proportion of the human population will be at risk in the future.

1.2.2 DENV prevalence in Australia

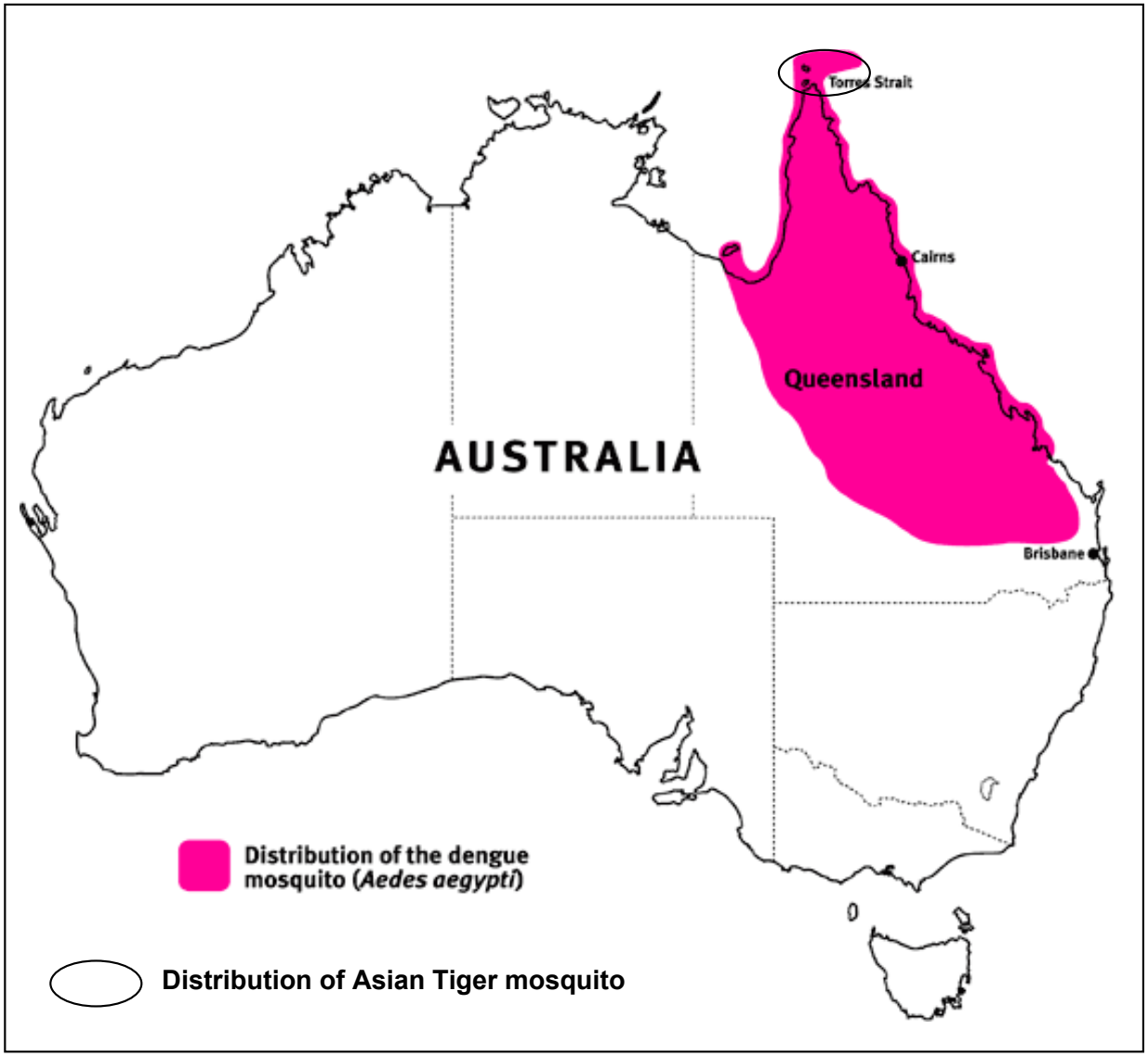
In Australia the first recorded outbreak of DF was in 1879 (McBride, 1999). The first fatality attributed to classical dengue occurred in Charters Towers in 1885 and the first fatality attributed to DHF occurred in the same town during the 1897 epidemic, where Hare (in 1898) recorded 60 fatalities (30 of those were children) (www.health.qld.gov.au). The largest outbreak reported so far occurred in Townsville/Charters Towers, the Torres Strait, and the Cairns/Mossman area of North Queensland in the early 1990's resulting in 1600 confirmed cases of dengue (www.health.qld.gov.au). Since then epidemic outbreaks have been restricted to North Queensland and the Torres Strait islands and a summary of DENV outbreaks reported in Queensland in the last ten years to date is summarised in Table 1.1.

Dengue and the mosquitoes *Aedes aegypti* (Fig 1.2B) are currently confined to the North Eastern part of Australia and the surrounding islands (www.health.qld.gov.au) (Fig 1.2A). In 2005 the presence of another dengue vector the *Aedes albopictus* (The Asian Tiger mosquito) was reported in the Torres Strait islands in the northern tip of Australia (www.health.qld.gov.au) (Fig 1.2C). *Aedes albopictus* is notorious for rapidly colonising new geographical areas including colder climates thereby posing further risk to vector and DENV spread to much larger parts of Australia.

Year	Location	Reported cases	DENV type
2008	Mossmon/Port Douglas	22	3
2007	Townsville	46	3
2006	Cairns/Townsville	37	2/3
2005	Townsville/Torres Strait Islands	74	4
2004	Torres Strait Island	1	2
2003-2004	Cairns/Townsville/Torres Strait Islands	892	2
2003	Cairns/Mareeba	9	1/2
2002	Kuranda/Townsville/Cairns	25	2/1/4
2001	Townsville	9	2
2000	Cairns	49	2
1997-1998	Cairns	12	2

Table 1.1. Dengue outbreaks reported in North Queensland in the last 10 years (adapted from www.health.qld.gov.au).

A



B

C

Fig 1.2. (A) Distribution of DENV and vector in Australia. **(B and C)** The two vectors *Aedes aegypti* and *Aedes albopictus.*(www.health.qld.gov.au/dengue/dengue_fever).

1.2.3 Vaccine development, prevention and control of DHF/DSS

Despite the likely increase in cases of DHF, there is no licensed vaccine available to protect against DENV-infection. However, there is currently a great focus on research into vaccine development, and an effective vaccine remains a global health priority. DENV vaccine development has been hindered by three major problems:

(1) The need to protect against all 4 DENV serotypes.

The observed association of more serious forms of DENV disease with secondary infections involving a heterologous serotype means that an effective vaccine will need to prevent infection with all four DENV serotypes and therefore require a complicated immunogen that induces high-level, long-lived immunity against all four DENV strains. Weak immune responses producing non-neutralising antibody would not be favourable as this may lead to serious forms of DENV pathology upon infection (due to ADE as discussed in section 1.8.2.1). Currently a number of live-attenuated tetravalent dengue virus vaccines containing each of the four serotypes are at clinical trial stages (http://clinicaltrials.gov). Meanwhile continual efforts are being made to develop alternate vaccine candidates such as chimeric (Guirakhoo *et al.*, 2000), DNA and inactive subunit vaccines (Kochel *et al.*, 2000; Konishi *et al.*, 2000; Raviprakash *et al.*, 2000). Table 1.2 shows a partial list of dengue vaccine candidates that are under development as reviewed in (Whitehead *et al.*, 2007).

(2) The lack of animal models to test the vaccine, as humans are the only hosts that manifest clinically apparent diseases (Rothman, 2004).

Non human primates (such as macaques, monkeys, baboons and chimpanzees reviewed in (Bente and Rico-Hesse, 2006) have been investigated as potential animal models. Even though these animals are susceptible to infection (showing detectable viremia and antibody response) they do not manifest clinically apparent or sub clinically detectable diseases as seen in humans.

Several mouse models have been described including BALB/c mice (Paes *et al.*, 2005), IFN α/β and IFN γ receptor gene knockout mice (AG129) (van den Broek *et al.*, 1995; Johnson and Roehrig, 1999; Shresta *et al.*, 2006) and the A/J mouse model (Huang *et al.*, 2000a), but again none mimic DHF/DSS symptoms as seen in humans and wild type viruses replicate to

Type	Vaccine developer	Clinical testing status
Live attenuated (Tetravalent)	WRAIR/ GSK Biologicals	Phase II
Live attenuated	Mahidol University/ Sanofi pasteur	No current testing
Live attenuated, chimeric (Monovalent)	NIAD,NIH	(DENV1-4), PhaseI/II
Live attenuated, chimeric (Tetravalent)	Acambis/Sanofi pasteur	Phase I
Live attenuated, chimeric	CDC/InViragen	Preclinical
Inactivated virus	WRAIR	Preclinical
Subunit	Hawaii Biotech	Begins 2007
DNA (Monovalent)	Navy Medical Research Centre	(DENV-1) Phase I

Table 1.2. A partial list of dengue vaccine candidates that are under development (reviewed in Whitehead *et al*., 2007).

very low titers therefore the use of mice as a true model for DENV in vaccine development is limited.

Use of immunodeficient SCID mice engrafted with human cells or cell lines such as HepG2 (An *et al.*, 1999) and K562 (Lin *et al.*, 1998) have led to sustained dengue viral infection. Immunodeficient NOD-SCID mice engrafted with human cord blood hematopoietic progenitor cells have also been found to be susceptible to DENV-infection showing signs of fever and thrombocytopenia (Bente *et al.*, 2005). However the main deficiency of these human engrafted models is lack of an immune response making them inadequate for human immunity studies.

Recently a new humanised RAG-hu mouse model has been reported to show productive DENV-infection associated with viremia, fever and generation of human anti-dengue antibodies (Kuruvilla *et al.*, 2007). An immunocompetent C57BL/6 mouse model has also been described, where 33% of mice infected with high titers of DENV exhibited haemorrhage at the subcutaneous tissue as seen in humans, but without the paralysis as normally seen in DENV-infected mice (Chen *et al.*, 2007). Thus these mice may have future potential use as an animal model in vaccine studies.

(3) Possibility of genetic drift.

Identification of rapid and dramatic changes in DENV sequences within the C, E, NS1 and NS2B genes suggests that DENV undergoes substantial sequence diversity (Sittisombut *et al.*, 1997; Wang *et al.*, 2002a; Wang *et al.*, 2002b; Wittke *et al.*, 2002), and therefore DENV vaccines may need to be regularly updated to remain active which would be a costly exercise (Aaskov, 2003).

Thus the designing of an ideal immunogen for dengue vaccination is a complicated process that would need to induce a high level of protection against all 4 DENV serotypes (without inducing more complex secondary infections due to the ADE phenomenon as discussed in section 1.8.2.1), to account for sequence diversity and provide life long immunity.

1.3 CLINICAL PRESENTATION

As discussed earlier, DENV-infection is characterised by a spectrum of disease ranging from clinically inapparent to death. The most common clinical manifestation is a non-specific febrile illness or classical DF. The incubation period for DF is usually 3 to 8 days. Infants and young children often have non-specific febrile illness with a maculopapular rash (Rigau-Perez *et al.*, 1998; McBride, 1999; Gibbons and Vaughn, 2002; Guzman and Kouri, 2002; Mairuhu *et al.*, 2004). Severe cases of DF are seen in older children and are characterised by an increase in temperature (≥ 39°C) for 5-6 days. The febrile period is accompanied by severe headache, retro-orbital pain, myalgia, arthralgia, nausea and vomiting. Patients generally recover after 7-10 days of illness. DHF is an acute febrile illness with haemorrhagic manifestations, thrombocytopenia (≤100,000 cells/mm^3) and evidence of an increased vascular permeability resulting in loss of plasma from the vascular compartment. When plasma leakage is so strong that shock occurs, it is referred to as DSS (WHO, 1997). About 250,000-500,000 cases of these latter two, more severe forms of disease, estimated to be about 5% of the DENV-infected population, are officially notified annually. WHO classification of symptomatic dengue infection is shown in Fig 1.3. The WHO case definition of DHF states that a patient must exhibit all four criteria to be classed as a DHF case.

These are: -

1. Acute sudden onset of high fever for two to seven days.
2. Haemorrhagic manifestations with at least a positive tourniquet test.
3. Platelet count <100 x 10^9/l.
4. Haemoconcentration (rising packed cell volume >20%) or other evidence of plasma leakage - for ascities, pleural effusions, low level of serum protein/albumin.

The severity of DHF is further classified into four grades according to the presence or absence of spontaneous bleeding and the severity of plasma leakage as follows: -

Grade I: Fever accompanied by non-specific constitutional symptoms; the only haemorrhagic manifestation is a positive tourniquet test and or/ easy bruising.

Grade II: Spontaneous bleeding in addition to manifestation of Grade 1 patients, usually in the forms of skin or other hemorrhages.

Grade III: Circulatory failure manifested by a rapid, weak pulse and narrowing of pulse pressure or hypotension, with presence of cold and clammy skin and restlessness.

Grade IV: Profound shock with undetectable blood pressure or pulse.

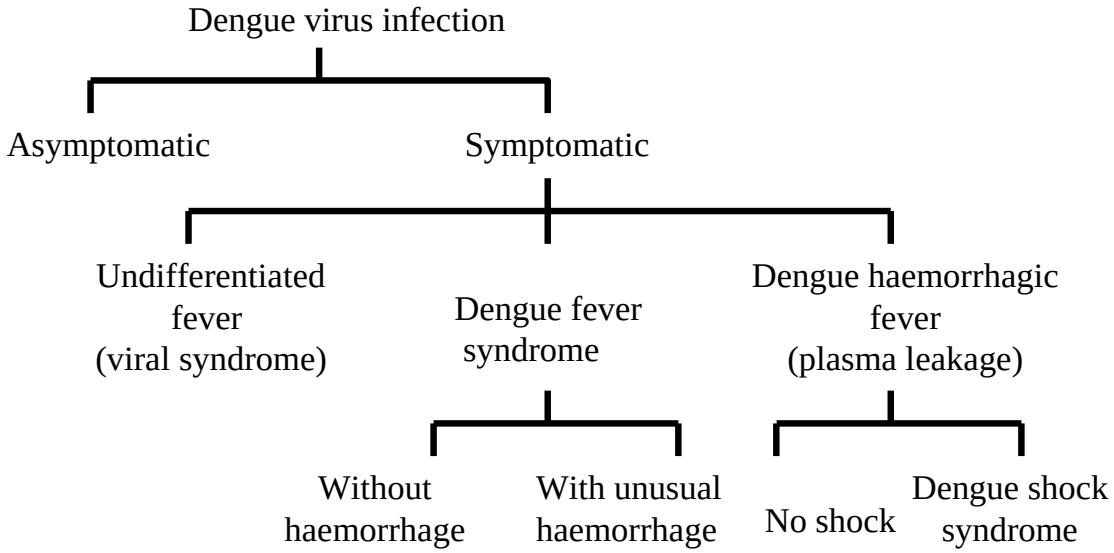


Fig 1.3. WHO classification of symptomatic dengue infection (WHO, 1997).

DSS refers to DHF grades III and IV. These classifications developed by WHO have been found to be clinically and epidemiologically useful in epidemics. These guidelines, however, are based on clinical experience mainly from Thailand and do not represent the findings from other countries affected by recent global expansion in dengue and thus there are strong suggestions that improvement to these guidelines are needed (Phuong *et al.*, 2004; Bandyopadhyay *et al.*, 2006; Deen *et al.*, 2006) for clinical management and epidemiological purposes.

1.4 VIRUS CLASSIFICATION

DENV belongs to the genus *Flavivirus,* of the family *Flaviviridae* that compromises over 70 viruses (Lindenbach and Rice, 2001). Most of the viruses belonging to this family are arthropod-borne human pathogens causing a variety of diseases including fevers, encephalitis and hemorrhagic fevers. The *Flaviviridae* family contains three genera: (1) *Flavivirus* (e.g. dengue virus, yellow fever virus, Japanese encephalitis virus (JEV) and tick borne encephalitis virus), Table 1.3 shows some of the common members of the *Flavivirus* genus. (2) *Hepacivirus* (hepatitis C virus (HCV)) and (3) *Pestivirus* (bovine viral diarrhea virus (BVDV), classical swine fever virus and border disease virus). The *Flaviviridae* family of viruses has a conserved genome structure containing a single molecule of linear positive sense (+ ve) ssRNA. The complete genome is 9500-12,500 nucleotides long. Members of the *Flavivirus* genus are distinguished by presence of a type I cap structure (m7GpppAmp) at the 5' end of the genome, a highly structured 3' untranslated region (UTR) (Brinton and Dispoto, 1988) and by the absence of a 3'-terminal poly (A) tract (Chambers *et al.*, 1990). It has also been shown recently that the 5' and 3' ends stack together to cause cyclisation of the genome which may be important for *flavivirus* RNA replication (Alvarez *et al.*, 2005; Filomatori *et al.*, 2006) (discussed in section 1.6.2.3). The other genera of the *Flaviridae* family (i.e hepaciviruses and pestiviruses) control translation by an internal ribosomal entry site (IRES) in the 5' UTR and have a shorter, less structured 3' UTR than *flaviviruses* (Pestova *et al.*, 1998).

1.5 DENV STRUCTURE

Like other *flaviviruses*, mature DENV virions consist of a ss positive sense (+ ve) RNA genome surrounded by an isometric nucleocapsid about 30 nm in diameter (Henchal and Putnak, 1990). The nucleocapsid is covered by a host derived lipid envelope into which the E

Flaviviruses (Antigenic group)	**Vector**	**Type/species**
Dengue virus	Mosquito	DENV 1 DENV 2 DENV 3 DENV 4
Japanese encephalitis group	Mosquito	Japanese encephalitis virus Kunjin virus West Nile virus Murray Valley encephalitis virus
Ungrouped	Mosquito	Yellow fever virus

Table 1.3. Some common *Flaviviruses* adapted from (Lindenbach *et al*., 2001).

and M proteins are inserted. The complete virion is 50 nm in diameter (Fig 1.4A). Cyro-electron microscopy studies have shown structural deviation between immature and mature DENV envelopes (Fig 1.4B,C) (Kuhn *et al.*, 2002; Zhang *et al.*, 2003). The immature DENV particle is covered with asymmetric trimers of prM-E hetrodimers that jut out like spikes on the surface (Fig 1.4B). In mature viruses, the E protein exists as homodimers that lie on the viral membrane in the form of rafts. These rafts are made of three parallel dimers arranged in icosahedral symmetry (Fig 1.4C).

DENV genome comprises of approximately 10,600 nucleotides. Most of the genomic RNA forms a non-interrupted open reading frame (ORF), which is flanked by untranslated regions (UTRs) at the 5' and 3' end (Huang *et al.*, 1997). Both the 5' and 3' UTR's have key roles in the regulation of translation and genomic RNA synthesis.

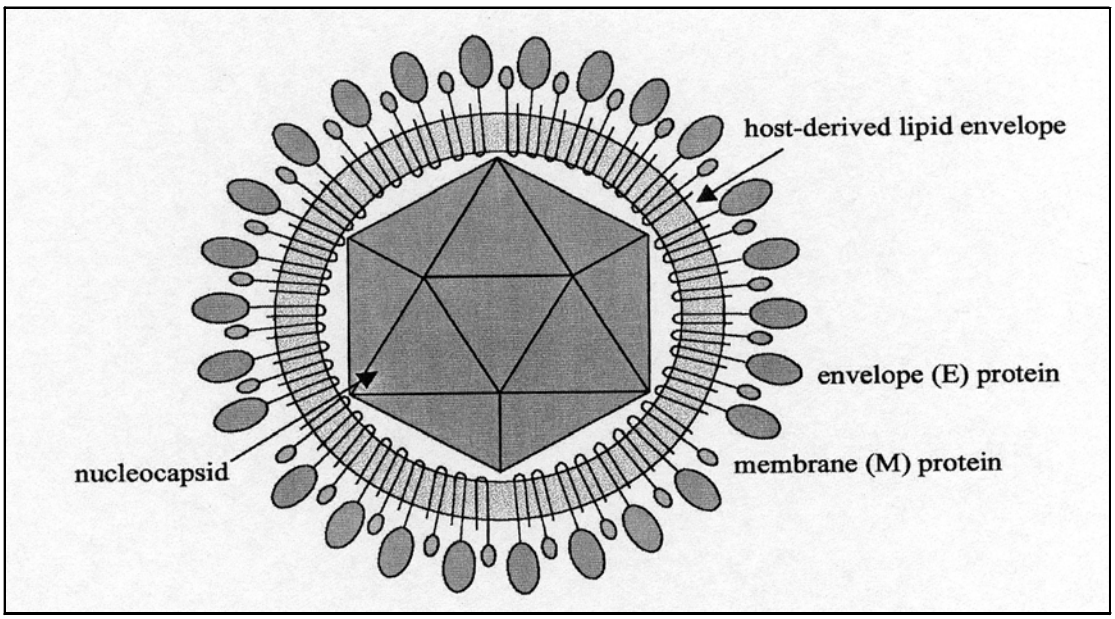
1.5.1 DENV structural and non structural proteins

The single ORF in the DENV RNA directs the synthesis of a long poly-protein (in the cytoplasm) that is subsequently co- and post-translationally modified by viral and host cell proteases to produce 10 viral proteins. These include three structural proteins, the capsid (C), the precursor (prM) to the membrane protein (M), the envelope glycoprotein (E), and seven non-structural (NS) proteins: NS1, NS2a, NS2b, NS3, NS4a, NS4b and NS5 (Fig 1.5). The processing of the DENV poly-protein includes a combination of viral serine proteases (NS2B-NS3) and host cellular proteases. Definitive roles for all the DENV proteins have not currently been fully characterised, and many of the DENV structural and non-structural proteins serve multiple functions in the viral life cycle as discussed below.

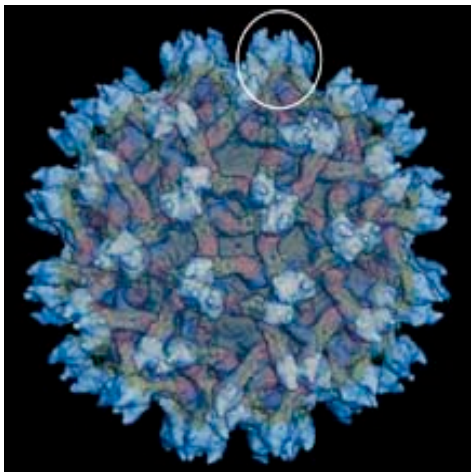
1.5.1.1 Structural proteins C, M and E

The structural C protein is highly basic and in the virion forms a nucleocapsid around the genomic RNA (Lindenbach and Rice, 2001). Nuclear magnetic resonation (NMR) techniques have been used to elucidate the structure of DENV-2 C protein (Jones *et al.*, 2003; Ma *et al.*, 2004) which shows that the C protein contains a hydrophobic cleft, of which the negative charged side is proposed to interact with the viral lipid bilayer and the positively charged region on the opposing side is thought to interact with viral RNA (Ma *et al.*, 2004).

A



B



C

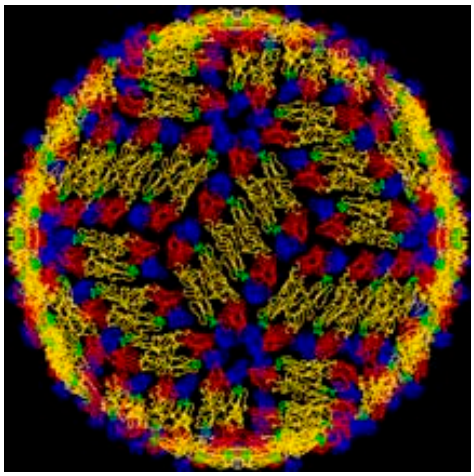


Fig 1.4. Immature and mature dengue viruses (A). Schematic representation of mature virions. E, envelope protein; M, membrane-associated protein; C, capsid protein (Henchel *et al*.,1990). **(B)**. The immature dengue particle with 60 spikes (circled) that jut from its surface (Zhang *et al*., 2003). **(C)**. The smooth structure of mature dengue virus (Kuhn *et al.*, 2002). One raft consists of three parallel dimers of the envelope protein, the different domains are represented by different colours (domain I, II and III are coloured red, yellow and blue respectively) and the fusion peptide is shown in green.

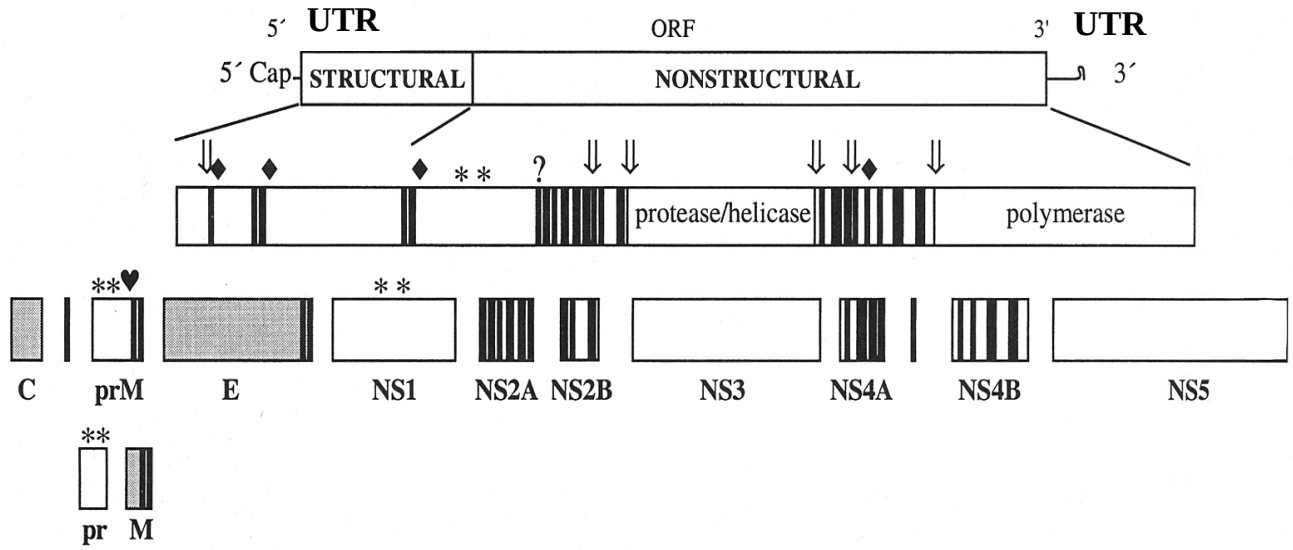


Fig 1.5. DENV RNA protein-coding regions and genome organisation from (Lindenbach *et al*., 2001). Untranslated regions of the genome (top), with their 5' and 3' terminal structures, are indicated by black lines. The single open reading frame encodes a polyprotein (middle) that is processed by the viral NS2B-NS3 protease and host protease to the mature viral proteins (bottom). Mature structural proteins are indicated by shaded boxes, and the non structural protein precursors by open boxes. Contiguous stretches of unchanged amino acids are shown by black bars. Asterisks denote proteins with N-linked glycans. Cleavage sites for host signalase (♦), the viral serine protease (⇩), furin or other golgi-localised protease (⇩), or unknown proteases (?) are indicated.

The nucleocapsid core is surrounded by an endoplasmic reticulum (ER) derived lipid bilayer containing the two other structural proteins E and prM. The prM is processed to mature M protein by cellular proteases shortly before the virion exits the cell (Stadler *et al.*, 1997). Studies have shown this maturation step is necessary to expose the E receptor binding domain allowing viral infectivity (Heinz and Allison, 2003).

The DENV E protein is the cell attachment protein involved in cell receptor binding (Anderson *et al.*, 1992; Chen *et al.*, 1996), and virus entry via membrane fusion (Lindenbach and Rice, 2001). The E protein is a 494 amino acid protein with two potential glycosylation sites and is present on the outer surface of the virus particle (Henchal and Putnak, 1990). The E glycoprotein can be divided into three structural and functional domains: - (1) the central domain (2) the dimerisation domain which encodes the fusion peptide and (3) the receptor binding domain. The DENV E protein is the major virion surface protein and thus plays an important role in virus attachment entry and is the major antigen involved in humoral immunity and protection. In both primary and secondary cases of DENV-infection antibody to E protein is most readily detected, suggesting E proteins are highly immunogenic (Churdboonchart *et al.*, 1991; Se-Thoe *et al.*, 1999; Valdes *et al.*, 2000). *Flavivirus* E protein is an essential component for initial viral infection (Henchal and Putnak, 1990; Chen *et al.*, 1996) and E reactive antibodies have been shown to neutralise virus infectivity by interfering with virus binding and penetration (He *et al.*, 1995; Hung *et al.*, 1999). Cells transfected with recombinant prM and E alone can form viral-like particles that do not contain C or genomic RNA (Purdy and Chang, 2005).

1.5.1.2 Non structural proteins

The most extensively characterised non structural proteins are NS1 (a glycoprotein), and two cytosolic proteins NS3 (a viral protease/helicase) and NS5 (the polymerase). In *in vitro* infections, **NS1** translocates into the ER where it associates as a homodimer which then interacts with membranous components (Winkler *et al.*, 1988; Winkler *et al.*, 1989). NS1 also has been shown to be associated with intracellular organelles where it is thought to aid in early steps of viral replication (Mackenzie *et al.*, 1996) and it is released as a soluble hexamer (sNS1) from infected mammalian cells (Flamand *et al.*, 1999). In a mouse model sNS1 has been shown to accumulate in the late endosomal compartment of hepatocytes and incubation of Huh7 cells (hepatoma cell line) with sNS1 for 24 hrs before infection with DENV has been shown to increase virus production up to 7 fold (Alcon-LePoder *et al.*, 2005). NS1 antibodies

are detected in DENV-infected patients and may be involved in pathogenesis as discussed in section 1.8.2.1.

NS3 protein is part of the replication complex and has several enzymatic functions. The amino terminus of NS3 is a serine protease domain which requires the DENV NS2B protein as a cofactor to cleave the DENV poly-protein with help of host proteases (Falgout *et al.*, 1991; Arias *et al.*, 1993). The carboxyl terminus of the NS3 exhibits nucleoside 5'-triphosphatase (NTPase) and helicase functions that are required for DENV RNA synthesis (Li *et al.*, 1999) and 5'-triphosphatase (RTPase) activity (Bartelma and Padmanabhan, 2002; Benarroch *et al.*, 2004). DENV NS3 protein has been shown to interact with human nuclear receptor binding protein (hNRBP), a host protein that modulates intracellular trafficking between the ER and the golgi compartment. The interaction of NS3 with hNRBP alters the cellular distribution of hNRBP and has been proposed to be important in viral maturation and the generation of virus-induced membrane structures from the ER (Chua *et al.*, 2004).

NS5 protein is the largest of the dengue proteins and is highly conserved among the *flaviviruses* (Mandl *et al.*, 1989). NS5 consists of a well characterised viral RNA dependent RNA polymerase (RdRp) activity in the carboxyl terminus (Tan *et al.*, 1996; Nomaguchi *et al.*, 2003) and methyltransferase activity in the amino terminus (Egloff *et al.*, 2002) which is the enzyme function responsible for viral RNA capping. NS5 complexes with NS3 in the cytoplasm to form a DENV replication complex. NS5 is responsible for stimulating the NTPase acitivity of NS3, which is necessary for unwinding of double stranded (ds) RNA substrates by the NS3 helicase activity during viral replication (Yon *et al.*, 2005). DENV NS5 protein has a putative nuclear localisation site (NLS) and can accumulate in the nucleus late in infection (Kapoor *et al.*, 1995). The function of nuclear localised NS5 is undefined but the impaired ability for replication of virus lacking nuclear localising NS5 suggest it is likely to be integral to viral infection (Pryor *et al.*, 2007).

NS2A, NS4A and NS4B are smaller hydrophobic proteins that are thought to be responsible for localisation of viral proteins and virion assembly (Lindenbach and Rice, 2001). They have also been implicated in blocking IFN mediated signal transduction as discussed in section 1.8.2.1.

1.6 DENV REPLICATION CYCLE

DENV replication is similar to that of other *flaviviruses* (Fig 1.6 (Lindenbach and Rice, 2001)). Briefly the steps involve **1**. Uptake of virus mediated by binding of the virus E protein to the cell membrane receptor, **2**. Receptor mediated endocytosis, **3**. Membrane fusion with the endosome and delivery of the nucleocapsid to the cytoplasm, **4**. Cytoplasmic uncoating of virus, **5**. Translation of the genomic RNA and production of a single long poly-protein with subsequent cleavage to form NS and structural proteins, **6**. RNA replication in membrane-associated replication complexes, **7**. Virion assembly and budding of progeny virions through *intracellula*r membranes into cytoplasmic vesicles, **8**. Virion transport and glycoprotein maturation, **9**. Vesicle fusion at the plasma membrane and release of mature virions into the *extracellular* compartment. A number of differences from the schematic *flavivirus* replication scheme shown in Fig 1.6 that are specific to DENV replication are discussed below. The structure of DENV genome and the roles of each protein and RNA structural region is shown in Fig 1.7.

1.6.1 DENV binding to receptors and entry

1.6.1.1 DENV receptors

DENV E protein attaches to a receptor, either Fc receptors on cells such as macrophages or B cells (Daughaday *et al.*, 1981; Littaua *et al.*, 1990) or an uncharacterised non-Fc receptor, to initiate infection. A number of putative non-Fc receptors have been identified in monocytes and macrophages which include a trypsin sensitive receptor (Daughaday *et al.*, 1981), LPS/CD14-associated binding protein (Chen *et al.*, 1999), 4 unnamed proteins with molecular weights 27, 45, 67 and 87 kDa (Moreno-Altamirano *et al.*, 2002) and a heat shock protein (HSP) 90 and 70 associated complex (Reyes-Del Valle *et al.*, 2005). In dendritic cells DENV entry has been shown to occur in an Fc receptor-independent manner (Wu *et al.*, 2000). Recently a non-Fc receptor for DENV on dendritic cells has been identified as DC-SIGN (CD209). DC-SIGN binds to all four DENV serotypes (Tassaneetrithep *et al.*, 2003) and is also a receptor for HCV and utilised during human immunodeficiency virus (HIV) infection of dendritic cells. Heparan sulphate and GRP78 (BiP) have been identified as putative DENV receptors in livers cells (Lin *et al.*, 2002a; Jindadamrongwech *et al.*, 2004).

Thus there is probably not one single DENV receptor, and DENV receptor binding varies between cell types.

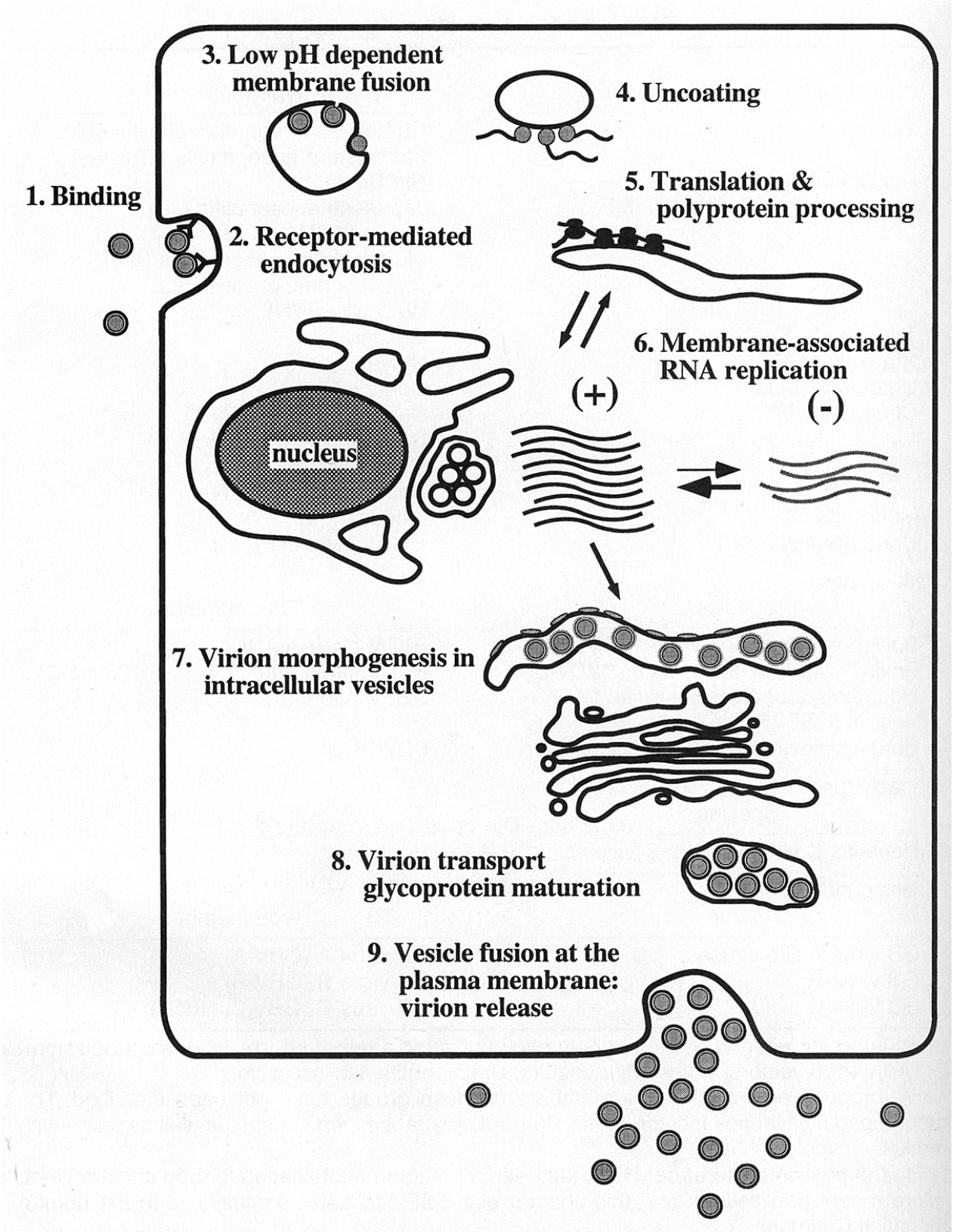


Fig 1.6. Life cycle of the *Flaviviridae* from (Lindenbach *et al.*, 2001).

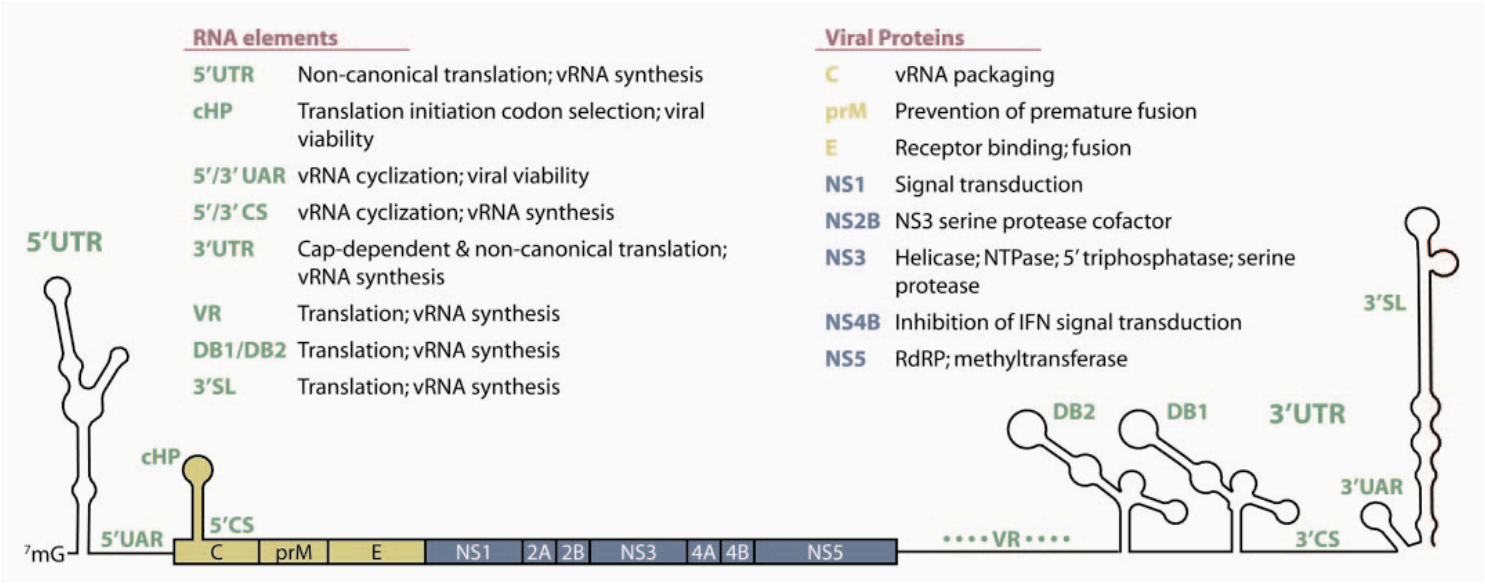


Fig 1.7. Structure of DENV RNA from (Clyde *et al*., 2006).

1.6.1.2 DENV entry

The E protein mediates virus binding to cell receptors and the subsequent low pH dependent fusion step (Randolph and Stollar, 1990). Attachment can occur at low temperatures (up to 4^0C) but penetration requires higher temperatures of 37^0C (Hase *et al.*, 1989). It is generally accepted that DENV gains entry into its target cell by receptor mediated endocytosis as seen with other *Flavivirus*. However there are reports of DENV entry via direct fusion with the plasma membrane in cultured C6/36 mosquito cells (Hase *et al.*, 1989). In this case DENV virions attach to host cells by their envelope spikes, the virion envelope overlaps the host plasma membrane and the plasma membrane dissolves at the attached sites, followed by penetration of the virion into the cytoplasm of C6/36 cells (Hase *et al.*, 1989). Similarly direct penetration into the cytoplasm through the plasma membrane of DENV-2 has been observed in peripheral blood mononuclear cells (PBMC) (Hase *et al.*, 1989). Electron microscopy has also shown that DENV can be taken up by phagocyotsis in monocyte/macrophages, with as many as 29-63% of virus particles engulfed by typical lamellipods and sequestered into intra-cytoplasmic vacuoles (Espina *et al.*, 2003). Thus the mode of entry for DENV may vary in different cell types.

1.6.2 DENV uncoating, translation, RNA transcription

1.6.2.1 Uncoating

Uncoating of virus occurs after exposure to acidic pH (below pH 6.5), and infection can be blocked by treatment of cells with agents that raise the pH of the intracellular endosomal compartment and prevent *Flavivirus* uncoating (Gollins and Porterfield, 1985; Gollins and Porterfield, 1986; Randolph and Stollar, 1990).

1.6.2.2 Translation

Following uncoating, nucleocapsids are disassembled, and genomic RNA is translated. Translation occurs in association with the ER derived membranes. Translation of the non structural proteins results in the formation of the replication complex that is essential for subsequent viral RNA synthesis and replication (Khromykh *et al.*, 2000).

1.6.2.3 Viral replication

Following initial translation of the infecting genomic RNA, the virus then produces viral mRNA for further protein production and viral genomic RNA for packaging and virion production. The positive (+ ve) genomic viral RNA initially operates as template for the synthesis of a negative (- ve) strand RNA to form a ds replicative form (RF) (Fig 1.8**)** (Chu and Westaway, 1985). Amplification of (+ ve) strand progeny genomes then occurs using the (- ve) RNA strand as a template in a semi-conservative manner where the nascent (+ ve) strand displaces the previously bound (+ ve) RNA, forming replicative intermediate (RI). The RI at any time consists of a (- ve) strand (template), a displaced (+ ve) strand and a base-paired nascent (+ ve) strand. As the release of the displaced (+ ve) strand progresses the nascent (+ ve) strand is extended but remains attached along its entire length to the (- ve) strand, and the whole RF molecule is regenerated. The displaced viral (+ ve) strand RNA either initiates further rounds of RNA replication or is translated and assembled with matured structural proteins to yield progeny virus particles, thus serving as both viral genome and mRNA. This RNA replication strategy produces 10-100 fold higher (+ ve) than (- ve) strand RNA (Cleaves *et al.*, 1981) although the mechanism by which this asymmetric synthesis occurs is not known. Additionally, the molecular mechanisms involved in the initiation of RNA replication or regulation of protein verses RNA synthesis during DENV replication is not understood.

Recent studies have shown that cyclisation of DENV RNA is a requirement for viral replication (Alvarez *et al.*, 2005; Filomatori *et al.*, 2006). 5' and 3' cyclisation sequences (5' and 3' CS), are thought to be responsible for mediating cyclisation of DENV RNA. Disruption of base pairing between the two regions has been shown to compromise viral RNA synthesis (You and Padmanabhan, 1999; You *et al.*, 2001; Holden *et al.*, 2006). Hence, a new model for synthesis of a DENV RNA (- ve) strand has been proposed (Filomatori *et al.*, 2006) in which the viral polymerase recognizes the 5' end of the RNA and through long-range RNA-RNA interactions the 5' promoter and the 3' promoter are brought together to facilitate RNA synthesis (Fig 1.9).

Accumulation of (- ve) strand viral RNA has been shown to be critical for productive cell infection by DENV and therefore the detection of (- ve) strand viral RNA is a reliable marker for active DENV replication (Diamond *et al.*, 2000a; Wang *et al.*, 2002c). Technically, however, specific quantitation of DENV (+ ve) and (- ve) RNA strands are problematic due to self-priming during cDNA synthesis (Tolou, 1994; Peyrefitte *et al.*, 2003), which may relate

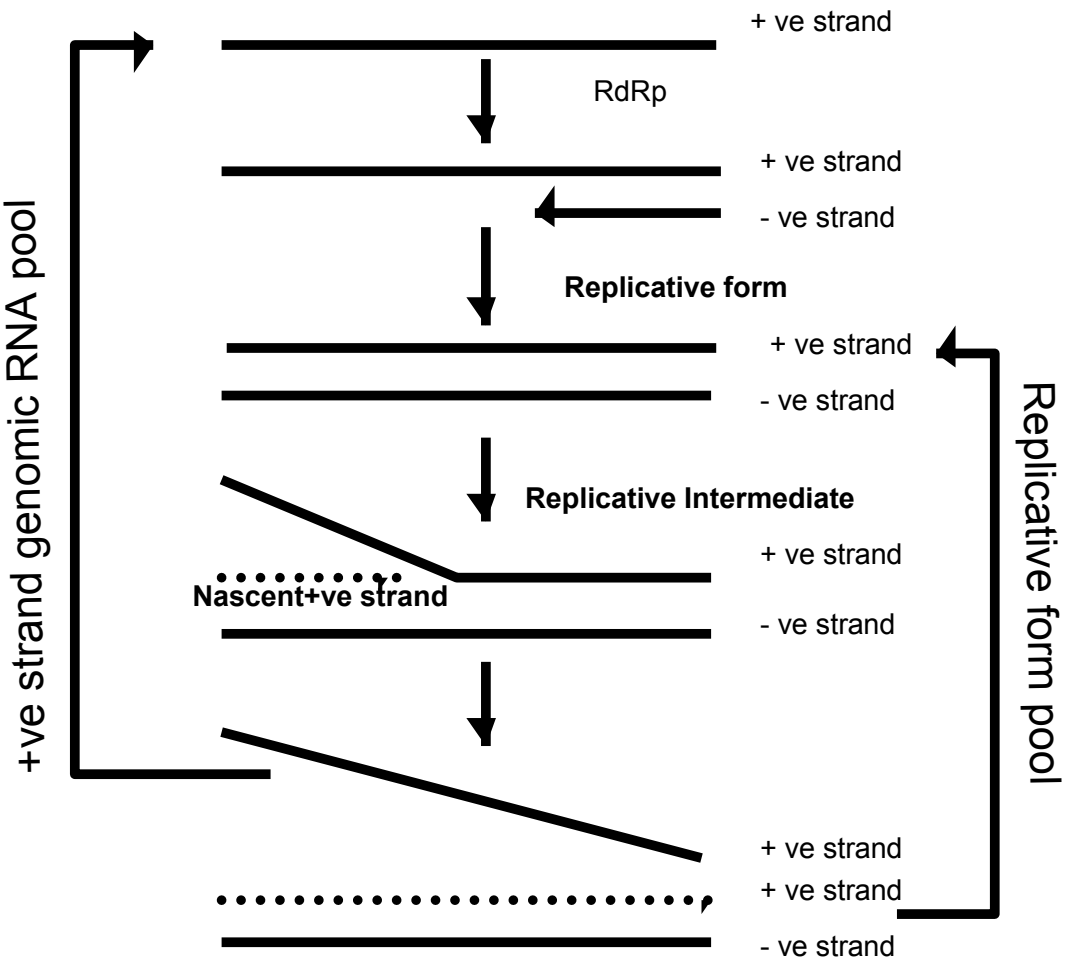


Fig 1.8. RNA replication by semi conservative RNA synthesis (Chu *et al*., 1985).

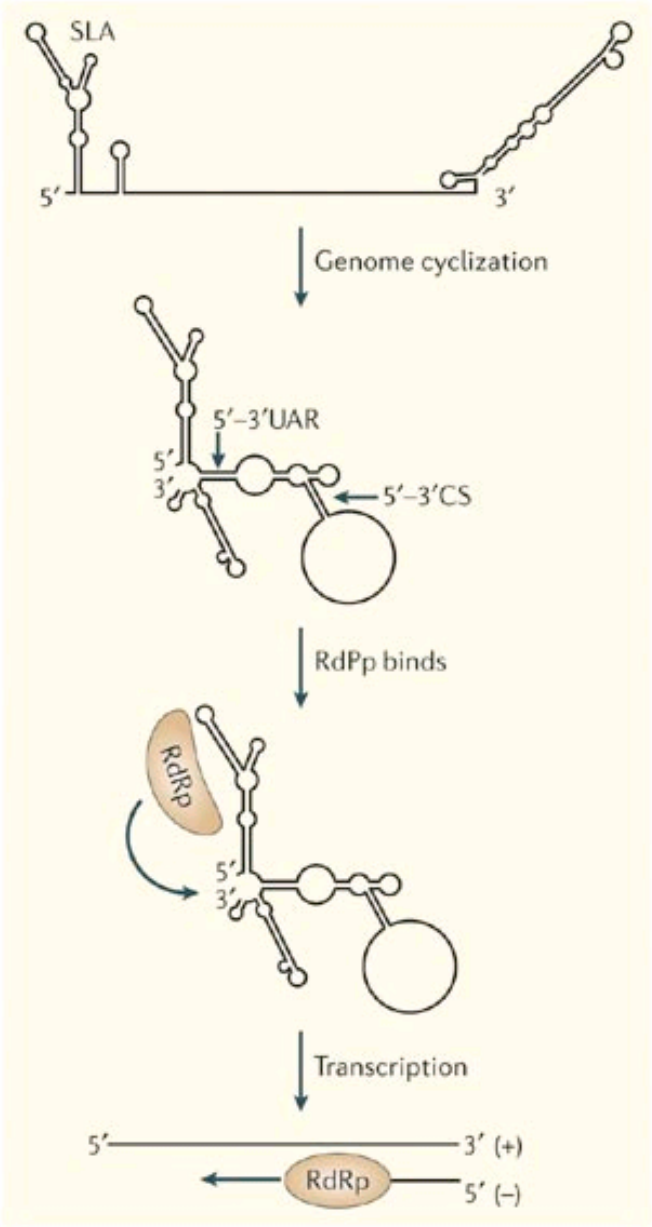


Fig 1.9. Model for DENV (- ve) strand RNA synthesis from Filomatori *et al*., 2006. The viral genome circularises in the absence of proteins mediated by 5' – 3' UAR and 5'-3' CS hybridisation. The viral RdRp binds to a 5' stem-loop (SLA), and by long-range RNA-RNA interactions the polymerase is transferred to the site of initiation at the 3' end of the genome.

to DENV RNA structure or cyclic replication mechanism (Fig 1.9) and this is addressed in chapter 3 of this thesis.

1.6.3 Assembly and release of DENV particles

Electron microscopy studies in cells such as C6/36 and Vero cells suggest that DENV morphogenesis occurs in association with intracellular membranes of the ER (Matsumura *et al.*, 1971; Matsumura *et al.*, 1977; Hase *et al.*, 1989; Mackenzie *et al.*, 1996). Here the poly-protein is produced and cleaved while in the membrane by cell and viral encoded (NS2B-3) proteases and assembled in a concerted processes (Lobigs, 1993; Stocks and Lobigs, 1995; Stocks and Lobigs, 1998). Following assembly in the ER the immature particle is shuttled through the trans-golgi network. The immature virion contains a spiked surface which has been shown to be the prM protein capping the E protein (Zhang *et al.*, 2003). Cleavage of prM from the E protein occurs before the virus is released from the plasma membrane by furin or furin-like proteases (Stadler *et al.*, 1997). Cyro-electron microscopy has been used to show that immature dengue particles undergo a reversible conformational change at low pH which makes them accessible to furin cleavage (Yu *et al.*, 2008). As a result of furin cleavage, the 60 spikes composed of three prM-E heterodimers are dissociated, consequently forming a smooth surface of mature virus composed of 90E homodimers (Fig 1.4C) (Kuhn *et al.*, 2002). Mature virions are released via exocytosis of secretory vesicles.

1.7 DENV TARGET CELLS

DENV can replicate in a wide range of cell types *in vitro*, including dendritic cells, monocytes and macrophages, B cells, endothelial cells and hepatocytes. Many kinds of cell lines have been used for viral propagation such as hepatoma cell lines (Huh7 and Hep3B) myelomonocytic cell lines (K562), B cell line (Raji) and T cell line (HSB-2) but the relevance to DENV replication *in vivo* is unclear (Kurane *et al.*, 1990; Lin *et al.*, 2000). Analysis of PBMC, liver, lymph nodes, spleen and bone marrow from DENV-infected patients has identified DENV antigens in monocytes, macrophages and B cells suggesting these cell types are natural target cells for DENV-infection in the body (Kurane *et al.*, 1994; Mangada and Igarashi, 1998; Couvelard *et al.*, 1999; Rosen *et al.*, 1999).

1.7.1 Macrophages

DENV has been recovered from PBMC derived from patient blood (Scott *et al.*, 1980; King *et al.*, 1999). The adherent cell population from PBMC which primarily consists of cells from the monocyte/macrophage lineage was found to yield the most infectious virus (Scott *et al.*, 1980). Additionally, DENV-infected mononuclear cells have been identified in pathological specimens from a number of tissues including skin, liver (Kupffer) cells and glass adherent peripheral blood leukocytes isolated from patients with DHF/DSS (Scott *et al.*, 1980; Halstead, 1989; King *et al.*, 1999). Cultured primary macrophages also support active DENV replication *in vitro* and release infectious viral progeny (Halstead *et al.*, 1977; Pryor *et al.*, 2001; Chen and Wang, 2002). Together these data suggest that macrophages are one of the main targets for DV replication *in vivo.*

1.7.1.1 Macrophages and DENV replication

Macrophages are generally present in every tissue of the body and provide innate immune surveillance. They are derived from myeloid precursors in bone marrow, spleen and fetal liver, where they migrate to enter the blood. Exposure to cytokines and chemokines changes their functional and phenotypic characteristics (Stout and Suttles, 2004), producing a heterogeneous cell population, that display a progression of functional changes in response to their microenvironment. For example, blood monocytes can be sorted as $CD14^{+}$, $CD16^{-}$ (major population) or $CD14^{+}$, $CD16^{+}$ (minor population). These circulating monocytes differentiate and migrate through blood vessel walls to various tissues where they mature into differentiated resident tissue macrophages, again with functional properties specific to the tissue they reside in e.g. normal macrophages in liver are Kupffer cells, in spleen are splenic macrophages and in kidney are mesangial cells.

Phagocytosis is one of the most important differentiated characteristics of macrophages. It can be grouped into receptor mediated (e.g. Fc receptors that recognise the Fc portion of the antibody) or receptor independent phagocytosis (Silverstein *et al.*, 1977). Splenic and peritoneal cavity macrophages from DENV-infected mice show a significantly reduced ability to phagocytose latex particles and opsonised sheep erythrocytes compared to macrophages from uninfected mice showing functional changes related to DENV replication (Gulati *et al.*, 1982; Chaturvedi *et al.*, 1983).

Monocyte derived macrophages (MDM) can be generated i*n vitro*, by differentiation of CD14+ monocytes and is a useful *in vitro* model of primary macrophage. DENV can productively infect MDM with high level production of infectious virus, independent of the stage of cell differentiation (Chen and Wang, 2002). DENV-infection of monocytes and MDM can also be enhanced by infection in the presence of antibody. The DENV virion can form a complex with non-neutralising antibody that can bind to the Fc receptors on MDM and be taken up as described in section 1.6.1.2 (Theofilopoulos *et al.*, 1976; Kliks *et al.*, 1989; Kliks, 1990; Kurane *et al.*, 1990). In this study we have used MDM as an *in vitro* model system for DENV replication, without antibody enhancement, as it has been previously shown in our lab and others that antibody enhancement is not required to obtain high level virus production (O'Sullivan and Killen, 1994; Pryor *et al.*, 2001).

1.7.2 Dendritic cells

Dendritic cells are also of interest as a primary target for DENV replication as they are exposed to virus early and recent studies have shown that immature dendritic cells support DENV-infection (Wu *et al.*, 2000; Ho *et al.*, 2001; Libraty *et al.*, 2001; Marovich *et al.*, 2001). Dendritic cells are also derived from cells of the monocytic lineage, and can exist as immature dendritic cells in blood or as specialised tissue dendritic cells in tissues such as lymph nodes or skin (Langerhans cells). Two subsets of human skin dendritic cell – (1) monocyte derived dendritic cells (resembling dermal/interstitial dendritic cells) and (2) human skin Langerhans cells have been shown to support DENV replication *in vitro* (Wu *et al.*, 2000). Interestingly, histology from a skin rash of a healthy volunteer who received attenuated tetravalent DENV vaccine showed that DENV had spread to Langerhans cells, thereby demonstrating DENV transmission and replication in this cell type *in vivo* (Wu *et al.*, 2000). These observations implicate dendritic cells as a potential initial target cell type for DENV-infections, following exposure to the virus early during the feeding of an infected mosquito.

1.7.3 B and T cells

In addition to the identification of monocytes and macrophages as the main circulating cell type infected by DENV the studies described in section 1.7.1 also identified DENV-infected, B cells. In particular the study of King *et a*l., 1999, which collected PBMC from acutely ill dengue patients, sorted these into different cell populations by flow cytometry and isolated

DENV by inoculation of cell samples into *Toxorhynchites splendens* mosquitoes (King *et al.*, 1999), principally isolated DENV from the B cell population. Comparative *in vitro* DENV replication studies have shown similar levels of infectious viral production from primary B cells and monocytes (isolated from PBMC of healthy donors) (Lin *et al.*, 2002b) and thus B cells may be another target cell for DENV *in vivo*. In contrast, although human T cell leukemia and lymphoma cell lines can be efficiently infected with DENV *in vitro* producing high levels of infectious virus (Kurane *et al.*, 1990), primary T lymphocytes ($CD4^+$, $CD8^+$) show only low levels of infection even at a high multiplicity of infection (MOI) of 20 (Mentor and Kurane, 1997) or no infection at all (Theofilopoulos *et al.*, 1976). Thus B cells but not T cells are probably also target cells for DENV *in vivo*.

1.7.4 Endothelial cells

Endothelial cells are involved in the severe DENV pathology associated with vascular leakage, but the changes in the endothelium are believed to be mediated by indirect immunopathology rather then direct viral induced cytopathic effect (CPE) on endothelial cells. Both human endothelial-like cell line (ECV304) (Avirutnan *et al.*, 1998) and primary cultures of human endothelial cells isolated from umbilical cord veins, can be infected by DENV *in vitro* (Andrews *et al.*, 1978; Killen and O'Sullivan, 1993; Anderson *et al.*, 1997b; Avirutnan *et al.*, 1998; Huang *et al.*, 2000b). To date there is no evidence of DENV-infection and endothelial cell damage during DENV-infection *in vivo*. Biopsy or autopsy specimens have shown no evidence of DENV-infection of endothelial cells (Halstead, 1989), and although endothelial cells are clearly important in DENV pathogenesis they are probably not a major target cell for viral replication *in vivo*.

1.7.5 Hepatocytes and Kupffer cells

Liver damage with elevation of aspartate (AST) and alanine (ALT) aminotransferase levels is a common complication of clinical DENV-infection in patients (Souza *et al.*, 2004), drawing recent attention to the involvement of the liver in DENV-infections. Hepatomegaly is evident in children with DHF and in Thailand up to 98% of children were reported to have DENV associated liver enlargement (Eram *et al.*, 1979; Nimmannitya, 1987; Wichmann *et al.*, 2004). In addition, viral antigens can be detected in hepatocytes and Kupffer cells and virus can be recovered from liver biopsy specimens of DHF patients (Rosen *et al.*, 1989; Hall *et al.*, 1991; Couvelard *et al.*, 1999). Kupffer cells are resident tissue macrophages of the liver, again

highlighting the importance of the macrophage as an *in vivo* target for DENV-infection. However, in contrast to the active DENV replication in blood derived macrophages, i*n vitro* studies have shown that DENV penetrates human primary Kupffer cells but no viral progeny is produced. Instead, DENV-infected Kupffer cells undergo apoptosis and are cleared by phagocytosis (Marianneau *et al.*, 1999). This finding suggests that Kupffer cells may protect against DENV-infection rather than acting as a site for DENV replication in the liver. DENV-infection of a range of hepatoma cell lines including Hep3B and Huh7 cells demonstrated active viral replication with reasonably high levels of DENV production and an increase in AST and ALT levels (Lin *et al.*, 2000). Infection, in these cell types also results in apoptosis and this has been suggested to be the key element in the pathophysiology of liver failure associated with DHF/DSS (Kangwanpong *et al.*, 1995; Marianneau *et al.*, 1996; Marianneau *et al.*, 1997; Lin *et al.*, 2000). It is also important to note that other immunological mechanisms caused by DENV-infection can induce liver damage such as activated T cells and cytokines (Gagnon *et al.*, 1999; Libraty *et al.*, 2002a). Thus DENV can infect liver cells and may be directly or indirectly involved in specific DENV-induced pathology in this tissue.

1.7.6 Other cell types

Involvement of primary mast cells in DENV replication has been suggested due to the presence of Fc receptors on this cell type, their location close to skin and association with recruiting and activating blood vessels (King *et al.*, 2002). Additionally primary mast cells can support active DENV replication *in vitro* (King *et al.*, 2002). Other cell types including primary skin fibroblast (Kurane *et al.*, 1992), cells of the bone marrow and bone marrow stromal cell types have been shown to support DENV replication *in vitro* (Rothwell *et al.*, 1996). However, the relevance of these cell types to DENV-infection and replication *in vivo* is undefined.

1.7.7 Target cells – summary

Despite the large number of cell types that can be infected with DENV *in vitro* and the potential DENV target cells *in vivo,* one cell type consistently identified as important and relevant for DENV-infection are cells of the monocyte lineage. *In vitro* infection of cultured primary monocyte/macrophages will be used in this study, along with DENV-infection of

hepatocyte cell lines in some experiments, thus using cell types relevant to DENV-infection *in vivo*.

1.8 PATHOGENESIS OF DENV-INFECTION

Despite the fact that DENV-infection causes one of the most important mosquito-borne viral diseases worldwide and the number of cases has increased over the last several decades, the mechanisms for induction of severe DENV pathogenic effects are still poorly understood. Serious forms of the disease such as DHF commonly occurs in areas where multiple serotypes of DENV are circulating leading to the ADE of infection hypothesis discussed in section 1.8.2.1. The tell-tale sign of DHF is vascular leakage, the onset of which is reported to occur after the peak of viremia, at the time of deffervescence (Kurane *et al.*, 1994). The vascular leakage following DENV-infection is generally short-lived and resolves without tissue damage, suggesting this effect is due to the actions of circulating factors rather than a direct viral effect. Though it is generally accepted that secondary infection (with increases in levels of viremia, antibody, cytokine and T cell responses) is the main risk factor for DHF, other factors such as viral virulence based on serotype (Gubler, 1998; Balmaseda *et al.*, 2006), viral genotype (Rico-Hesse *et al.*, 1997; Messer *et al.*, 2003) and host characteristics (Halstead *et al.*, 2001; Guzman *et al.*, 2002) are also thought to be involved in determining the severity of the disease.

1.8.1 The effect of DENV serotypes and genotypes on DENV pathogenesis

DENV2 and DENV3 genotypes are more often associated with greater severity of disease, including DHF/DSS (Rico-Hesse *et al.*, 1997; Messer *et al.*, 2003). Similarly the DENV-2 Asian-type genotypes have been found to associate with greater disease severity than the American-type DENV-2 or the South Pacific genotypes (Rico-Hesse *et al.*, 1997). Additionally, Thai DENV-2 strains (Asian genotype) replicate to higher titers than the American genotype DENV-2 strains in both MDM and dendritic cells (Pryor *et al.*, 2001; Cologna and Rico-Hesse, 2003), thereby showing an association of ability to cause disease *in vivo* with replicative capacity in important cell types *in vitro*. Mutation of asparagine at position 390 in the E protein, commonly found in Asian-type genotypes, to aspartic acid, as present in the American-type genotype reduced virus output in both MDM and dendritic cells, again suggesting genotype differences may play a role in the severity of DENV-infections (Pryor *et al.*, 2001; Cologna and Rico-Hesse, 2003).

1.8.1.1 The role of viral proteins in DENV pathogenesis

The levels of DENV **NS1** protein in the serum from infected individual's has been shown to correlate well with viremia and the development of DHF (Libraty *et al.*, 2002b; Avirutnan *et al.*, 2006). Thus it has been suggested that high levels of NS1 could be used as a marker for disease severity or the progression of pathology in infected individuals. The NS1 glycoprotein is essential for virus replication but how it influences pathogenesis is still not clear. However antibody to DENV NS1 protein has been shown to cross-react with antigens on the surface of endothelial cells inducing them to undergo nitric oxide (NO) mediated apoptosis (Lin *et al.*, 2003) and cross-reactions between NS1-specific monoclonal antibodies and cellular antigens have also been reported (Falconar, 1997). Thus vascular damage induced by anti-NS1 antibodies could play a role in DENV pathogenesis.

The IFN response is an important anti-viral mechanism and many viruses have established mechanisms to evade this innate anti-viral response (discussed in section 1.8.2.3). Three DENV proteins **NS2A, NS4A and NS4B** all block IFN signalling to different extents thus promoting viral replication (Munoz-Jordan *et al.*, 2003) (discussed in section 1.8.2.3).

The DENV **NS5** protein (the viral polymerase) which plays a key role in DENV replication can induce expression of the anti-viral IL-8 gene and secretion of IL-8 from NS5 transfected HEK-293 cells (Medin *et al.*, 2005).

Thus some DENV proteins can play key roles in indirectly inducing pathogenesis or subverting host protective responses to potentially allow viral evasion and successful infection.

1.8.2 The host response and DENV pathogenesis

1.8.2.1 Role of antibodies in pathogenesis

Antibodies against both E, prM and NS1 proteins are capable of protecting mice from DENV-infection (Kaufman *et al.*, 1987; Henchal *et al.*, 1988; Kaufman *et al.*, 1989; Wu *et al.*, 2003) and are usually detected in DENV patient sera. During natural DENV-infection, neutralising antibodies are generated that provide solid immunity against the infecting primary serotype only. Additionally the immune response to the primary serotype is cross-protective against other serotypes for only a short period after infection. After this initial period of cross-

protection individuals become susceptible to secondary infection with a heterologous serotype, which is often associated with more severe disease.

The antibody-dependent enhancement (ADE) theory (Halstead *et al.*, 1977) was first proposed to explain the observation that severe manifestations of DHF/DSS occur more often in a secondary than primary DENV-infection. The ADE theory postulates that some DENV specific antibodies, either cross-reacting antibodies from a previous DENV-infection or sub-neutralising levels of serotype-specific antibodies, can interact with DENV without neutralising the virus, and enhance Fc_{γ}-mediated receptor binding and uptake by monocytes and macrophages which as discussed earlier, are target cells for DENV-infection (Fig 1.10). This hypothesis is supported by evidence that infants between six and twelve months of age with passively acquired heterotypic maternal DENV antibodies, experience a more severe form of DENV-infection in epidemic cases (Kliks *et al.*, 1988; Halstead *et al.*, 2002; Nguyen *et al.*, 2004). By approximately 6 months of age, levels of maternal antibody to DENV decrease below a protective level, potentially leaving these infants at a risk for development of DHF and DSS in the absence of a prior DENV-infection and DENV-specific cellular immunity (reviewed in (Whitehead *et al.*, 2007)). Complete decay of maternal antibodies ultimately leads to decreased rates of more serious forms of DENV-infection.

In addition to enhanced viral uptake and potential enhanced viremia resulting from ADE, it has also been proposed that ADE can lead to activation of macrophages and DENV specific memory T lymphocytes induced during the primary infection, which then release a number of cytokines and chemical mediators that can induce vascular permeability and may be responsible for the capillary leak syndrome in severe DENV pathogenesis (Fig 1.11).

Viral entry by ADE can also stimulate cell-signalling changes. ADE has also been observed for a number of other viruses. In Ross River virus (RRV), an arthropod-borne alphavirus, infection in the presence of enhancing antibody results in down-regulation of anti-viral genes and disruption of anti-viral activation pathways (such as NF-kB complex formation and production of TNF-α mRNA). This is accompanied by unrestricted RRV replication (Lidbury and Mahalingam, 2000; Mahalingam and Lidbury, 2002). Similarily DENV ADE has recently been shown to up-regulate the production of anti-inflammatory cytokines (IL-6 and IL-10) but suppresses pro-inflammatory cytokine production (IL-12, TNF-α and IFN-γ) in THP-1 cells (Chareonsirisuthigul *et al.*, 2007). Thus ADE may not only facilitate the viral

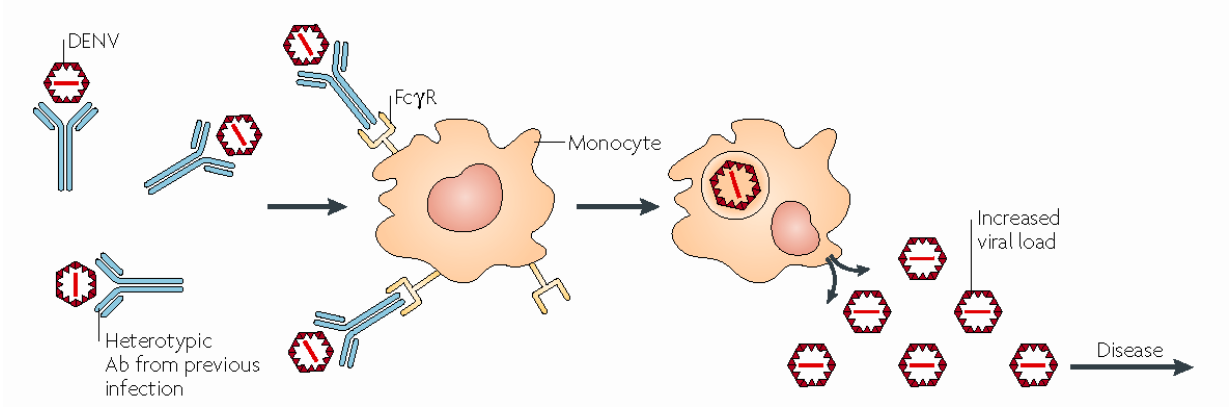


Fig 1.10. Model for antibody-dependent enhancement of dengue virus replication. Antibody (Ab) dependent enhancement of virus replication occurs when heterotypic, non neutralising Ab present in the host from a primary DENV infection binds to an infecting DENV particle during a subsequent heterotypic infection but cannot neutralise the virus. Instead, the Ab-virus complex attaches to the Fc receptors on circulating monocytes, thereby facilitating the infection of Fc cell types in the body not readily infected in the absence of antibody. The overall outcome is an increase in the overall replication of virus, leading to the potential for more severe disease (Whitehead *et al*., 2007).

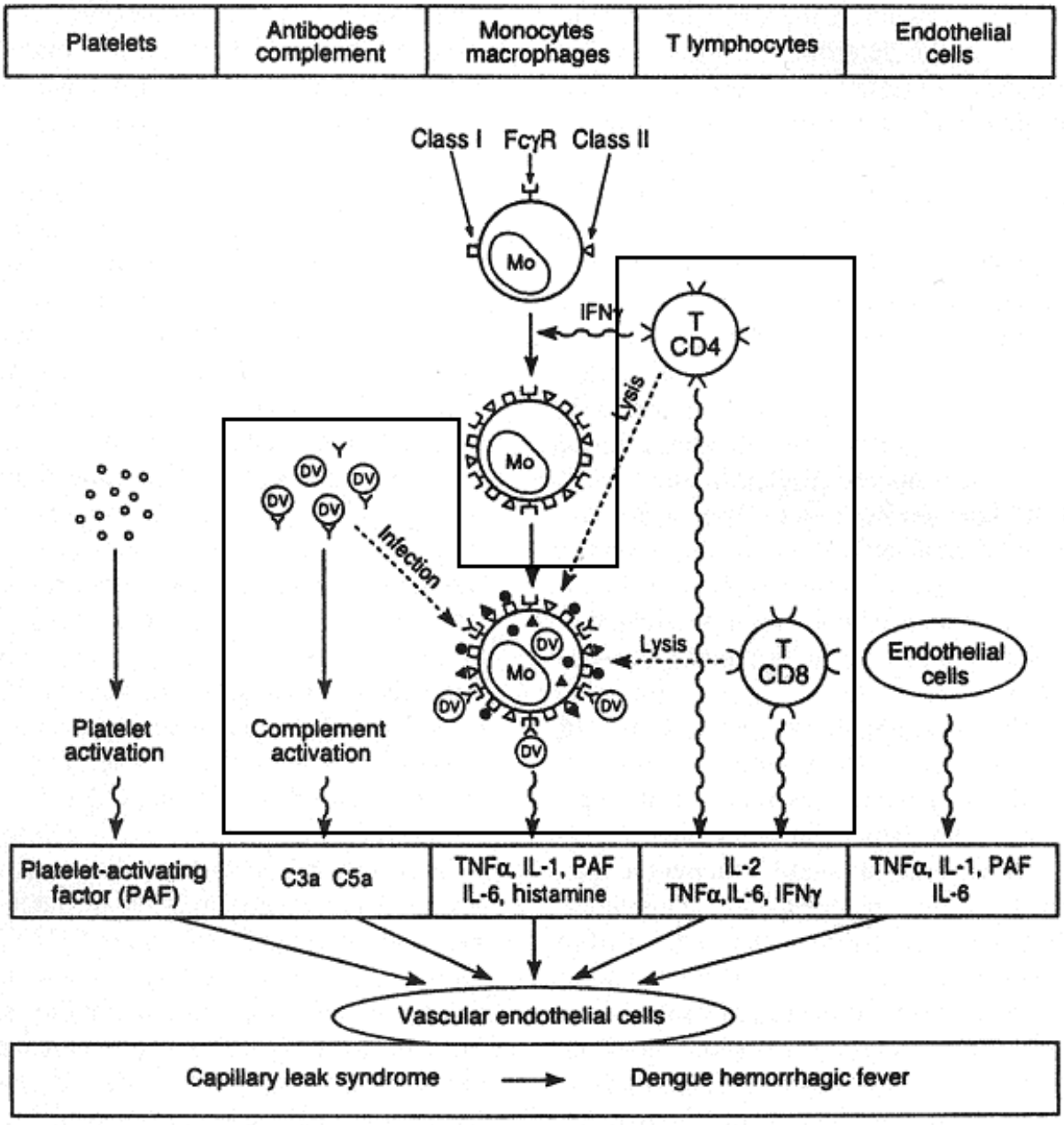


Fig 1.11. Diagram of immunopathogenesis of dengue haemorrhagic fever induced by cytokines and other chemical mediators that increase vascular permeability. Boxed area highlights the ADE proposal and central role macrophages play in DV pathogenesis. Adapted from (Kurane *et al*., 1994).

entry process, but may also modify innate and adaptive intracellular anti-viral mechanisms and hence allowing unrestricted DENV replication.

Only 5% of infected individuals with secondary infection develop DHF and DSS and the more serious forms of the illness have also been reported in patients with primary infection (Scott *et al.*, 1976; Morens *et al.*, 1987; Martinez *et al.*, 1993), and thus the ADE theory alone does not fully explain the mechanisms predisposing to induction of severe DENV pathology.

1.8.2.2 Role of circulating mediators in immunopathogenesis

The absence of severe pathological changes in fatal DENV cases (Halstead, 1989) combined with the fact that most surviving DHF/DSS patients recover rapidly (WHO, 1997) suggest that DHF and DSS are secondary to the action of cytokines and chemical mediators that are capable of producing severe illness with minimal structural injury. Evidence of circulating changes such as marked T cell activation in patients with primary DENV-infection and the presence of high levels of cytokines has also been directly associated with the disease (Kurane *et al.*, 1991; Hober *et al.*, 1993; Green *et al.*, 1999a), and these are discussed below.

T Lymphocytes

DENV specific $CD4^+$ and $CD8^+$ T cells are detected in infected individuals (Bukowski *et al.*, 1989; Kurane *et al.*, 1989), and activated DENV-specific T cells have been suggested to play an important role in immunopathogenesis (Mathew *et al.*, 1998; Gagnon *et al.*, 1999; Zivna *et al.*, 2002; An *et al.*, 2004). Markers of T cell activation, including levels of soluble CD4, soluble CD8, soluble IL-2 and soluble TNFR, are all elevated in DENV-infected patients (Kurane *et al.*, 1991; Hober *et al.*, 1996a; Green *et al.*, 1999a). DENV-specific memory $CD4^+$ and $CD8^+$ T cell are induced on primary DENV-infection and activated DENV specific $CD4^+$ T cell clones lyse non-antigen presenting HepG2 cells suggesting a role of T cell immune response in liver injury during DENV-infection (Gagnon *et al.*, 1999). Additionally during DENV-infection $CD4^+$ T cells *in vitro* produce IFNγ, TNF-α and TNF-β. Interestingly IFN-γ has been shown to increase Fcγ receptors on monocytes thus potentially increasing viral infection by virus antibody complexes (Kontny *et al.*, 1988).

Soluble mediators

High serum levels of soluble CD8 (sCD8), sCD4, IL-2 receptors (sIL-2R) are seen in children with DHF when compared to DF (Kurane *et al.*, 1991). TNF receptors (sTNFR-II)

are higher in individuals with DENV induced shock than those with DHF but no shock (Bethell *et al.*, 1998). Plasma sCD8, sIL-2R and sTNFR-II are higher in children with DHF than in those with DF (Green *et al.*, 1999a) and plasma sTNFR-II levels peak at 2 days before the onset of plasma leakage with levels correlating with the magnitude of plasma leakage. These observations strongly suggest that release of soluble factors that are associated with immune activation is an important determinant of the severity of disease.

Complement activation

Complement activation by dengue antigen and antibody complexes has been hypothesised to be involved in pathogenesis with high levels of cleaved activated complement forms C3a and C5a detected in DSS patients (Avirutnan *et al.*, 1998) and higher serum levels of complement (C), C3, C4, and C5 are detected during DENV-infections in particularly grade 3 and grade 4 diseases (Bokisch *et al.*, 1973). C5a is a potent chemo-attractant of neutrophils and monocytes (Hopken *et al.*, 1996) and C3a is known to induce histamine release from mast cells, which enhances vascular permeability (Bjork *et al.*, 1985) and thus enhanced complement activation has the potential to both enhance infection and induce the release of vasoactive factors (Fig 1.11).

Platelet activating factors and NO

Platelet activating factor, a known enhancer of inflammatory reactions, is also produced at higher levels during DHF (Yang *et al.*, 1995). NO radical has also been shown to be produced by DENV-infected macrophages, which can suppress DENV replication *in vitro* and has also been suggested to cause disease via oxidative stress (Charnsilpa *et al.*, 2005; Neves-Souza *et al.*, 2005). Serum from DENV-infected individuals also show increases in a range of cytokines discussed below at different stages of infection.

Cytokines

Increased levels of cytokines, including IL-2, IL-6, IL-8, IL-10, IL-13, IL-18, TNF-α, IFN-γ have been observed in patients with DENV-infection compared with uninfected individuals (Kurane *et al.*, 1991; Hober *et al.*, 1993; WHO, 1997; Avirutnan *et al.*, 1998; Raghupathy *et al.*, 1998; Green *et al.*, 1999a; Chaturvedi *et al.*, 2000; Juffrie *et al.*, 2001; Mustafa *et al.*, 2001; Gagnon *et al.*, 2002; Suharti *et al.*, 2003; Nguyen *et al.*, 2004). Further DHF patients have even higher levels of TNF-α, IL-6, IL-8, IL-10, IL-13 and IL-18 compared to DF patients (Hober *et al.*, 1993; Avirutnan *et al.*, 1998; Raghupathy *et al.*, 1998; Green *et al.*,

1999b; Chaturvedi *et al.*, 2000; Juffrie *et al.*, 2001). A summary of cytokine changes during DF and DHF is presented in Table 1.4.

In vitro a number of cells and cell lines, including DENV-infected monocytes, B lymphocytes T cells, mast cells and dendritic cells, produce cytokines or chemokines in response to DENV-infection. The cytokines produced by each cell type is summarised in Table 1.5. Two factors of particular note are the cytokines IFN-γ and TNF-α.

The importance of the IFN response in DENV pathology is highlighted by the severe course of disease in IFN knockout mice and the viral subversion of this pathway (section 1.8.2.3). TNF-α is also central to DENV pathology (see 1.8.2.4). Proposed pathogenesis of DENV-infection of monocytes and dendritic cells as a result of the two cytokines TNF-α and IFN-γ and NO radicals are shown in Fig 1.12 (Clyde *et al.*, 2006).

1.8.2.3 IFN response in DENV-infection

IFNs play an important role in the first line of defence against viral infections and are important regulators of innate and adaptive responses. It is well established that IFN levels markedly increase in response to viral infection including that of DENV and that some viruses are able to counteract IFN anti-viral effects (Diamond *et al.*, 2000a; Polyak *et al.*, 2001). HCV, a member of the family *Flaviviridae,* is one of the most well studied viruses in regards to IFN because IFN is used as the main anti-viral therapeutic against HCV infections. HCV has evolved multiple mechanisms to inhibit anti-viral activity. Expression of HCV proteins can block IFN-α induced gene transcription responses by inhibiting signal transduction through the JAK-STAT pathway which is critical for IFN signalling (Heim *et al.*, 1999). Both HCV, NS5A and E2 proteins have been shown to interact with and inhibit the function of protein kinases, (reviewed in (Katze *et al.*, 2002)). HCV core protein expressed in HepG2 and Huh7 cells inhibits IFN-α induced nuclear STAT1 import (Melen *et al.*, 2004) and the HCV NS3/4A protease inhibits IRF-3 phosphorylation which subsequently inhibits RIG-I dependent signalling in the IFN pathway (Foy *et al.*, 2003; Breiman *et al.*, 2005). Thus, several HCV proteins can inhibit at multiple stages of the IFN signalling pathway.

The IFN response is also a very important anti-viral pathway to reduce DENV-infection. DENV challenge of knock out mice that lack IFN receptors develop limb paralysis and die in comparison to the mild disease seen in wild type mice (Johnson and Roehrig, 1999; Shresta *et*

Cytokines	DF	DHF
IL-Iβ	→	→
IL-2	↑↑	↑
IL-4	↓	↑↑
IL-6	↑	↑↑
IL-8	↓	↑↑
IL-10	↓	↑↑
IL-12	↑↑	↓
IL-13	↓	↑↑
IL-18	↑	↑↑
TNF-α	↑↑	↑↑
IFN-γ	↑↑	↑
TGF-β	↓	↑↑
hCF	↑	↑↑

→, no change; ↓,decrease; ↑,increase,↑↑,marked increase

Table 1.4. Cytokines levels in patients with dengue (Chaturvedi *et al*., 2000).

cell type/ cell line	Cytokine released when infected with DENV	Reference
macrophages	TNF-α, IFN-α, IL-Iβ, IL-8, IL-12, MIP-1α, RANTES	(Chen *et al*, 2002,Hober *et al*., 1996 ,Lee *et al*., 1996,Espina, *et al*., 2003,Carr *et al*., 2003)
dendritic cells	TNF-α, IL-10, IFN-α,IL-12 p70,	(Palmer *et al*., 2005, Marovich *et al*., 2001, Ho *et al*., 2001,Libraty *et al*., 2001)
B cells	TNF-α, IL-6	(Lin *et al*., 2002)
T cells	TNF-α, TNF-β, IFN γ,	(Gagnon *et al*., 1999, Mangada *et al*., 2005, Kurane *et al*., 1989)
endothelial cells	TNF-α, IL-8, IL-6, RANTES	(Avirutnan *et al*.,1998,Huang, *et al*., 2000)
Kupffer	TNF-α, IFN-α,IL-6,	(Marianneau *et al*., 1999)
mast	IL-1β, IL-6,RANTES, MIP-α, MIP-β	(King *et al*., 2000, King *et al*., 2002)
skin fibroblast	IFN-β, IL-6, GM-CSF	(Kurane *et al*., 1992)

Table 1.5. Cytokines released by DENV permissive cells and cell lines – *in vitro*

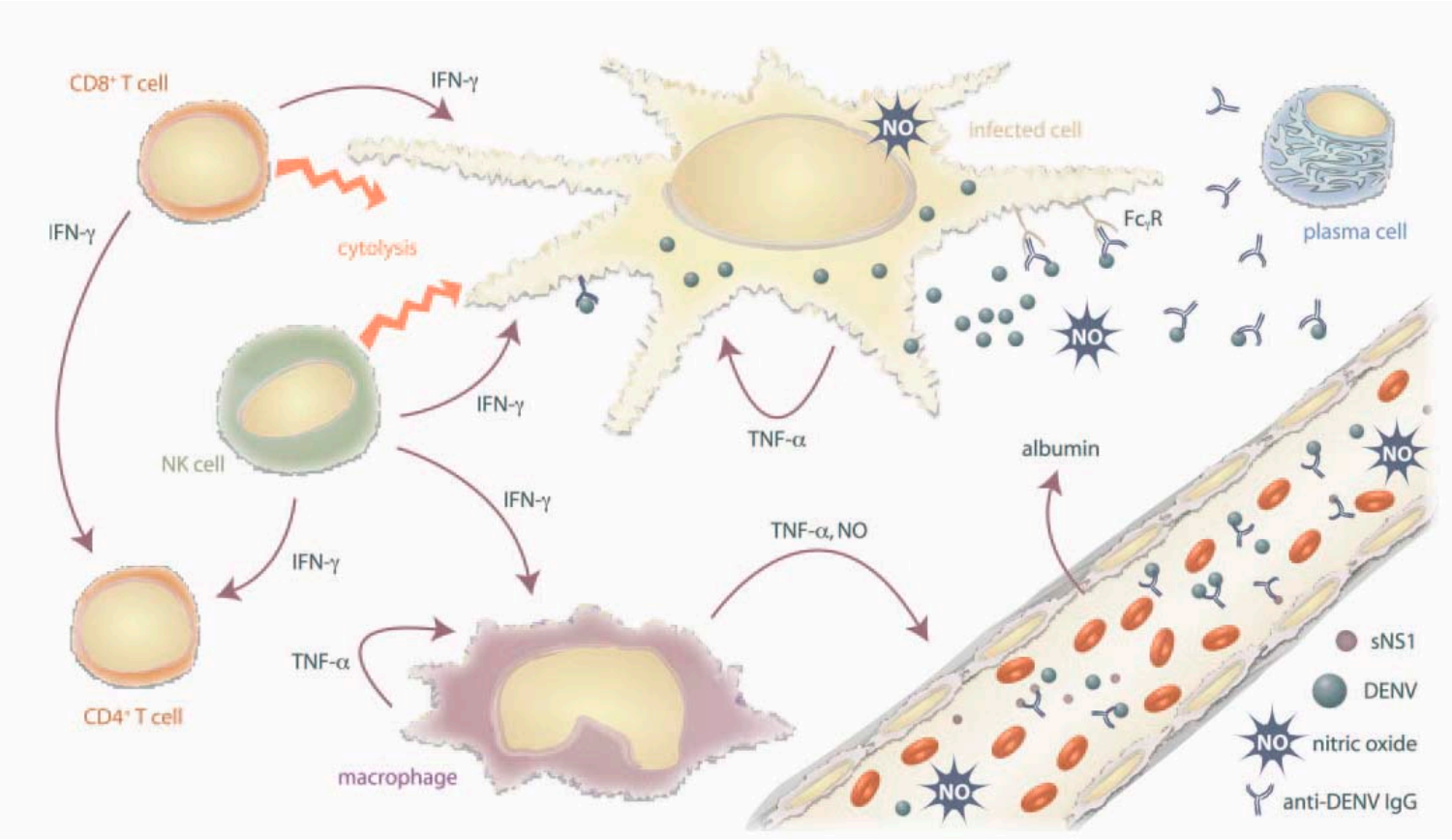


Fig 1.12. Pathogenesis of DENV infection after infection of dendritic cell/macrophage/monocyte line. TNF-α and NO are produced by macrophages and can activate endothelial cells which is responsible for increasing vascular permeability. IFN-γ activates macrophages as well as CD4+ T cells. High levels of DENV have been shown to circulate as immune complexes. (Clyde *et al*., 2006).

al., 2004). *In vitro* pre-treatment of cultured cells with IFN reduces subsequent DENV replication through inhibition of DENV RNA translation (Diamond *et al.*, 2000b; Diamond and Harris, 2001). These studies suggest that the innate immune response is activated by DENV-infection to produce IFN that has anti-viral effects on DENV replication. However there is growing evidence to suggest that similar to that seen for HCV, DENV has evolved mechanisms to evade the anti-viral effects of IFN. Treatment of DENV-infected cells with IFN as little as four hours after infection resulted in almost complete loss of the IFN anti-viral effect (Diamond *et al.*, 2000b) suggesting that once infection is established the anti-viral effects of IFN become less effective. Expression of either DENV NS2A, NS4A or NS4B can block IFN-stimulated signal transduction cascade by interfering with STAT1 function (Munoz-Jordan *et al.*, 2003). NS4B in particular is strongest in blocking the IFN induced signal transduction in response to IFN-β and IFN-γ in monkey kidney cells. The other two proteins NS4A and NS2A also block IFN signalling but to a much lesser extend than NS4B and all three proteins in synergy block IFN signalling completely. DENV-infection itself can inhibit IFN-α mediated signal transduction by decreasing phosphorylation of STAT2 (Jones *et al.*, 2005) which are key component of the IFN-α signalling pathway. Thus collectively these studies show that DENV-infection and expression of some DENV proteins can inhibit the effects of IFN, a key mediator of the innate anti-viral response, and thus once infection and viral protein production is established IFN, has reduced effects on the established replication of DENV.

1.8.2.4 TNF-α and DENV-infection

TNF-α production is increased during DENV-infection in vivo and in vitro

Increased circulating TNF-α levels are found in patients with DENV-infection, with even higher levels associated with more serious forms of disease as summarised in Table 1.4 (Hober *et al.*, 1993; Green *et al.*, 1999a; White, 1999; Kittigul *et al.*, 2000). Exposure of monocytes/macrophages to DENV particles or DENV proteins has been suggested to be responsible for the enhanced production of TNF-α in DENV-infected patients (Hober *et al.*, 1996b). Increased TNF-α release in response to DENV-infection of monocyte/macrophage cultures has been observed in our lab and by others (Lee *et al.*, 1996; Chen and Wang, 2002; Carr *et al.*, 2003; Espina *et al.*, 2003). As shown in Table 1.5 many cells types release TNF-α when infected or exposed to DENV *in vitro.*

TNF-α plays a role in pathogenesis in mouse models of DENV-infection

DENV challenge of BALB/c mice (haplotype H-2d) with a mouse adapted DENV strain results in clinical symptoms of severe DENV disease, including hemorrhagic shock with 100% mortality rate (Atrasheuskaya *et al.*, 2003). In this mouse model of DENV-infection levels of TNF-α increased abruptly and steeply shortly before death, and treatment of these animals with anti-TNF-α antibody reduced mortality rate to 40%. Recent studies using a new DENV strain D2S10, which mimics human DENV disease including viremia and increased vascular permeability in mice, have shown that neutralisation of TNF-α acitivity prevented early death of infected mice confirming that TNF-α is one of the key cytokines responsible for severity of DENV diseases and lethality in this model of DENV infection (Shresta *et al.*, 2006). Using an immunocompetent mouse model of DENV-infection (C57BL/6) it has been recently shown that high virus levels, macrophage infiltration and increased TNF-α levels are three important factors associated with haemorrhage in DENV-infection (Chen *et al.*, 2007).

TNF-α plays a role in endothelial cell activation and permeability in vitro

In vitro studies have shown that exposure of human endothelial cells to culture fluids from DENV-infected monocytes or macrophages triggers endothelial cell activation and induction of endothelial cell permeability (Anderson *et al.*, 1997a; Carr *et al.*, 2003) which can be reversed by treating with anti-TNF-α antibodies (Anderson *et al.*, 1997a; Cardier *et al.*, 2005).

Together these data show that circulating TNF-α levels are high in DENV-infection and are involved in the pathogenesis of disease, potentially by direct effects on the endothelium; however, the effect of these high levels of TNF-α on DENV replication are unknown.

Anti-viral effects of TNF-α in other viral systems

TNF-α is a powerful pro-inflammatory cytokine with pleiotropic properties. It is known to have anti-viral effects on several viruses and can contribute to virus clearance (reviewed extensively in (Herbein and O'Brien, 2000)). For example TNF-α is associated with viral clearance in Hepatitis B virus (HBV) transgenic mice by inhibiting HBV gene expression and accelerating the degradation of HBV mRNA (Gilles *et al.*, 1992; Guilhot *et al.*, 1993; Chisari, 2000). Endogenously released TNF-α plays an integral part in cytomegalovirus (CMV) clearance, since administration of neutralising TNF-α antibodies to immunocompetent and

CD8 T cell deficient mice abolished the anti-viral activity of CD4 T cells (Pavic *et al.*, 1993). TNF-α has also been shown to suppress HIV-1 replication in PBMC and alveolar macrophages (Lane *et al.*, 1999), and TNF-α can protect mice from infection with herpes simplex virus type 1 (HSV1) (Rossol-Voth *et al.*, 1991). Further, TNF-α knockdown mice showed increased susceptibility to primary corneal infection with HSV1 and increased virus spread, while TNF-α deprivation also significantly prompted the re-activation of HSV1 in latently infected trigeminal ganglia explants (Minagawa *et al.*, 2004). Challenge of mouse embryonic fibroblast cells with West Nile virus, another *Flavivirus*, has shown that cells from TNF-α deficient mice are more susceptible to West Nile virus infection than cells from wild type mice, suggesting a role for TNF-α in protection against initial West Nile virus infection (Cheng *et al.*, 2004). TNF-α also has been implicated in transiently changing permeability of the blood-brain barrier and hence allowing West Nile virus to cross the central nervous system (Wang *et al*., 2004).

In relation to DENV, however, possible anti-viral effects of TNF-α on DENV replication are less defined. TNF-α is released from MDM after DENV-infection, and the peak of this release coincides with peak of virus production *in vitro* (Carr *et al.*, 2003; Espina *et al.*, 2003). Other cells of the immune system such as B and T cells that functionally interact with monocytes and macrophages during viral infection can also release TNF-α when exposed to DENV (Lin *et al.*, 2002b; Mangada *et al.*, 2002; Mangada and Rothman, 2005). Thus TNF-α released from cells of the immune system may contribute to the elevated circulating (endocrine) levels of TNF-α that may in turn induce viral pathogenesis in addition, TNF-α may act in an autocrine or paracrine manner to modulate viral replication in infected cells. Thus TNF-α can be anti-viral in other viral systems and elevated TNF-α has been implicated to play a role in pathogenesis of DENV but the potential regulatory role of TNF-α in DENV replication needs to be investigated. This is the aim of chapter 4 of this thesis.

1.8.2.5 Host proteins

Viral replication within the infected host cell can alter host cell gene expression. Many changes in circulating mediators and release of cytokines/chemokines from DENV-infected host cells have been characterised (see section 1.8.2.2) leading to the suggestion that DENV disease is primarily a result of immunopathogenesis and that host factors are an important determinant of pathogenesis. In addition to well-characterised changes in circulating host

factors, DENV induced alterations in host *intracellular* responses may also be important. Microarray analysis of RNA extracted from human umbilical vein endothelial cells (HUVEC) that were infected *in vitro* with DENV have shown up to 269 genes that are induced and 126 that are suppressed during DENV-infection (Warke *et al.*, 2003; Liew and Chow, 2006). Similarly DNA microarray analysis of whole blood cells from DENV-infected patients found down-regulation of transcription of IFN-inducible genes in DSS patients compared to response in DHF (Simmons *et al.*, 2007). Recent microarray analysis of both DENV-infected cell line (HepG2) and in DENV patient blood samples have identified a highly up-regulated IFN stimulation gene termed Viperin. Further over-expression of this gene in A549 cells protected against DENV-infection demonstrating high levels of anti-viral activity (Fink *et al.*, 2007). Another novel anti-viral protein called Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) has been shown to be up-regulated in DENV-infected immune cells (monocytes, dendritic and B cells) and HUVECs by assessing gene expression changes using Affymetrix GeneChips (Warke *et al.*, 2008).

Thus microarray analysis has identified many changes in host cell gene expression and the full role of these proteins in disease pathogenesis requires further study. Complementing these microarray studies, part of this thesis (Chapter 5) identifies changes in DENV-infected cells using proteomic studies.

1.9 SUMMARY

DENV-infections of humans is characterised by immunopathology (i.e. diseases caused by the host's response to infection). Understanding how the infected host cell regulates and responds to DENV-infection may lead to better understanding of the mechanisms of DENV pathology and to therapeutic avenues to counter DENV disease.

We know that

- Monocytes and macrophages are an important target cell for DENV replication *in vivo* and *in vitro*.
- TNF-α has an anti-viral role in a number of other viruses and plays a role in inducing DENV pathology but the potential anti-viral role of TNF-α against DENV replication is not known.

- While there are many reports of changes in the release of circulating factors during DENV-infection only limited studies, mainly by microarray analysis, have assessed the *intracellular* host response to DENV-infections.

This study looks at the potential anti-viral role of TNF-α and host response at a cellular level to DENV replication in an important cell type the MDM

1.10 HYPOTHESIS AND AIMS

DENV-infection of MDM induces host cell responses including the release of TNF-α that may enhance or counteract viral replication.

We specifically aim to:

(1) Investigate the role of TNF-α, in regulating DENV replication.

(2) Identify other novel host-cell responses to DENV-infection in MDM.

1.11 OUTCOMES

Throughout my PhD I have achieved

- Successful establishment of a RT real-time PCR assay to quantitate (+ ve) and (- ve) strand DENV RNA (Chapter 3).
- Further characterisation of the DENV – MDM *in vitro* infection model (Chapter 4).
- Successful application of the DENV quantitative RT real-time PCR technique and MDM infection model to analyse TNF-α regulation of DENV replication in MDM (Chapter 4).
- Establishment of techniques for 2D analysis of changes in the proteome in DENV-infected cells (Chapter 5)

This project has the following major novel findings:

1. Addition of exogenous TNF-α or blocking of endogenous TNF-α from DENV-infected MDM has no effect on DENV replication.
2. DENV-infected MDM do not respond normally to TNF-α-stimulation
3. DENV-infection of MDM induces GRP78 and HSP70 expression.

Points 1 and 2 have been published in Wati *et al*., Journal of Virology, 2007 vol. 81 (18), 10161-71.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Antibodies and dilutions used

TNF-α blocking experiments

Mouse anti-human TNF-α monoclonal antibody (2.5 μg/ml) (Pharmingen, catalogue number 18630D)

Mouse IgG isotype control (2.5 μg/ml) (Pharmingen catalogue number 20800D)

DENV primary detection antibody

Mouse monoclonal DENV anti-E antibodies (1/50) (a gift from Prof. Peter Wright, Monash University)

DENV positive patient sera (1/5000)

Secondary conjugated antibody for confocal microscopy

Goat anti-mouse Alexa Fluor 546 (1/200) (Molecular Probes)

Goat anti-rabbit Alexa Fluor 488 (1/200) (Molecular Probes)

Goat anti-rabbit Alexa Fluor 647 (1/200) (Molecular Probes)

Rabbit anti-goat Cy^{TM}2 (1:150) (Jackson Immunoresearch Laboratories)

Donkey anti-mouse Cy^{TM}5 (1:400) (Jackson Immunoresearch Laboratories)

Other antibodies for confocal microscopy

Rabbit anti-HSP70 polyclonal antibody (1/100) (Chemicon catalogue number AB3470)

Goat anti-GRP78 polyclonal antibody (1/50) (Santa Cruz Biotechnology catalogue number C-20, a gift from Prof James Paton, University of Adelaide)

Rabbit p65 antibody (1/50) (Abcam catalogue number ab7970, a gift from Dr Michael Beard, University of Adelaide)

Western blot primary antibody

Rabbit anti–HSP70 polyclonal antibody (1/200) (Chemicon catalogue number AB3470)

Mouse anti-actin monoclonal antibody (1/100000) (Chemicon catalogue number MAB1501R)

Western blot secondary conjugated antibody

ImmunoPure goat anti-mouse IgG, (H+L), perioxidase conjugated (Pierce Biotechnology catalogue number 31430)

ImmunoPure goat anti-rabbit IgG, (H+L), perioxidase conjugated (Pierce Biotechnology catalogue number 31460)

2.1.2 Cells

African green monkey kidney (Vero) cells (a gift from Dr Tuckweng Kok, IMVS, Adelaide) human hepatoma cells Huh7 and HepG2 cells (a gift from Dr Karla Helbig, Hepatitis C Lab, University of Adelaide), monocytic cell lines K562 and U937 (a gift from Mr John Mackrill, School of Molecular Biosciences, University of Adelaide) were used for DENV-infection studies, mouse fibroblast cells L929 (a gift from Dr Kerrie Diener, Hanson Institute, Adelaide) were used in the TNF-α bioassay and *Aedes albopictus* C6/36 (American Type Culture Collection, ATCC) were used to prepare DENV stocks.

2.1.3 Commercial kits

OptEIA^{TM} human TNF-α ELISA set (Pharmingen catalogue number 2637KI)

RNeasy mini kit (Qiagen catalogue number 74104)

RC DC^{TM} protein assay kit I (Biorad catalogue number 500-0121)

2.1.4 Commonly used buffers and solutions

Cell culture media and buffer

MDM adherence media

Dulbecco modified Eagle medium (DMEM, Gibco BRL) supplemented with 20% (v/v) Fetal Bovine Serum (FBS) (JRH, Biosciences), 1.2 μg/ml penicillin, 1.6 μg/ml gentamicin, 2 mM L-glutamine and 10 mM Hepes

MDM culture media

DMEM supplemented with 10% (v/v) FBS, 7.5% (v/v) human serum (Australian Red Cross Blood Bank), 1.2 μg/ml penicillin, 1.6 μg/ml gentamicin, 2 mM/L-glutamine and 10 mM Hepes

Hanks buffers

Hanks balanced salts (HBSS) – without $CaCl_2$ and $MgSO_4$ (USBiological catalogue number H1800)

Hanks balanced salts (HBSS+) – with $CaCl_2$ and $MgSO_4$ (Sigma catalogue number H1387)

Media for culturing HepG2, Huh7 and Vero cells

DMEM supplemented with 10% (v/v) FBS (5% (v/v) for vero cells), 1.2 μg/ml penicillin, 1.6 μg/ml gentamicin, 2 mM L-glutamine and 10 mM Hepes

Media for culturing K562 and L929 cells

RPMI 1640 media (Gibco BRL) supplemented with 10% (v/v) FBS, 1.2 μg/ml penicillin, 1.6 μg/ml gentamicin, 2 mM L-glutamine and 10 mM Hepes

Media for culturing C6/36 cells (Eagle's basal medium)

Earles salts (BME) (Gibco BRL) supplemented with 7.5 % (v/v) FBS, 1.2 μg/ml penicillin, 1.6 μg/ml gentamicin, 2 mM L-glutamine, 1mM Sodium pyruvate, 0.1 mM of MEM non essential amino acids solution (Gibco)

Buffers for DNA/RNA

DNA gel loading buffer

60% (w/v) Glycerol, 100 mM EDTA (pH 8.0); 100 mM Tris base (pH 7.5), 0.416% (w/v) Bromophenol blue and 0.416% (w/v) Xylene cyanol

DNase buffer (1x) for RNA

0.1 M sodium acetate, 5 mM $MgSO_4$, pH 5.0

Ethidium bromide stock solution

10 mg/ml ethidium bromide (Sigma) dissolved in dH_2O. Stored at 4^0C in a dark bottle.

Phenol

Tris base-equilibrated phenol was prepared according to supplier's instructions (Sigma)

TBE (1x)

90 mM Tris base, 90 mM boric acid, 2.4 mM disodium EDTA (pH 8)

Buffers for 2D gel analysis

Cell Lysis buffer

10 mM Tris-HCl, pH 7.4, 5 mM $MgCl_2$, 0.5% Triton X-100 (v/v) (Sigma), 20 μg/ml Aprotinin (Sigma) and 1 mM DTT (Sigma)

Equilibration Base Buffer

6M Urea, 2% (w/v) SDS, 0.05M Tris-HCl, pH 8.8, 20% (v/v) glycerol

Rehydration Buffer (containing multiple surfactant solution)

5M urea, 2M Thiourea, 40 mM DTT, 2% (v/v) Chaps, 2% (v/v) SB 3-10 (3-Decyldimethylammonio–propanesulfonate) (Fluka), 40 mM Tris base, 0.0002% Bromophenol blue, 0.2% (w/v) ampholytes 3-10 (Biorad).

Buffers for western blots

Blocking Buffer

5% (w/v) skim milk powder, diluted in 0.1% (v/v) Phosphate buffered Saline (PBS)-Tween

Tris glycine electrophoresis buffer

25 mM Tris base, 250 mM glycine, 0.1% (w/v) SDS (pH 8.3)

Western blot stripping solution

50 mM Tris pH 7.5, 2% SDS (w/v), 0.25% (v/v) Beta mercaptoethanol

Western blot transfer buffer

24 mM Tris base, 186 mM glycine, 20% (v/v) methanol (pH 8.0)

10% SDS PAGE gels

Two phase SDS PAGE gels (9 x 8cm), consisted of a 12.5% (v/v) acrylamide resolving gel and 5% (v/v) acrylamide stacking gel as described in Sambrook *et al.*(1989). The formulation of acrylamide to cross-linker Bis acrylamide ratio used was 29:1 as per Biorad Acrylamide and Bisacrylamide solution preparation instructions.

Others

PBS

140 mM NaCl, 3 mM KCl, 1 mM KH_2P0_4, 8 mM Na_2HPO_4 (pH 7.4)

2.1.5 Oligonucleotide sequences

All oligonucleotides were synthesised by Geneworks (www.geneworks.com.au). Dried oligonucleotide pellets were resuspended in sterile water to final stock concentrations of 200 pmol/µl before further dilutions to working concentrations (generally 20 pmol/µl). The sequences and nucleotide positions of the oligonucleotide primers used in this study are presented in Table 2.1.

Table 2.1 Primers

Name	Sequence 5'→ 3'	Co-ordinate
CAPECORI F[a]	CACGAATTC/AGCTCAACGTAGTTCTAACAG	50-70
CAPBAMHI R[a]	CGTGGATCC/GATCATGTGTGGTTCTCCGTT	477-457
CAPF 5' F[a]	GCAGATCTCTGATGAATAACCAAC	86-109
CAPR 3'[a]	GTTCTGCGTCTCCTGTTCAAG	398-378
DENV5.1 [a]	GCAGATCTCTGATGAATAACCAAC	86-109
CAP 3.1 [a]	GTTCTGCGTCTCCTGTTCAAG	398-378
CAP 5.2 [a]	AGCTCAACGTAGTTCTAAC	50-68
DENV3.2 [a]	TTGTCAGCTGTTGTACAGTCG	187-167
CYCLOPHILIN F[b]	GGCAAATGCTGGACCCAACACAAA	383-406
CYCLOPHILIN R[b]	CTAGGCATGGGAGG GAACAAGGAA	737-714
TNF F[c]	CCCCAGGGACCTCTCTCTAATC	358-379
TNF R[c]	GGTTTGCTACAACATGGGCTACA	455-433
TAG[d]	CGGTCATGGTGGCGAATAA	

[a] (Robyn Taylor, University of Adelaide) – Genbank accession number AF038403

[b] (Peter Brautigan, Institute of Medical and Veterinary Science) - Genbank accession number NM_021130

[c] (Boeuf *et al.*, 2005) Genbank accession number NM_000594

[d] (Peyrefitte *et al.*, 2003)

2.1.6 TNF-α siRNA

TNFα siRNA was synthesised by Invitrogen. Dried siRNA pellets were resuspended in DEPC-treated water (Invitrogen) to 20 µM concentration and stored at -20^{0}C. The sequence of siRNA is shown in Table 2.2

Table 2.2 TNFα siRNA sequences

Catalogue number	Sequence 5'→ 3'
HSS110854(3 RNAi)	UCG AGA AGA UGA UCU GAC UGU CUG G
	CCA GGC AGU CAG AUC AUC UUC UCG A
HSS110855(3 RNAi)	UUA UCU CUC AGC UCC ACG CCA UUG G
	CCA AUG GCG UGG AGC UGA GAG AUA A
HSS110856(3 RNAi)	AGA CUC GGC AAA GUC GAG AUA GUC G
	CGA CUA UCU CGA CUU UGC CGA GUC U

2.2 CELL CULTURE

2.2.1 Isolation and culture of primary MDM

Monocytes from healthy donor buffy coat blood packs (Australian Red Cross Blood Bank) were isolated by adherence as previously described (Pryor *et al.*, 2001). Briefly, buffy coats (usually 20-50 ml) were diluted to 100 ml with PBS and 25 ml was gently layered onto 20 ml lymphoprep (Nycomed Pharma) in four 50 ml centrifuge tubes. Gradients were centrifuged at 2000 rpm in a Heraeus Multifuge 3 S-R for 20 mins with low brake. The PBMCs form a visual band that was carefully collected and washed 3 times with PBS to completely remove lymphoprep and minimise platelet contamination. Cells were enumerated and resuspended at 1 x 10^7 cell/ml in pre-warmed MDM adherence media (section 2.1.4) in 75 cm^3 tissue culture flasks (Nunc) at 37^0C in 5% CO_2 for 1 hr. Non-adherent cells were further depleted of monocytes by 3-4 rounds of adherence, as above. Adherent monocytes were washed 3-6 times with HBSS+ (section 2.1.4) to remove any non-adherent cells and cultured in MDM culture media (section 2.1.4) overnight. Day one post isolation monocytes were gently detached by cell scraping in HBSS (section 2.1.4). Adherent cells from 4 buffy coat packs were pooled, re-plated and cultured in fresh MDM culture medium (section 2.1.4) for 5 days. On the fifth day after isolation, cells were strongly adherent and had differentiated into macrophages (MDM). Cells were detached and seeded in 48 well plates at a concentration of 2 x 10^5 cells per well and allowed to adhere for one day. This method of MDM isolation has been previously shown to yield cells that are 85-90% $CD14^+$ positive by flow cytometry with macrophage morphology by giemsa staining (Pryor *et al.*, 2001).

2.2.2 Cell lines and cell culture

Vero, Huh7 cells, HepG2, L929, U937 and K562 cells were maintained in 75 cm^3 tissue culture flasks (Greiner, Germany) at 37^0C in 5% CO_2. These cell lines were passaged every 2-3 days. Vero, HepG2, and Huh7 were cultured in DMEM (section 2.1.4), K562, U937 and L929 cells in RPMI (section 2.1.4) at 37^0C in 5% CO_2. C6/36 cells were grown in Eagle's basal medium (section 2.1.4) at 28^0C in 5% CO_2.

2.3 DENV-INFECTION OF CELLS

2.3.1 Preparation and titration of DENV stock

The strain of DENV-2 used was a laboratory clone of the dengue virus strain New Guinea C (MON601) obtained from Prof Peter Wright, Monash University (Gualano *et al.*, 1998; Pryor *et al.*, 2001) (Appendix 1). To generate DENV stocks pMON601 was *in vitro* transcribed using mMessage mMachine®T7 Kit (Ambion catalogue number AM1344) as per manufacture's instructions. 40 ng of *in vitro* transcribed DENV RNA was transfected into BHK cells using 4 µl of Enhancer reagent and 8 µl TransMessenger reagent (TransMessenger Transfection Reagent kit; Qiagen catalogue number 301525). *In vitro* transcription and transfection was performed by Mr Carl Coolen, Research assistant, IMVS. Supernatant from transfected cells were collected, filtered and amplified in C6/36 cells. Infected cells were observed daily by light microscopy for visual CPE. This was usually visible at day 4 post-infection. After visualisation of CPE, infected cells were resuspended in fresh media and supernatant collected daily for 3 days to produce 3 separate stocks of infectious DENV. The collected supernatant was clarified by centrifugation, filtered, aliquoted into 1 ml aliquots and stored at -80^0C. The viral titer was determined by plaque assay using Vero cells as described in section 2.3.2.

2.3.2 Quantitation of infectious virus by plaque assay

Cell culture supernatants from infected cells were assayed for infectious DENV by plaque assay. Vero cells were plated at 3 x 10^5 cells per well in 6 well plates (Falcon) containing 3 ml of media (section 2.1.4) and cultured overnight. Cells were washed with serum free DMEM and infected with 300 µl of serially diluted (10^{-1}-10^{-6}) samples. Following infection, inoculum was removed, cells washed using serum free DMEM and overlayed with 4 ml of a 1:1 mix of 2% metaphor agarose (BioWhittaker Molecular Applications) prepared in water (sterilised by autoclaving) and 2 x DMEM + 10% (v/v) FBS. Agarose was allowed to set and

plates were inverted and incubated at 37^0C with 5% CO_2, for 5 days. On day 5, cells were overlayed with 2 ml of 1:1 mix of 2 x DMEM containing 10% (v/v) FBS, 0.03% (w/v) neutral red (MP Biomedicals) and 2% (w/v) metaphor agarose. Agarose was allowed to set, inverted and incubated till plaques were visible (approximate 5 days). Plaques were counted at dilutions yielding 10-100 pfu/well and levels of infectious virus were quantitated as plaque forming units per ml (pfu/ml). The reproducibility of plaque assay was determined by multiple measurement of the same sample (n=3) in two different experiments, yielding 5-10% variation from the mean, the plaque assay routinely detected > 50 pfu/ml.

2.3.3 Culture and infection of MDM and cell lines with DENV-2 (MON601)

MDM infection

MDM were plated the day prior to infection at 2 x 10^5 cells per well in a 48 well plate (described in section 2.2.1), washed with serum free DMEM and then infected with DENV at a MOI of 5, in a volume of 100 µl for 90 mins at 37^0C with intermittent rocking of the plate as previously described (Pryor *et al.*, 2001). Mock-infected controls were performed by infecting, as above, with equal volumes of heat inactivated virus (80^0C for 20 mins). After infection, the cells were washed, cultured in 400 µl MDM culture media (section 2.1.4) and one-half the volume of fresh medium was sampled and replaced at each time point (unless stated otherwise).

Infection of the adherent cell lines:- HepG2 and Huh7 cells

HepG2 and Huh7 cells were seeded at 2 x 10^5 cells per well in a 12 well plate, one day before infection. Cells were washed as described for MDM infection, and infected with DENV (MOI) of 5, in a volume of 200 µl of serum free DMEM for 90 mins at 37^0C with intermittent rocking of the plate as described for MDM. Mock-infected controls were performed as described for MDM (section 2.3.3) using an equal volume of heat inactivated virus (80^0C for 20 mins). After infection, the cells were washed, cultured in 1 ml HepG2 and Huh7 media (section 2.1.4) and one-half the volume of fresh medium was sampled and replaced at each time point.

Infection of PMA differentiated U937 cells

U937 cells were seeded in a 24 well plate at 5 x 10^5 cells/well in the presence of PMA (6ng/ml) to stimulate differentiation into macrophage-like cells. 48 hrs after PMA addition

the cells became adherent and were washed twice with serum free medium and infected with DENV at a MOI of 10 in a 150 µl volume for 2 hrs at 37^0C with intermittent rocking. Mock-infected controls were performed as above for HepG2 and Huh7 cells. After infection, the cells were washed, cultured in U937 culture media (section 2.1.4) and one-half of the volume of fresh medium was replaced at each sample point. In a separate experiment cells were trypsinised at 48 hrs post-infection and fixed onto slides at concentrations of 10^5 cells per well for confocal staining (section 2.6.7).

Infection of non adherent K562 cells

K562 (non adherent) cells at concentrations of 5 x 10^6 cells were washed and suspended in 500 µl of media containing 1% (v/v) FBS in four separate wells (for sampling at 4 different time points) and infected with DENV at a MOI of 5 in 500 µl volume for 2 hrs at 37^0C with intermittent rocking. Mock-infected controls were performed as above using an equal volume of heat inactivated virus (80^0C for 20 mins). Cells were washed twice and resuspended at a concentration of 2 x 10^5 cells in K562 cell media (section 2.1.4) in cell culture flasks. At time points 0, 24, 48 and 72 hrs all media containing cells were removed from each well, clarified by centrifuging at 6000 g, and supernatant stored at -80^0C until ready for plaque assay. Cells were also fixed onto slides at concentrations of 10^5 cells per well for confocal staining (section 2.6.7).

2.3.4 Characterisation of DENV-infected MDM

2.3.4.1 Preparation of opsonised Sheep Red Blood Cells (SRBC)

SRBC (obtained from Garry Penny, University of Adelaide) were washed 3 times by resuspending in PBS and centrifuging at 2000 rpm in a Heraeus Multifuge 3 S-R for 10 mins. A 0.2% suspension of the SRBC in HBBS+ was incubated with healthy human sera diluted 1:1000 (Australian Red Cross Blood Bank) at 37^0C for 1 hr with intermittent rocking. SRBC suspension were washed twice with HBBS+ and resuspended in 10 ml of DMEM and used for phagocytosis assay.

2.3.4.2 Phagocytosis

MDM were either DENV or mock-infected as described in section 2.3.3 and 24 hrs post-infection cells were washed twice with warm HBBS (section 2.1.4). 200 µl DMEM without

FBS or human sera + 200 µl of opsonised SRBC (2.3.4.1) was added to the cells and incubated at 37^0C for 24 hrs. Cells were then washed 5 to 6 times with warm HBBS to remove any SRBC that were not ingested by MDM and either stained with haematoxylin (Sigma) and observed using a light microscope or fixed and stained for DENV antigens using DENV anti E monoclonal antibodies and Alexa Fluor 546 (as described in section 2.5.8) and observed using a Fluorescent microscope.

2.4 EXTRACTION OF RNA AND REVERSE TRANSCRIPTION (RT) REAL-TIME PCR TECHNIQUES

2.4.1 Generation of DENV capsid construct and *in vitro* transcribed RNA

The DENV-2 capsid (CAP) region was PCR amplified from full length, infectious clone MON601 (Appendix 1) with primers CAP*ECOR1* and CAP*BAMHI* 3', and cloned into pGEM-3Zf(-) (Promega) which contained a T7 forward promoter and an SP6 reverse promoter (Appendix II). Cloning was performed by Ms Robyn Taylor, Honours student, University of Adelaide. For generation of positive (+ ve) strand RNA, the pGEM-DENV-2CAP was linearised with *Hind III* and *in vitro* transcribed with T7 RNA polymerase. For generation of negative (- ve) strand RNA, the pGEM-DENV2capsid was linearised with *EcoRI* and *in vitro* transcribed with SP6 RNA polymerase. Both *in vitro* transcription reactions utilised Ambion maxiscript^{TM} following the manufacturer's instructions. The *in vitro* transcribed RNA was purified using an RNeasy RNA extraction kit (Qiagen) as per manufacturer's instruction and quantified by a spectrophotometer. RNA was immediately aliquoted and stored at -70^0C for use as standards, and RNA copy number calculated using the following equation:

$$\text{RNA standard copy number} = \frac{\text{Amount of standard RNA (grams)} \times C}{\text{RNA size (base)} \times M}$$

Where: C= Avogadro's constant (6.022×10^{23} molecules)

M=Molecular weight of a single base (330 daltons)

2.4.2 RNA extraction

Total RNA was isolated from DENV-infected or mock-infected cells using Trizol (Invitrogen) following the manufacturer's instructions. The RNA was DNase treated with 2 units of RNase free DNase1 (Roche) in 0.1 M sodium acetate, 5mM $MgSO_4$ pH 5 with 10 U RNase

inhibitor (Roche), incubated for 15 mins at 37^0C. The DNA free RNA was extracted with an equal volume of phenol/chloroform (BDH), ethanol precipitated at -20^0C with 1/10 volume of 3M sodium acetate (BDH) and 2 volumes of ethanol (Ajax, Finechem). Extracted RNA was washed with 70% ethanol air-dried and resuspended in RNase free water with 10 U RNase inhibitor and quantified by a spectrophotometer.

2.4.3 Tagged RT real-time PCR for (+ ve) and (- ve) DENV RNA strands

Isolated RNA was reverse transcribed (RT) and tagged as follows: RNA was denatured at 65^0C for 3 mins in presence of 20 pmole of DENV specific primer attached to a 19-mer-long sequence (Tag) as described in Peyrefitte *et. al*., 2003 (Table 2.1). The primer sequence for the DENV (+ ve) strand RNA was Tag(DENV3.2) and for the DENV (- ve) strand RNA was Tag(DENV5.1) (Table 2.1). 10 µl of denatured RNA (approx 100 ng) was added to an RT mixture containing 10 U M-MuLV (New England Biolabs (NEB)), 10 U RNase inhibitor, 0.5 mM each dNTP (Promega) in 1 x M-MuLV buffer (New England Biolabs (NEB)) and RNase free water up to 20 µl. Known amounts of (- ve) and (+ ve) strand *in vitro* transcribed DENV RNA were RT in parallel with the extracted RNA from infected cells to quantitate RNA copies in the samples. RT reactions were performed at 37^0C for 1 hr followed by 95^0C denaturation.

The tagged DENV cDNA was then subjected to real-time PCR as follows: 2 µl of (1:100) diluted cDNA sample was amplified in 1x Quantitect SYBER Green PCR mix (Qiagen) and 0.5 µM of each primer. The DNA primer pair for (+ ve) strand RNA was primer Tag and DENV5.1, and for the (- ve) strand was Tag and DENV3.2. Real-time PCR were performed in a Rotor gene 3000 real-time thermal cycling system (Corbet Research). The cycling parameters were: 50^0C, 2 min; 95^0C, 15 min, (95^0C, 20 sec; 58^0C, 20 sec; 72^0C, 20 sec (acquiring to SYBER Green)) cycled 35 X, 72^0C 30 sec, melt 72-99^0C in 1^0C increments. Data was analysed using Rotor-Gene DNA sample analysis system software; version 4.6.

2.4.4 RT-real time PCR for cyclophilin A mRNA

The RT real-time PCR was normalised against cyclophilin A mRNA levels quantitated in total RNA. This involved RT of RNA, as above except 0.5 µg oligo $(dT)_{15}$ (Promega) was used as primer for cDNA synthesis. RT real-time PCR was performed using primers

cyclophilin (F) and cyclophilin (R), as for DENV except that the cycling parameters involved 40 cycles (94^0C, 20 sec; 60^0C, 20 sec; 72^0C, 30 sec (acquiring to SYBER Green)). Known concentrations of total RNA quantitated by spectrophotometer were used as standards. Cyclophilin mRNA levels did not vary over a three day period in DENV-infected and uninfected macrophages (Appendix III).

2.4.5 RT-real time PCR for TNF-α mRNA

RNA was extracted as described in section 2.4.2 and TNF-α mRNA quantitated. TNF-α mRNA primer sequences used are shown in Table 2.1. RT-PCR was performed by addition of 10 µl of denatured RNA (approx 100 -2000 ng) to an RT mixture containing 20 pmoles of TNF-α (F) primer, 10 U M-MuLV, 10 U RNase inhibitor, 0.5 mM each dNTP (in 1 x M-MuLV buffer) and RNase free water up to 20 µl. RT reactions were performed at 37^0C for 1 hr followed by 95^0C denaturation. 2 µl of (1:100) diluted cDNA sample were used in a real-time PCR reaction containing 1x Quantitect SYBER Green PCR mix (Qiagen) and 0.5 µM of each TNF-α primer. Real-time PCR were performed as described for DENV in section 2.4.3, with the cycling parameters as follows: 50^0C, 2 min; 95^0C, 15 min, (95^0C, 20 sec; 58^0C, 20 sec; 72^0C, 20 sec, 75^0C, 20 sec (acquiring to SYBER Green)) cycled 35 X: 72^0C 30 sec, melt 72-99^0C in 1^0C increments. Representative amplification profile is shown in Appendix IV. Data was analysed as described for DENV by normalising against cyclophilin mRNA (section 2.4.4).

2.5 ANALYSIS OF THE EFFECT OF TNF-α ON DENV-REPLICATION

2.5.1 Treatment of cells with cytokines

HepG2 cells were seeded at 2 x 10^5 cells per well in a 12 well plate, and adherent MDM were seeded at 2 x 10^5 cells per well in a 48 well plate. To pre-treat cells with IFN-α or TNF-α, the medium was removed and the required amount of cytokine added in medium containing 2% FBS and incubated for 4 or 24 hrs prior to infection. IFN-α (Intron A, Schering-Plough Ltd) (gift from Dr Karla Helbig, Hepatitis C Lab, University of Adelaide) was used at 100 IU/ml and TNF-α (Prospec-TechnoGene Ltd) was used at 500 ng/ml. Cells were washed, infected with DENV at a MOI of 5 as described in section 2.3.3. Following infection the cells were again washed and resuspended in fresh media containing 2% FBS (designated as time-point 0).

For post-treatment of cells with IFN-α or TNF-α, the cells were infected as described in section 2.3.3 and cultured for 24 hrs, medium was removed, and the required amount of cytokine as above was added in medium containing 2% FBS and incubated for a further 24 hrs. At 48 hrs post-infection supernatant was collected and assayed for infectious virus production (pfu/ml) and RNA extracted from cell lysates to quantitate DENV RNA.

2.5.2 Blocking TNF-α using TNF-α antibodies

Adherent MDM were seeded at of 2 x 10^5 cells per well in 48 well plates and infected with DENV at MOI 5, as described in section 2.3.3. 24 hrs post-infection all the supernatant from the wells were replaced with MDM culture medium (section 2.1.4) containing either 2.5 μg/ml purified mouse anti-human TNF-α antibody (Pharmingen), purified mouse IgG isotype matched control (Pharmingen), or without any antibody. MDM culture media +/- antibody was again completely replenished at 48 hrs. At 24, 48 and 78 hrs post-infection supernatant was quantitated for virus release (plaque assay), and cells lysed at 78 hrs post-infection and viral RNA quantitated by RT-PCR.

2.5.3 Transfection of siRNAs into MDM

Pre-designed Stealth™ duplex oligonucleotide siRNAs were obtained from Invitrogen. The sequences of the sense and anti-sense strands are shown in Table 2.2. The negative control siRNA used was Stealth™ RNAi negative control duplex "medium GC content" (Invitrogen).

2.5.3.1 siRNA transfection efficiency in MDM

siRNA transfection efficiency was determined by transfecting Block-iT™ fluorescent oligo (Invitrogen) using lipofectamine™ (Invitrogen) as described by the manufacturers. Briefly MDM were seeded at concentrations of 2 x 10^5 cells per well (in 48 well plates), 24 hrs prior to transfection, then incubated in MDM growth media that did not contain any antibiotics. Cationic lipid complexes were prepared by incubating 20 pmoles Block-iT™ fluorescent oligo with 1 μl lipofectamine (Invitrogen) in serum and 100 μl antibiotic free DMEM and then added to cells. After 4 hrs incubation, the cells were washed and replaced with fresh complete MDM culture media (section 2.1.4), 24 hrs siRNA post-transfected cells were observed under the fluorescent microscope and greater than 90% of cells had taken up the fluorescent siRNA indicating high transfection efficiency (Appendix V).

2.5.3.2 Blocking of TNF-α mRNA using siRNA

The day before transfection, 5 day old adherent MDM were seeded at concentrations of 2 x 10^5 cells per well in a 48 well plate in MDM growth media without antibiotics. Cationic lipid complexes were prepared by incubating 1.6 μM siRNA duplexes with 0.6 μl lipofectamine RNAiMAX (Invitrogen) in 40 μl of DMEM without serum, and then added to the cells. After 4 hrs incubation, the cells were washed and replaced with 200 μl fresh MDM culture medium (section 2.1.4) without antibiotics for at least 18 hrs to allow recovery, followed by a second round of siRNA transfection as described above. After 4 hrs of incubation the cells were washed and cultured in MDM culture media (section 2.1.4).

2.5.4 DENV-infection and lipopolysaccharide (LPS) stimulation of siRNA transfected cells

MDM were transfected with TNF-α siRNA as above and 18 hrs after the second transfection (section 2.5.3.2) the cells were infected with DENV at MOI of 5 or mock-infected as described in section 2.3.3. At 32 hrs post-infection supernatants were collected. The levels of infectious virus released were determined by plaque assay and TNF-α production was quantitated by ELISA (Pharmingen, OptEIA^{TM} Human TNF-α Set) in accordance with the manufacturer's recommendation. RNA was also extracted from these cells and analysed for TNF α mRNA by RT-PCR (section 2.4.5).

To validate the efficacy of siRNA knockdown of TNF-α protein, MDM were transfected with TNF-α siRNA and 18 hrs after the second transfection (section 2.5.3.2) TNF-α production was stimulated by addition of 10 ng/ml LPS (Sigma) for 4 hrs. Supernatant was collected and cells were thoroughly washed using HBSS (section 2.1.4) and refreshed with fresh MDM growth media. LPS exposure (as above) was repeated daily up to day 4 post-transfection. The supernatants were assayed for TNF-α bioactivity using a L929 cytotoxic bioassay (as in section 2.5.5).

2.5.5 L929 Cytotoxic Bioassay

L929 cytotoxicity was analysed by modifying the method from (Cseh and Beutler, 1989) based on TNF-α killing of L929 cells. L929 cells were plated in 96 well plates at a density of 10^4 cells per well in RPMI media supplemented with 10% FBS, and incubated overnight. Known concentration of TNF-α (320 pg/ml) and LPS-stimulated supernatants (section 2.5.4)

were serially diluted 2 fold in RPMI media containing 2% FBS and 2 µg/ml actinomycin D (Sigma). Spent medium was removed from overnight cultured L929 cells and 100 µl of TNF-α standards or diluted samples were added to the wells. The cells were re-incubated for 18 hrs, fixed with 100% methanol, and viable cells were stained using 0.5% crystal violet (BDH) diluted in 25% methanol. After 5 mins of staining the monolayers were washed extensively with water, and dye adherent to plates was solubilised using 1% (w/v) SDS (BDH). Absorbance was read at 540 nm, standards were graphed and concentrations of TNF-α in samples were determined (Appendix VI-A). Specificity of this assay for TNF-α was shown by antibody blocking of the bioactivity of LPS-stimulated supernatants, from TNF-α siRNA transfected macrophages or 50 000 pg/ml of TNF-α individually co-incubated with 2.5 µg/ml TNF-α monoclonal antibodies for 2 hrs prior to L929 cytotoxicity assay (Appendix VI-B).

2.5.6 Quantitation of TNF-α-stimulated NF-kappa B (NF-kB) mediated luciferase (LUC) reporter gene transcription

Huh7 cells were seeded at concentrations of 1.4 x 10^5 cells per well in a 6 well plate, then co-transfected with two different reporter plasmids (2 µg each) and 10 µl Superfect (Qiagen) as per manufacturer's instructions. The first plasmid pTK81NF-kBLUC (a gift from Dr Andrew Bert, Human Immunology, IMVS, Adelaide, SA 5000) was a modified version of pT81-LUC (Nordeen, 1988) and contained 5 tandem repeats of a 27 bp NF-kB responsive element derived from the immunoglobin k gene enhancer (aacagagGGGACTTTCCgaggccatct) upstream of the TK promoter. The second plasmid pRL-TK (Promega) contained a renilla LUC gene under a control of a constitutive TK promoter (Appendix VII). After 18 hrs, the transfected cells were trypsinised, distributed equally into 4 wells of a 24 well plate and allowed to adhere for 18 hrs. Duplicate wells were infected with DENV at MOI 5 or mock-infected as described in section 2.3.3. 24 hrs after infection, 10 ng/ml of TNF-α or fresh medium alone was added and incubated for 6 hrs before the cells were harvested and lysed in passive lysis buffer (Promega). LUC activity was quantitated in cell lysates using Promega's Dual Luciferase Reporter Assay System Kit and luminescence measured (using Turner Designs: Td20/20 Luminometer version 2020-IE 1098). The activity of firefly LUC was normalised to the activity of renilla LUC (to control for different transfection efficiencies), or to the total protein concentration quantitated by a Biorad DC Protein assay (section 2.5.7). Both methods of normalisation yielded the same result. As a control the IFN-α responsive plasmid pISRE-LUC (Clontech Labs, Appendix VIII) was also transfected into cells, the

transfected cells were stimulated with 100 IU IFN-α, and the LUC activity was measured and normalised as described above.

2.5.7 DC™ Protein Assay (Biorad catalogue number 500-0121)

BSA standards (Biorad) were diluted in PBS (0-1 ug/ml) and samples were either tested neat or diluted 1:5 and 1:10 in PBS. A working reagent A was first prepared as described by manufacturer which involved adding 5 µl of DC reagent S to 250 µl of DC reagent A. 5 µl of BSA standards or samples were pipetted into 96 well Nunc Flat bottom plates with 25 µl of working reagent A. 200 µl of reagent B was added to each well. The plates were incubated for 15 mins and absorbance read at 595 nm using a plate reader (EL 808, Ultra microplate reader, Bio-Tek). A standard curve was extrapolated and concentration of protein was calculated.

2.5.8 Analysis of TNF-α stimulation of NF-kB nuclear localisation using confocal microscopy

MDM and Huh7 cells were seeded at 1 x 10^5 and 1 x 10^4 cells per well respectively in a 16 well Lab-Tek chamber glass slide and infected at MOI of 5. MDM two days after infection, or Huh7 cells 1 day after infection, were treated with or without TNF-α (10 ng/ml) for 30 mins. Cells were fixed in 1% formalin and permeabilised with 0.05% IGEPAL CA-630 (Sigma). MDM were then blocked with 4% (v/v) human sera, 4% (v/v) goat sera and 0.4% (w/v) bovine serum albumin (BSA). Huh7 cells were blocked with 4% (v/v) goat and 0.4% (w/v) BSA diluted in HBSS (section 2.1.4) containing 5% FCS and 0.02% Sodium Azide (Ajax chemicals). After 30 mins of blocking, the MDM were incubated with mouse monoclonal DENV anti-E antibodies (1/50) and rabbit anti-NF-kB p-65 (1/50). Huh7 cells were incubated with DENV positive patient sera (1/5000) and rabbit NF-kB anti-p-65 (1/50). Following 45 mins incubation the MDM were incubated with 1/200 conjugated secondary goat anti-mouse Alexa Fluor 546 (Molecular Probes) and goat anti-rabbit Alexa Fluor 488, while the Huh7 cells were incubated with 1/200 of goat anti-human Alexa Fluor 546 and goat anti-rabbit Alexa Fluor 488. Slides were mounted with ProLong Antifade Mounting Medium (Molecular Probes), allowed to dry and examined by confocal microscopy. Confocal images were captured at 400 x magnification using a Biorad Radiance 2100 confocal microscope. Specificity of staining was shown using uninfected cells stained with DENV antibodies and DENV positive cells stained with a species matched irrelevant antisera.

2.6 PROTEOMICS

2.6.1 Sample preparation for proteomic analysis

K562 cells were DENV or mock-infected as in section 2.3.3. At 72 hrs post-infection, the cells were washed twice with PBS and resuspended in 1 ml of cold cell lysis buffer (section 2.1.4). Nucleic acids were degraded following addition of 0.2 mg/ml of DNase I and RNase A and incubation on ice for 20 mins. Cells were sonicated (MiSonix sonicator XL 2007-setting 3) for 1 min in 10 sec bursts to prevent over heating of samples. Proteins from the disrupted cell suspensions were precipitated by addition of 4 volumes of ice-cold acetone at -20^0C for 2 hrs. The protein precipitate was collected by centrifugation (13 000 g, 30 min at 4^0C) and residual acetone was removed by evaporation. Purified proteins were dissolved in rehydration buffer (section 2.1.4) containing multiple surfactant solution and clarified by centrifugation (13 000 g, 15 mins at 20^0C). Aliquots of supernatant were then stored at -80^0C. Protein concentration was determined by RC DC protein assay (2.6.2).

2.6.2 RC DC™ Protein Assay kit 1 (Biorad catalogue number 500-0121)

The RC DC assay system is compatible with the 2D rehydration buffer components and therefore used to determine accurately the protein concentration in samples for proteomics. RC DC protein assay was performed as per manufacturer's instructions. Briefly, BSA standards (Biorad) were diluted in PBS (0-1 µg/ml) and samples were either tested neat or diluted 1:10 and 1:20 in PBS. A working reagent A was first prepared as described by manufacturer which involved adding 5 µl of DC reagent S to each 250 µl of DC reagent A. 25 µl of BSA standards and samples were pipetted into microcentrifuge tubes (the standard and samples were tested in duplicate). 125 µl RC reagent I was first added into each tube, vortexed and incubated for 1 min. Next 125 µl RC reagent II was added into each tube and vortexed. The tubes were centrifuged at 15 000 x g for 5 mins. The supernatant was discarded and pellet was dissolved in 127 µl of reagent A. Tubes were vortexed and incubated till the precipitate completely dissolved and 1 ml of DC reagent B was added to each tube. The tubes were incubated for 15 mins and read at 750 nm. A standard curve was extrapolated and concentration of protein was calculated.

2.6.3 Two-dimensional electrophoresis (2D-PAGE)

Isoelectric focussing (IEF) was performed on 11 cm precast IPG strips with pH range of 3-10 using a Protean IEF cell (Biorad). Briefly, 0.7 mg cell protein as determined by RC DC protein assay (section 2.6.2) in rehydration buffer was used to passively rehydrate each 11 cm IPG strip overnight. IEF was run using a linear voltage increased to 10 000 volts over 3 hrs. Focussing occurred for 60 000 volts per hr with 50 μA per strip current limit, with the temperature maintained at 20^0C. The 6 IPG strips (3 from replicate DENV-infections and 3 from replicate mock-infections) were run concurrently. After IEF, the IPG strips were subjected to a two-step equilibration which involved 20 mins incubation in a 2% DTT equilibration base buffer with gentle agitation followed by 20 mins incubation in a 2.5% iodoacetamide equilibration base buffer. Separation of proteins in the second dimension was performed using 12% precast criterion gels (Bio-Rad). The proteins were electrophoresed at 80 volts for 1 hr, washed 3 times in double distilled water and stained with Biosafe ^{TM} Coomassie G250 stain (Biorad) for 1 hr followed by removal of excess stain by washing gels in double distilled water for 30 mins. Stained gels were stored in double distilled water at 4^0C until analysis.

2.6.4 Image acquisition and analysis

The stained gels from section 2.6.3, were scanned using a GS-800 densitometer and analysed using PD-Quest software (Biorad). Firstly a match set of protein spots using 6 gels from the 3 DENV-infected cells and 3 mock-infected cells was created with PD-Quest that represented the protein spot pattern consistently detected in all 3 DENV-infected and mock-infected gels. Spot density was normalised within each gel based on the total density of spots detected in each gel using PD-Quest software. Difference in presence of unique proteins in either subset was first analysed using the PD-Quest software. The mean protein spot density between DENV-infected and mock-infected groups were futhur analysed using Student's t-test. Protein spots showing a statistically significant quantitative change between DENV-infected and mock-infected samples were identified, excised from the gels and examined by mass spectrometry.

2.6.5 MALDI TOFF/TOFF mass spectrometry and protein identification

Excised protein spots from section 2.6.4 were processed by Dr Chris Bagly at the Adelaide Proteomics Centre, School of Molecular and Biomedical Science, University of Adelaide. Briefly, the excised protein was trypsin digested and subjected to mass spectrometry (MS)

using MALDI TOFF/TOFF MS. The spectra and mass lists were exported to Bio Tools (version 3.0, Bruker Daltonik GmbH) and submitted to the in-house or on-line Mascot database-searching engine (Matrix Science: http://www.matrixscience.com). The specifications were as follows: Taxonomy: Homo sapiens (human), Database: SwissProt 51.3, Enzyme: Trypsin, Fixed modifications: Carbamidomethyl (C), Variable modifications: Oxidation (M), Mass tol MS: +/- 100ppm, missed cleavages:1. The MOWSE and probability scores calculated by the software were used as the criteria for protein identification.

2.6.6 Western blot for HSP70

40 µl (approx 0.1 mg protein) of whole cell lysate (from section 2.6.1) of infected and uninfected K562 cells used in 2D–PAGE analysis were separated in 10% SDS PAGE gels alongside BenchMark^{TM} Prestained Protein Ladder (Invitrogen). The proteins were separated at 80 volts for 1-2 hrs. Gels were equilibrated in Transfer Buffer (section 2.1.4) for 30 mins and the proteins were transferred onto nitrocellulose membrane (Amersham) using Biorad Transblot apparatus at 0.5 mA/cm^2 for 60 mins. The membrane was washed in PBS-Tween (0.1% Tween 20) three times for 5 mins. The nitrocellulose membranes were blocked for 1 hr in blocking buffer (section 2.1.4). The blocking buffer was replaced with fresh blocking buffer containing rabbit HSP70 antibodies diluted 1:200 (Chemicon catalogue number AB3470) and was incubated at room temperature for 1 hr with continuous rocking. The membranes were rinsed 3 times in PBS-Tween and then washed 3 times for 15 mins. Secondary peroxidase conjugated goat anti-rabbit IgG (Pierce Biotechnology catalogue number 31460) diluted 1:50 000 in PBS-Tween was added to the membrane and incubated at room temperature for 1 hr with continuous shaking. The membrane was rinsed again and washed as above with PBS-Tween. Bound antibody complexes were detected by chemiluminescence using ECL substrates (Amersham). Substrates were mixed and poured onto the filter for 5 mins. Excess fluid was drained from the nitrocellulose membrane and was exposed to X-Omat BT Film (Kodak) for 10 mins, and the film was developed using Ilfospeed 2240 automated developer. To normalize the levels of protein, the membrane was stripped in warmed stripping buffer (section 2.1.4) and thoroughly washed in PBS-Tween. Membranes were blocked and subjected to western blot, as described above, with (1:1000) mouse actin antibodies (Chemicon catalogue number MAB1501R), and bound proteins detected with 1:100 000 conjugated peroxidase goat anti-mouse IgG (Pierce Biotechnology catalogue number 31430) and ECL substrates as described above.

2.6.7 Immuno-staining and confocal microscopy

2.6.7.1 Detection of DENV antigens in K562 and U937 cells

K562 and U937 cells were infected or mock-infected as described in section 2.3.3. At peak of infection previously determined by plaque assays (i.e. 48 hrs for U937 cells and 72 hrs for K562 cells) cells were fixed and stained as described in section 2.5.8. K562 were blocked with 4% human sera, 4% goat sera and 0.4% BSA, and U937 cells were blocked with 4% goat and 0.4% BSA diluted in HBSS containing 5% FBS and 0.02% Sodium Azide (Ajax chemicals). The K562 cells were incubated with mouse monoclonal DENV anti-E antibodies (1/50). U937 cells were incubated with DENV positive patient sera (1/5000). Following 45 mins incubation the K562 cells were incubated with 1/200 of goat anti-mouse Alexa Fluor 546 (Molecular Probes), while U937 cells were incubated with goat anti-human Alexa Fluor 488. Cells were mounted onto glass slides as and examined using confocal microscope described in section 2.5.8. The percentage of DENV positive cells were enumerated by counting total cells using light microscopy and infected cells using confocal microscopy.

2.6.7.2 Detection of HSP70 and GRP78 in DENV-infected and mock-infected K562 and MDM cells

K562 cells stained at day 3 post-infection as in section 2.6.7.1 and showing 100% infection were also stained for HSP70 and GRP78. Similarly day 2 post-infected and mock-infected MDM were fixed, blocked as described above (with exception for GRP78 staining where 4% goat sera in blocking buffer was replaced with 4% rabbit sera) and stained for HSP70, GRP78 protein and DENV. Primary rabbit anti-HSP70 antibody (1:100) (Chemicon) was used to detect HSP70, in MDM and K562 cells and primary mouse monoclonal DENV anti-E antibodies (1/50) was used to detect DENV-infected MDMs. The secondary antibody to detect HSP70 was conjugated goat anti-rabbit Alexa Fluor 488 (1/200) (Molecular probes) and to detect DENV virus was conjugated donkey anti-mouse Cy^{TM}5 (1:400) (Jackson Immunoresearch Laboratories). Primary goat anti-GRP78 antibody (1/50) (Santa Cruz Biotechnology C-20) was used to detect GRP78 in MDM and K562 cells and mouse monoclonal DENV anti-E antibodies (1/50) was used to detect DENV-infected MDMs. The secondary antibody to detect GRP78 in K562 and MDM cells were conjugated rabbit anti-goat Cy^{TM}2 (1:150) (Jackson Immunoresearch Laboratories) and DENV anti E antibodies was conjugated donkey anti-mouse Cy^{TM}5 (Jackson Immunoresearch Laboratories). Stained cells were mounted onto glass slides as and examined using confocal microscope described in

section 2.5.8. Note - DENV anti-mouse monoclonal antibodies were used instead of DENV positive patient sera in K562 and macrophages due to high background issues.

2.7 LIST OF SUPPLIERS

Abcam, Cambridge Science Park, Cambridge, UK

ABI, Applied Biotechnology Inc., Division of Perkin-Elmer Corporation

Ajax, Finechem, Bay Rd, Taren Point, NSW, Australia

Ambion, Woodward St, Austin, TX, USA

American Tissue Culture Collection, Manassas, Virginia, USA

Amersham, Amersham International PLC, Little Chalfont, Buckinghamshire, UK

Australian Red Cross Blood Bank, Adelaide, Australia

BDH, BDH Chemicals Australia Pty Ltd, Kilsyth Vic, Australia

New England Biolabs, Ipswich, MA, UK

Bio-Rad, Bio-Rad Laboratories, Hercules CA, USA

Bio-Tek Instruments Inc., Highland Park, Winooski, VT, USA

Biowhittaker Molecular Applications, Rockland, Maine, USA

Boeringer, Boeringer Mannheim, Mannheim, Germany

Chemicon, Temecula, California, USA

Clonetech labs, Mountain view, CA, USA

Fluka, Buchs, Switzerland

Geneworks, Geneworks Pty Ltd, Adelaide, SA, Australia

Gibco BRL, Grand Island, USA

Greiner, Frickenhausen, Germany

Intron A, Schering-Plough Ltd, USA

Jackson Immunoresearch Laboratories, West Baltimore Pike, West Grove, USA

JRH, Biosciences, Lenexa, Kansas, USA

Kodak, Eastman Kodak, Rochester NY, USA

Lab Tek, Nalge Nunc International, Naperville, IL, USA

Millipore Coorporation, Bedford, MA, USA

Misonix, Inc., New Highway, Farmingdale, NY, USA

Molecular Probes, Eugene, Oregon, USA

MP Biomedicals, Irvin, CA., USA

Nunc, Nunc A/S Roskilde, Denmark

Nycomed Pharma AS, Oslo, Norway

Perkin-Elmer Corporation, Roche Molecular Systems, Inc., Branchberg NY, USA

Perkin Elmer Life Sciences, Boston, MA, USA

Pierce Biotechnology, Rockford Illinois, USA

Pharmacia, Pharmacia Biotech, Uppsala, Sweden

Pharmingen, BD, North Ryde, NSW, Australia

Promega, Promega Corporation, Madison WI, USA

Prospec-TechnoGene Ltd, Rehovot, Israel

Qiagen, Doncaster, Victoria, Australia

Roche Ltd, Basel, Switzerland

Santa Cruz Biotechnology, CA, USA

Sigma, Sigma Chemical Company, Sigma-Aldrich Pty Ltd, St. Lois MO, USA

Turner Designs Inc, Sunnyvale, CA, USA

USBiological, United States Biological, Swampscott, Massachusetts, USA

CHAPTER 3

DEVELOPMENT OF AN IMPROVED RT REAL-TIME PCR TO DETECT DENV (+ ve) AND (- ve) STRAND RNA

3.1 INTRODUCTION

The DENV genome consists of ss (+ ve) sense RNA. During viral replication the incoming virus genome synthesises a (- ve) strand RNA molecule, utilizing the virus (+ ve) strand RNA as a template. This (- ve) strand then is present in infected cells as part of a ds complex known as the replicative intermediate (RI) or replicative form (RF) (Cleaves *et al.*, 1981) (Fig 1.8). The newly synthesised (- ve) strand RNA within the RI is used as a template for synthesis of a (+ ve) strand RNA molecule via semi-conservative RNA synthesis, that is then either translated into the DENV poly-protein or packaged to form new virions (Cleaves *et al.*, 1981).

The (- ve) strand RNA is detected from 3 hrs after infection (Peyrefitte *et al.*, 2003) and is present throughout the DENV replication cycle (Cleaves *et al.*, 1981) making it a good marker for active viral infection. In contrast, the (+ ve) strand RNA is present in DENV-infected cells in larger amounts at a ratio of approximately 10:1 to that of (- ve) RNA strand (Cleaves *et al.*, 1981) and is detectable both in the presence or absence of active replication. However, the quantitation of (- ve) strand RNA as a marker of active replication has been hampered by lack of strand-specificity in PCR based assays.

In vitro reverse transcription (RT) of DENV RNA (either *in vitro* transcribed RNA or RNA extracted from DENV-infected cells) **without complementary primers** generates DENV specific cDNA (Tolou, 1994; Peyrefitte *et al.*, 2003), suggesting non primer-mediated generation of DENV specific cDNA during the RT step. This non primer-mediated RT amplification has been shown to occur using *in vitro* transcribed DENV RNA of both (+ ve) and (- ve) strands (Tolou, 1994). Why or how this non primer-mediated RT occurs is not fully understood but a similar phenomenon has also been reported for HCV RNA, another well studied virus of the *Flaviviridae* family (Lanford *et al.*, 1994; Lerat *et al.*, 1996; Mellor

et al., 1998). For HCV it has been suggested that the non primer-mediated RT occurs due to the secondary structure in the complex HCV 5' non coding region or due to random priming via interaction with cellular nucleic acids (Gunji *et al.*, 1994; Lanford *et al.*, 1994). Thus, RT-PCR assays using conventional primer-driven methods to detect strand-specific DENV RNA are not conclusive, since non primer-mediated RT can produce either false detection or over estimation of (- ve) strand RNA. Since the present study aimed to look at DENV replication under different conditions, a reliable system was needed for detecting strand-specific DENV RNA, particularly (- ve) strand as a specific marker of active replication.

This chapter describes the development of a RT real-time PCR to specifically detect (+ ve) and (- ve) strand DENV RNA, which was used to determine levels of DENV replication in later chapters. The first part of this chapter describes a range of optimisation methods in an attempt to prevent non primer-mediated RT, while the second part describes a tagged RT real-time PCR protocol first described by Peryfitte *et al*., 2003, to specifically detect DENV RNA (+ ve) and (- ve) strands by incorporating a tagged primer into DENV-specific cDNA during the RT process.

3.2 RESULTS

3.2.1 Generation of *in vitro* transcribed DENV RNA

The pGEM-DENV-2CAP clone was generated by honours student Ms Robyn Taylor, University of Adelaide (Taylor, 2003). This plasmid construct allowed *in vitro* transcription of DENV strand-specific RNA to establish RNA copy number standards for quantification of (+ ve) and (- ve) DENV RNA (Fig 3.1). Briefly, a 428 base pair (bp) region of the DENV laboratory strain (MON601) capsid sequence (nucleotide #s 50-477, Accession# AF038403) (Appendix I) was cloned into pGEM-3ZF(-) plasmid vector between Eco RI and Bam HI (Appendix II).

The pGEM-DENV-2CAP construct was *in vitro* transcribed to produce (+ ve) or (- ve) transcripts (as outlined in Fig 3.1 and described in materials and methods section 2.4.1). The *in vitro* transcribed RNA was treated with DNaseI and quantitated by spectrophotometry, and copy numbers were calculated as outlined in section 2.4.1. The transcripts were then RT with DENV primers 3.1 or 5.1 as outlined in Fig 3.2. Controls for the RT-PCR included the RT reaction with (i) no primers (ii) with the same sense primers (referred to as **wrong primer**

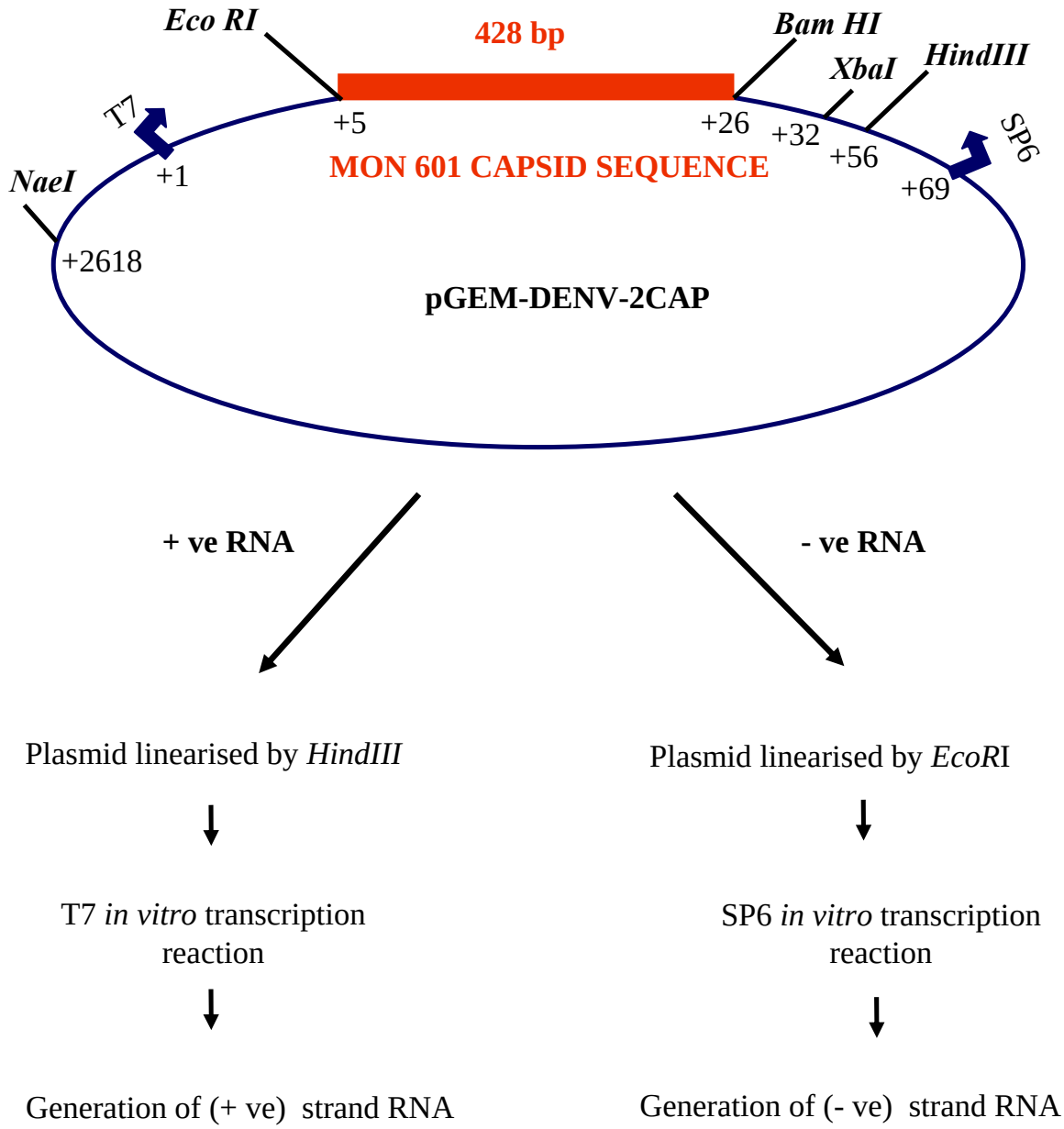


Fig 3.1. *In vitro* transcription strategy for generation of strand-specific DENV RNA from pGEM-DENV-2CAP. pGEM-DENV-2CAP containing 428 bp sequence of DENV-2 capsid protein (nucleotides #s 50 to 477, Accession AF038403) was used to *in vitro* transcribe (+ ve) or (- ve) strand RNA with T7 or SP6 polymerase, respectively. The synthesised RNA was quantified by spectrophotometer, copy numbers were calculated and the RNA was used as standards in RT real-time PCR.

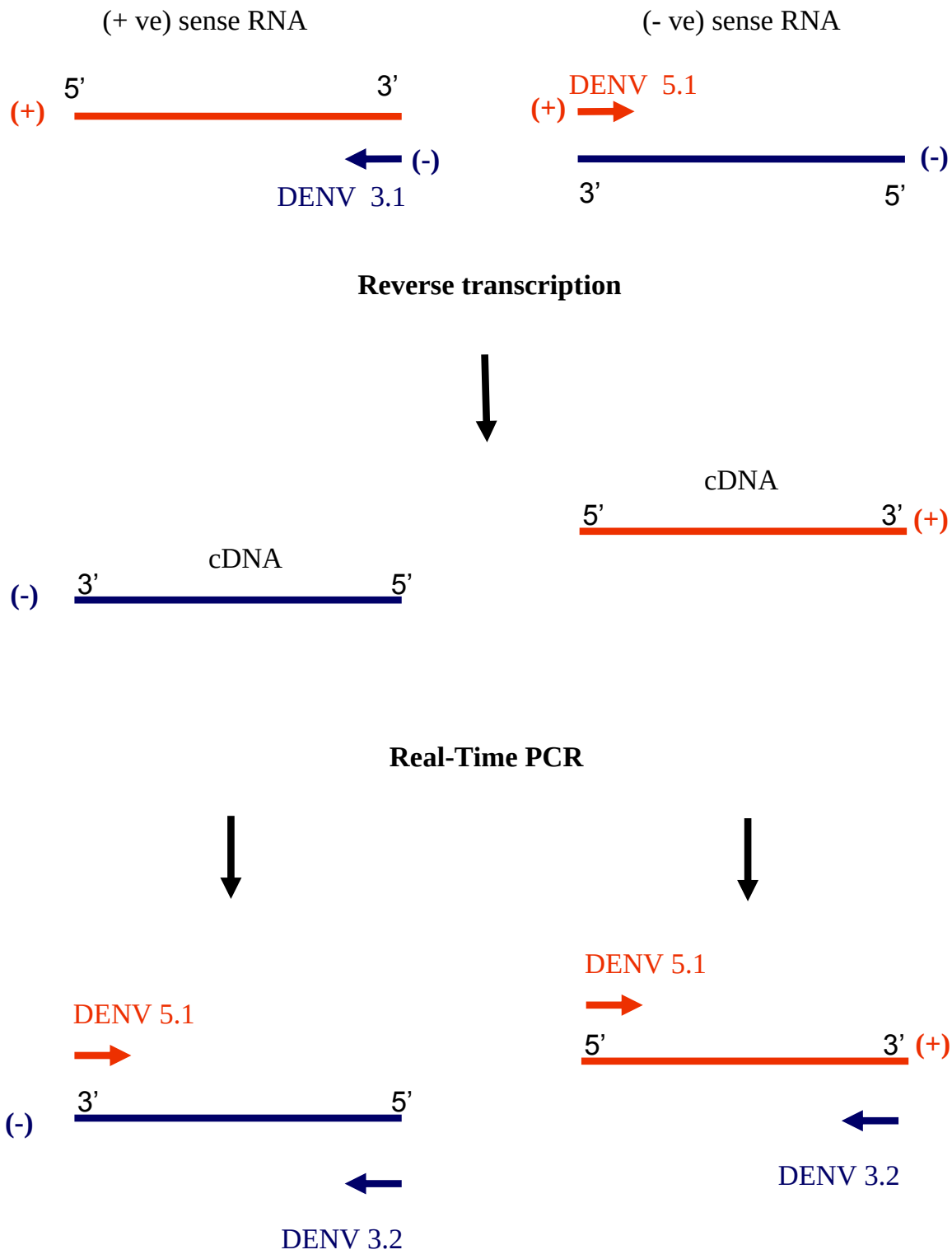


Fig 3.2. Conventional two step RT real-time PCR. *In vitro* transcribed (+ ve) or (- ve) RNA is reversed transcribed with complementary primers 3.1 and 5.1 respectively. The resultant cDNA is subjected to real-time PCR using DENV specific primers DENV 5.1 and 3.2 and products detected by incorporation of syber green.

throughout this chapter) and (iii) no RT enzyme (to test for complete removal of pGEM-DENV-2CAP DNA). The synthesised cDNA was amplified by real-time PCR as outlined in Fig 3.2. In initial reactions, all the controls (ie no RT controls, no primer and wrong primer controls) produced high levels of PCR products with identical melting curves to authentic DENV PCR products from authentically primed (+ ve) and (- ve) strand (results not shown). This suggested (i) DENV DNA contamination from pGEM-DENV-2CAP (ii) false priming during *in vitro* transcription or producing both (+ ve) and (- ve) strand DENV RNA (iii) non primer-mediated RT. These three scenarios were investigated.

3.2.2 DNA contamination of RT reaction from pGEM-DENV-2CAP

The PCR signal in the control reactions without any RT enzyme suggested DNA contamination in the *in vitro* transcribed RNA (both (+ ve) and (- ve) strand RNA). Thus the *in vitro* transcribed DENV RNA was further DNase treated with 100 units of DNaseI for an extra 15 mins beyond that recommended by the manufacturer (Ambion *in vitro* transcription kit). No PCR product was observed in the no RT controls in the RT real-time PCR (Fig 3.3 lanes 4 and 8) and thus this extra DNaseI treatment totally eliminated any DNA contaminants in the *in vitro* transcribed products. However, the no primer controls and wrong primer controls still produced DENV specific PCR products (Fig 3.3, lanes 2-3 and 6-7), suggesting non specific priming during *in vitro* transcription or in the RT reaction.

3.2.3 Lack of strand specificity – Possible false priming arising during *in vitro* transcription

The production of DENV PCR products without specific priming may have been a consequence of (i) production of the opposite DENV RNA strand during the *in vitro* transcription reaction or (ii) production of small nucleic acids during the *in vitro* transcription reaction that can act as subsequent primers in the RT reaction. These possibilities were investigated.

3.2.3.1 Removal of all unlinearised pGEM-DENV-2CAP DNA, 3' overhangs and the SP6 promoter

Unlinearised vector could lead to complete continuous transcription of the circular pGEM-DENV-2CAP templates that could subsequently self-prime during RT. Thus pGEM-DENV-2CAP was digested with *HindIII,* and the linearised fragment was then gel purified on 0.7% agarose gels to eliminate all unlinearised pGEM-DENV-2CAP DNA. This extra purification

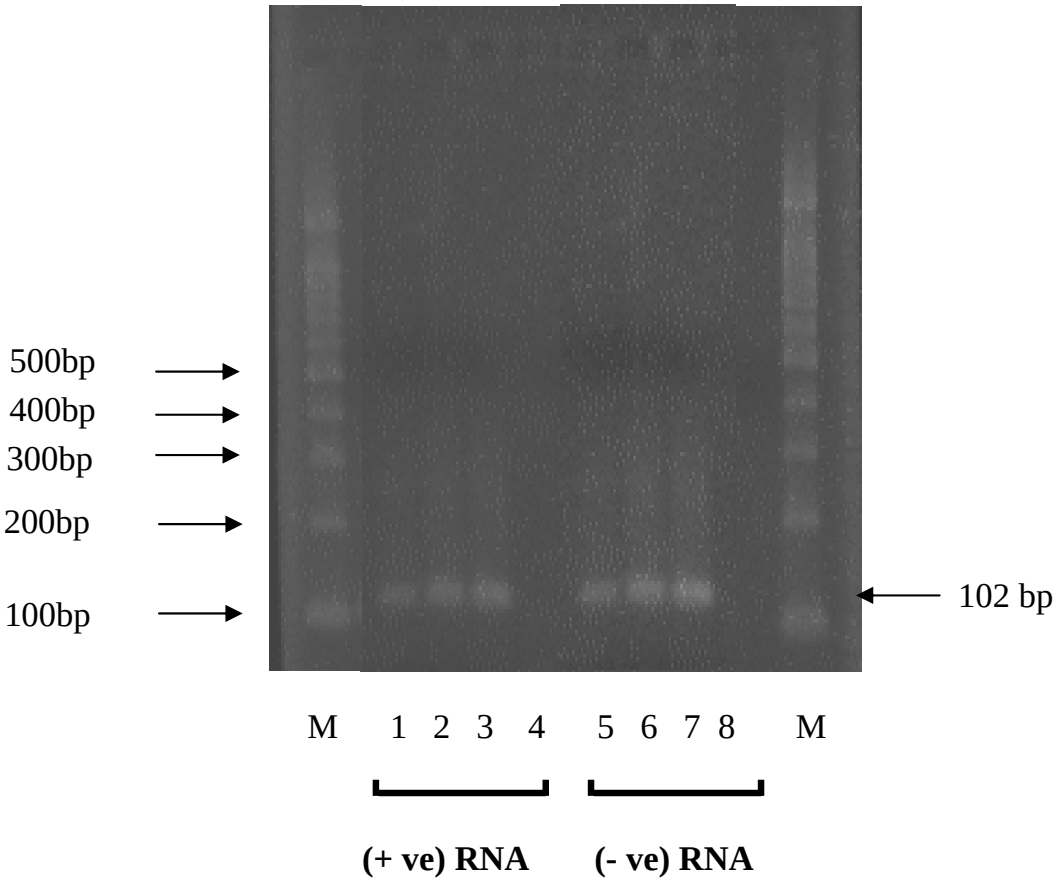


Fig 3.3. PCR amplification of DENV RNA without specific priming of the RT reaction. *In vitro* transcribed RNA for both + ve (0.02 pg) and - ve (0.017 pg) strand RNA were amplified by RT real-time PCR with varying primers during RT step. The amplified products were run on 2.5% agarose gel and stained with ethidium bromide. The expected DENV specific PCR product of 102 bp was clearly visible in all lanes except those that did not have any RT enzyme (4 and 8).

M = GelPilot 100 bp marker (Qiagen)
1 and 5 = complementary primer
2 and 6 = wrong primer
3 and 7 = no primer
4 and 8 = complementary primer, **NO RT** enzyme
M = GelPilot 100 bp marker

step alone was not sufficient to reduce non primer-mediated generation of DENV PCR products (data not shown).

Additionally the presence of 3' overhang after restriction enzyme digestion can result in transcription of wrong RNA strand (see Ambion maxiscript™ guide). Accordingly, two enzymes *Hind III and Xba I* were used to ensure complete linearisation of pGEM-DENV2-CAP. Additionally, these restriction enzymes leave a 5' overhang rather than 3' overhang. The double cut plasmid was gel purified and used to *in vitro* transcribe DENV RNA. Again these measures had no effect on the subsequent production of non primer-mediated PCR products (data not shown).

The linearised plasmid pGEM-DENV-2CAP contained two promoters, T7 and SP6, and it was possible that the presence of the SP6 promoter might generate the opposite RNA strand during *in vitro* transcription using the T7 polymerase. Therefore pGEM-DENV-2CAP was digested with two restriction enzymes *Hind III* and *Nae I* (Appendix II) producing an 1050 bp product that included the 428 bp DENV-2 capsid and T7 promoter, and a 2563 bp product that contained the SP6 promoter and the remaining plasmid. The 1050 bp DNA fragment lacking the SP6 promoter was excised from the gel, purified using Qiagen gel purification and used for *in vitro* transcription of the (+ ve) strand RNA. This still did not reduce the observed non primer-mediated production of DENV PCR products, as the wrong primer in the RT reaction also gave PCR products with the same melting peak and concentration as that seen with complementary primers (results not shown).

In summary, these measures to ensure strand-specific *in vitro* transcription had no subsequent effect on the generation of non primer-mediated DENV specific PCR products, suggesting non specific priming during the RT reaction rather than during *in vitro* transcription.

3.3.3.2 Purification of *in vitro* transcribed RNA template

Any small nucleic acids, potentially produced during the DNase treatment of the input vector following *in vitro* transcription, could act as a random primer during the RT reaction. Thus, following *in vitro* transcription and DNase treatment, the RNA product was purified twice by RNA extraction using the RNeasy column by following manufacturer's instructions (Qiagen). This step should completely remove all small fragments of RNA or DNA, which should not co-purify on the RNeasy column due to their size (less than 50 bp). This extra purification step had no effect on reducing non primer-mediated generation of DENV specific PCR

products, as RT reactions with the wrong primer still gave PCR products with same melting peak and concentration as that seen with complementary primers (results not shown).

The measures described in section 3.2.3 had no effect on improving the strand specificity of the RT-PCR and therefore were not incorporated in subsequent methods for detecting strand-specific DENV RNA.

3.2.4 Lack of strand-specificity – possible non primer-mediated generation of RT products during the RT reaction

3.2.4.1 Increased temperatures and different RT enzymes to increase specificity of RT

Non primer-mediated generation of RT products during the RT reaction can be reduced by increasing the temperature to reduce any RNA secondary structure. AMV RT has the capacity to function at high temperature, and cDNA synthesis at temperatures up to 70^0C using AMV reverse transcriptase has been shown to improve the specificity of RT-PCR (Fuchs *et al.*, 1999). Increasing the temperatures of RT up to 48^0C has been also shown to reduce non primer-mediated generation of RT products in *in vitro* transcribed DENV RNA (Tolou, 1994). Therefore AMV-RT was utilised and different temperatures for the RT reaction (42^0C, 50^0C, 55^0C, 58^0C ,60^0C and 65^0C) were investigated. Increasing the temperatures of RT to 58^0C, 60^0C and 65^0C decreased non primer-mediated generation of DENV specific PCR products in some experiments, but it also decreased the sensitivity of the RT-PCR. Additionally, results were not reproducible (data not shown).

Omniscript (Qiagen) RT enzyme has been suggested to be superior to AMV RT enzyme. However, use of Omniscript RT yielded no improvement to the specificity of the PCR since DENV specific PCR products still produced products without priming in the RT reaction.

3.2.4.2 Other optimisation methods to reduce non primer-mediated generation of DENV(+ ve) and (-ve) strand RNA

Other technical measures were investigated to reduce the non primer-mediated generation of PCR products from DENV RNA:

(i) A range of RT enzyme concentrations (ranging from 0.02-10 units) were used in the RT reaction.

(ii) *In vitro* transcribed RNA and RNA extracted from infected cells were heated at 65^0C with or without primers before addition of RT to the reaction.

(iii) RT reaction products were ethanol precipitated to purify the cDNA before adding to the real-time PCR reaction.

(v) A range of primer combinations were used in the RT and real-time PCR reactions (Fig 3.4).

None of the above measures had any affect on reducing the DENV PCR product observed without priming of the RT reaction (i.e. non primer-mediated priming), and thus none of these protocols were used for detection of DENV RNA in subsequent procedures.

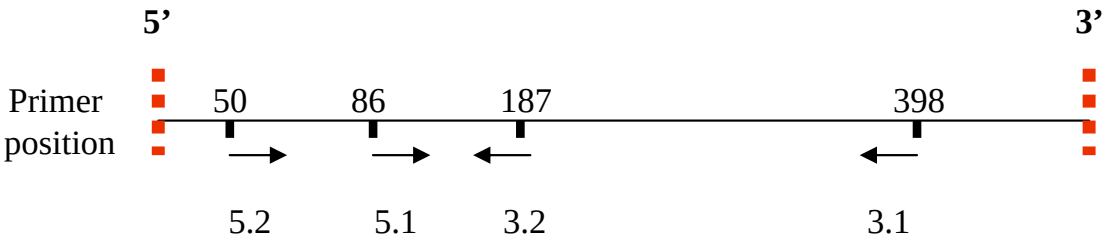
3.2.5 Tagged RT real-time PCR for quantitation of DENV (+ ve) and (- ve) strand RNA

A DENV specific, tagged RT real-time PCR was developed according to the system first described by Peyrefitte *et al.,* 2003. This method does not prevent the production of non primer-mediated DENV specific cDNA during RT, but the subsequent PCR reaction only amplifies products synthesised from the strand-specific tagged primers used during RT. This ensures quantitative measurement of only the strand-specific cDNA generated under controlled reverse transcription conditions.

3.2.5.1 Development of a tagged RT real-time PCR

Primers that were previously used for RT in optimisation methods above i.e DENV3.2 for (+ ve) strand and DENV5.1 for (- ve) strand were re-designed with the addition of a 19-mer sequence Tag (CGGTCATGGTGGCGAATAA) to the 5' end. The cDNAs generated from *in vitro* transcribed DENV RNA following RT using the Tag primers, were diluted 1:100 and 2 µl was amplified in real-time PCR reactions using a DENV specific primer DENV5.1 for (+ ve) strand and DENV3.2 for (- ve) strand and a primer containing the 19-mer sequence Tag only. Schematic diagrams of tagged RT real-time PCR for (+ ve) and (- ve) are shown in Figs 3.5. RT real-time PCR from *in vitro* transcribed RNA and RNA extracted from DENV-infected cells showed specific production of DENV PCR product only in those RT reactions that were primed with a tagged DENV primer (Fig 3.6 and 3.7). Reaction products were not observed without primer or RT or with the wrong primer in the RT reaction (Fig 3.6 or 3.7C). Linear amplification curves were achieved, across 100 fold dilution range (Fig 3.6 or 3.7A-C). Melt curves were representative of a single product (Fig 3.6 or 3.7 D) that was confirmed by agarose gel electrophoresis (data not shown). Thus the tagged-RT real-time PCR protocol successfully yielded strand-specific quantitation of DENV RNA.

A



B

RNA strand	RT -primer	Real-time primer combinations
(-)	5.2	5.2/3.2, 5.1/3.2
(-)	5.1	5.1/3.2
(+)	3.2	3.2/5.1, 3.2/5.2
(+)	3.1	3.2/5.1, 3.2/5.2

Fig 3.4. Assessment of RT and PCR primer combinations for elimination of false priming in the DENV capsid region. (A) The relative positions of the primers are shown. **(B)** Primer combinations used are listed.

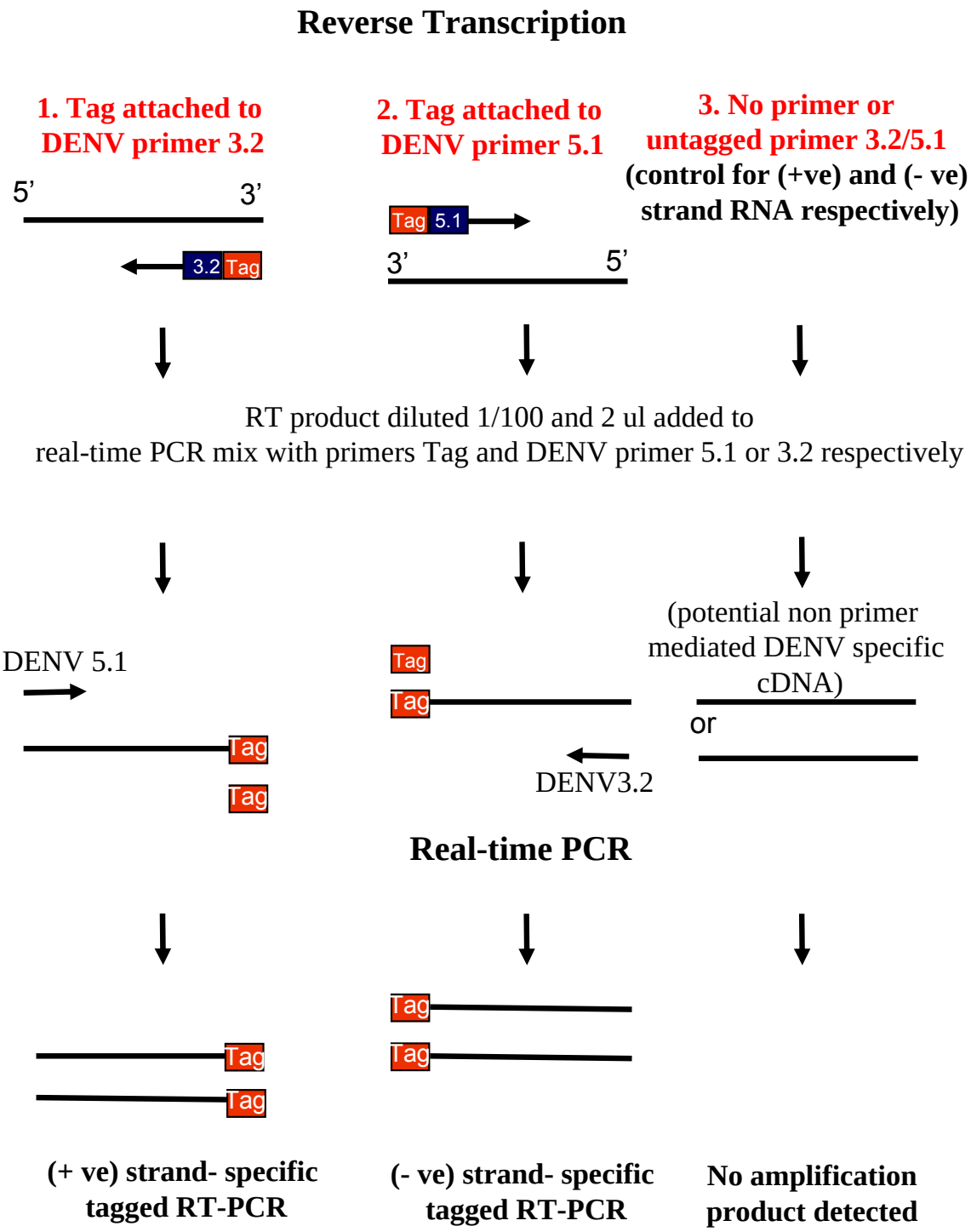
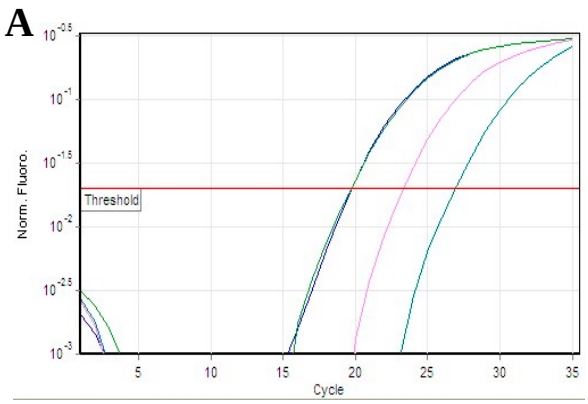
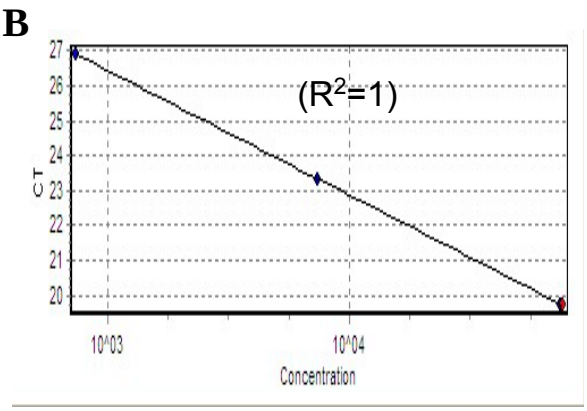


Fig 3.5. Schematic diagram showing (+ ve) or (- ve) strand-specific tagged RT real-time PCR adapted from Peyrefitte *et al*., 2003 and used to detect specific RT products synthesised using tagged RT primers to avoid detection of non primer-mediated DENV specific cDNA.





C

	pg RNA *in vitro* transcribed (+ ve) strand or extracted from cells	Ct	Calculated concentration (copies/reaction)
standards	0.002 Tagged complementary primer	19.74	74,115
	0.0002 Tagged complementary primer	23.32	7,376
	0.00002 Tagged complementary primer	26.89	741
0.02pg in vitro transcribed RNA	Tagged complementary primer	19.74	74,115
	no primer control	> 35	0
	wrong primer control	> 35	0
	no RT control	> 35	0
RNA from DENV infected HepG2	Tagged complementary primer	19.71	75,616
	no primer control	> 35	0
	no RT control	> 35	0

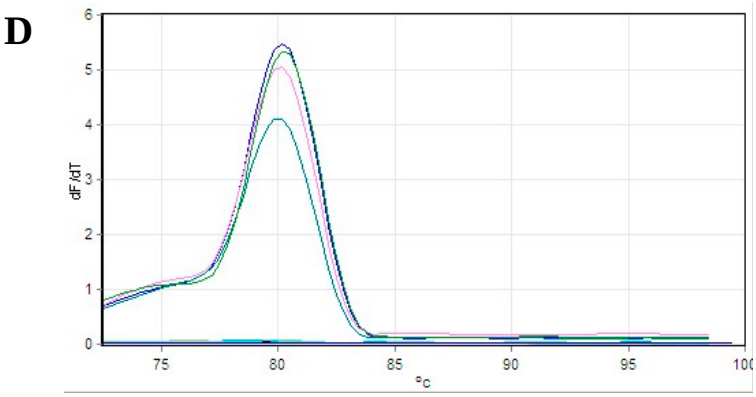
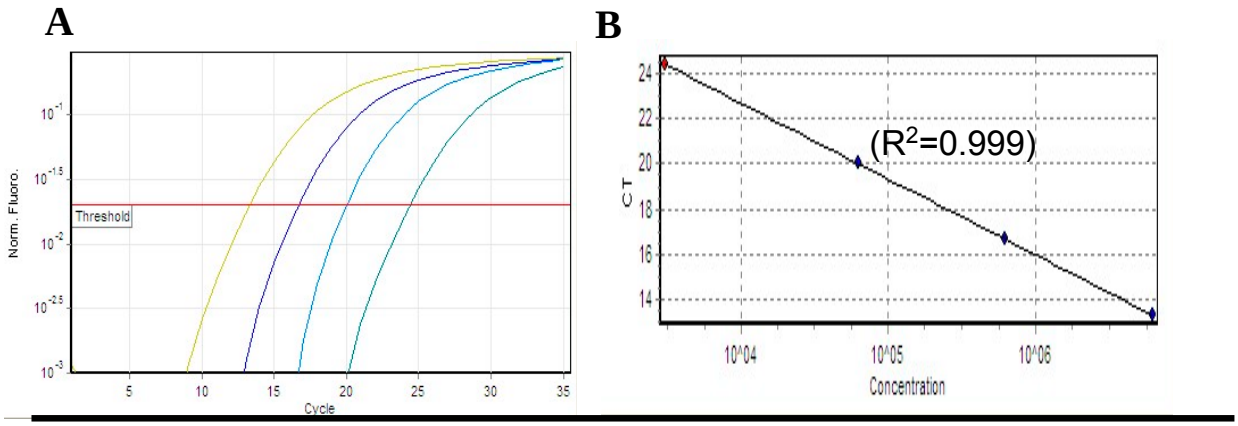


Fig 3.6. Use of tagged RT primers prevents detection of falsely primed products from (+ ve) strand RNA. Quantitation curves **(A)** and calculated copy numbers **(C)** based on standard curve **(B)** are shown for RT real-time PCR of *in vitro* transcribed RNA and RNA extracted from infected HepG2 cells. Melting curves **(D)** show one peak at 80^0C.



C

	pg RNA *in vitro* transcribed (+ ve) strand or extracted from cells	Ct	Calculated concentration (copies/reaction)
standards	1.7 Tagged complementary primer	13.31	6,265,042
	0.17 Tagged complementary primer	16.68	618,723
	0.017 Tagged complementary primer	20.01	62,650
0.017pg *in vitro transcribed RNA*	Tagged complementary primer	20.01	62,650
	no primer control	> 35	0
	wrong primer control	> 35	0
	no RT control	> 35	0
RNA from DENV infected HepG2	Tagged complementary primer	24.39	3,055
	no primer control	> 35	0
	no RT control	> 35	0

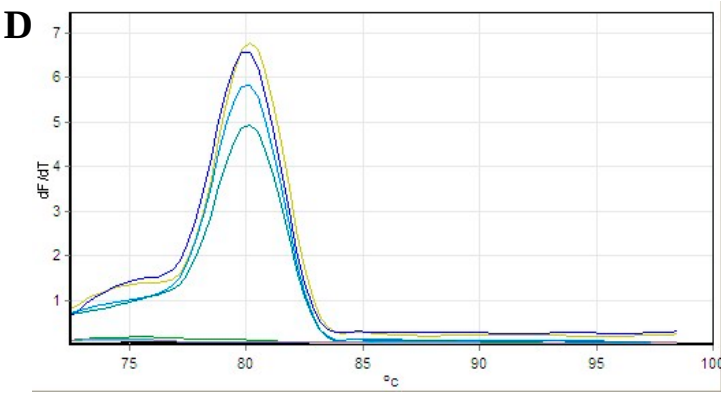


Fig 3.7. Use of tagged RT primers prevents detection of falsely primed products from (- ve) strand RNA. Quantitation curves **(A)** and calculated copy numbers **(C)** based on standard curve **(B)** are shown for RT real-time PCR of *in vitro* transcribed RNA and RNA extracted from infected HepG2 cells. Melting curves **(D)** show one peak at 80^0C.

Theoretically, residual primers (Tag-DENV) from the RT reaction may also prime in the subsequent PCR reactions and amplify the falsely primed DENV cDNA by virtue of the DENV specific portion of the Tag-DENV primer. To prevent this, Peyrefitte *et. al*., 2003 purified the tagged cDNA on a column, removing residual Tag RT primers based on size. This is costly and time consuming, and we chose to investigate diluting the RT reaction products prior to PCR amplification.

Following the Tag-RT reaction, the RT products were diluted 1:10 or 1:100 and 2 µl of the diluted product was amplified in either (i) a normal real-time PCR with both Tag and DENV specific primers or (ii) just one DENV specific primer alone as outlined in Fig 3.8. If any residual Tag-DENV primer from the RT reaction was taking part in subsequent PCR, then PCR products would be generated after adding only a single DENV specific primer.

Using *in vitro* transcribed (+ ve) or (- ve) strand RNA no PCR products were generated unless both Tag and DENV specific primers were added to PCR reactions (Fig 3.9), demonstrating that after dilution of 1:10 or 1:100, the remaining Tag-DENV primer from the RT reaction was insufficient to prime the PCR reaction. Thus the Tag primer RT-PCR approach circumvented the amplification of falsely primed DENV cDNA and yielded valid quantitations of *in vitro* transcribed (+ ve) and (- ve) strand RNA.

3.2.5.2 Specificity of detection of (- ve) strand RNA from infected cells using the tagged RT real-time PCR

We next examined the specificity of the tagged RT real-time PCR for detection of (- ve) strand from DENV-infected cells. The (- ve) strand RNA should not be present at 0 hrs post-infection, where there is exclusively (+ ve) strand input genomic RNA. Results from multiple experiments in MDM (Fig 4.1, chapter 4) K562 and HepG2 (Fig 3.10) confirm detection of (+ ve) but not (- ve) strand RNA immediately following DENV-infection. Similar analysis could not be performed for (+ ve) strand RNA as it is present throughout the entire infection period as seen in K562 and HepG2 DENV-infected cells (Fig 3.10).

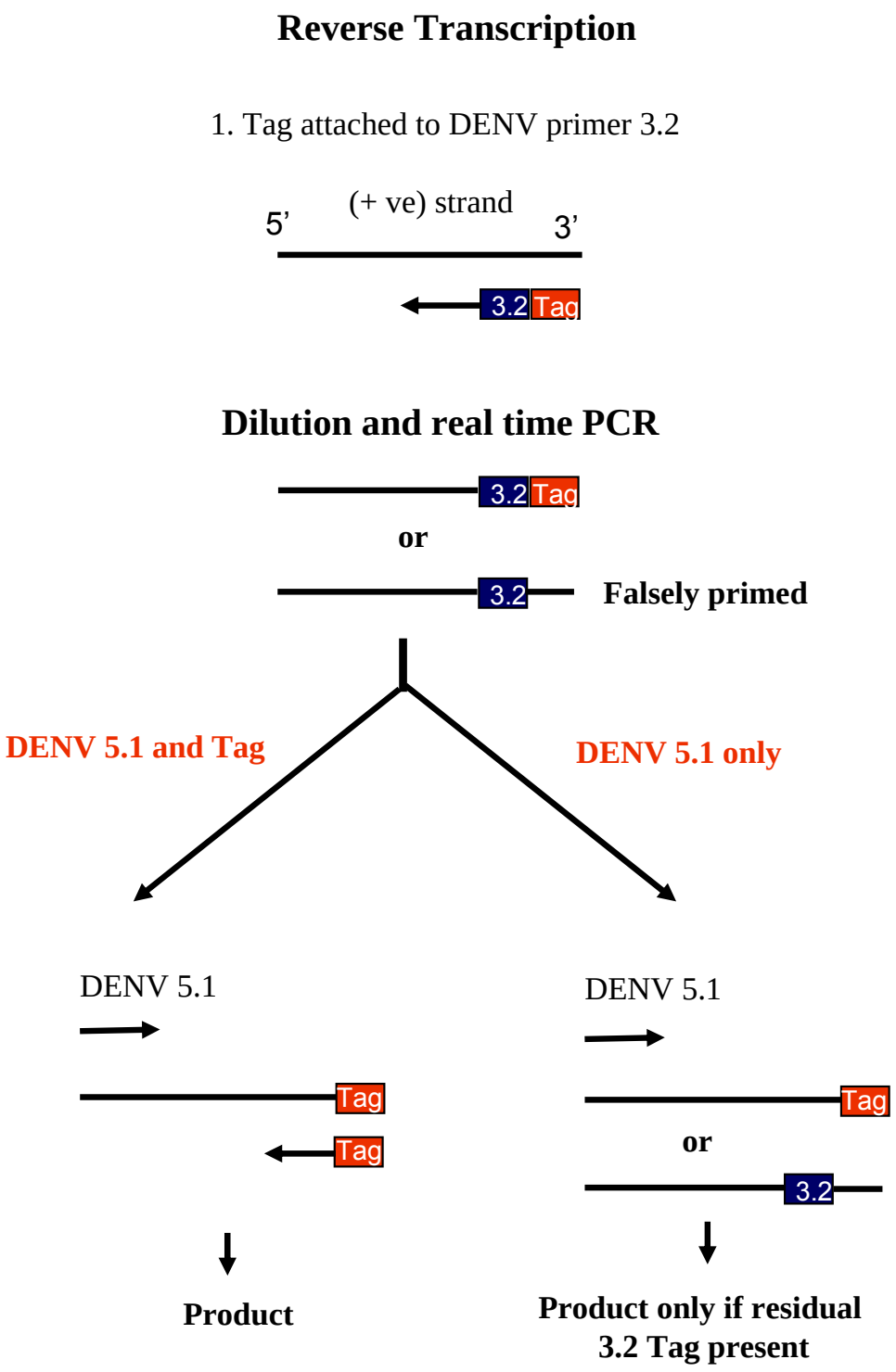
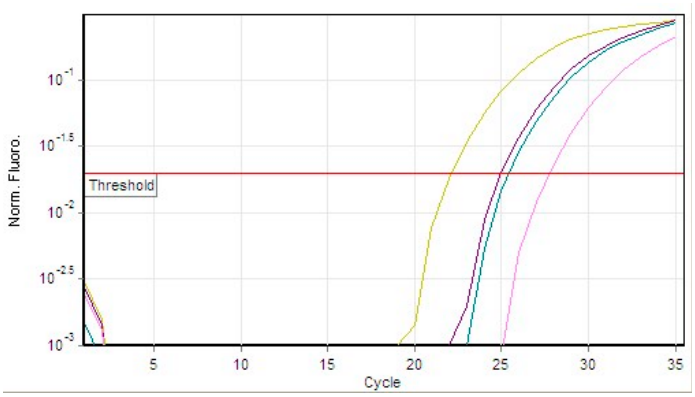


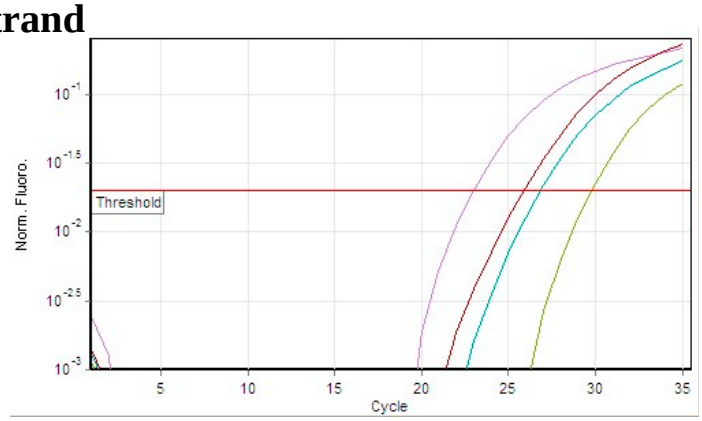
Fig 3.8. Strategy to check that residual Tag RT primers do not serve as PCR primers to amplify falsely-primed cDNAs. Addition of only one PCR primer i.e. DENV primer 5.1 or DENV primer 3.2 theoretically will only amplify products in presence of residual tagged RT primers from RT reactions. Similar strategy was validated for (– ve) strand RNA reverse transcribed with Tag DENV primer 5.1 and PCR amplified with DENV primer 3.2 and Tag or DENV primer 3.2 alone.

A. (+ ve) strand



	pg (+ ve) strand *in vitro* transcribed RNA	Ct	Calculated concentration (copies/reaction)
standards	0.02 (PCR primers Tag + 5.1)	22.13	75,089
standards	0.002 (PCR primers Tag + 5.1)	25.00	7,186
standards	0.0002 (PCR primers Tag + 5.1)	27.75	750
0.002pg *in vitro transcribed RNA*	PCR primers Tag + 5.1 (1/100)	25.42	5,087
0.002pg *in vitro transcribed RNA*	PCR primers 5.1-1/10	> 35	0
0.002pg *in vitro transcribed RNA*	PCR primers 5.1-1/100	> 35	0

B. (- ve) strand



	pg (- ve) strand *in vitro* transcribed RNA	Ct	Calculated concentration (copies/reaction)
standards	0.0017 (PCR primer Tag + 3.2)	22.98	5,633
standards	0.00017 (PCR primer Tag + 3.2)	25.88	787
standards	0.000017 (PCR primer Tag + 3.2)	29.77	56
0.00017pg *in vitro transcribed RNA*	PCR primer Tag + 3.2 (1/100)	26.82	416
0.00017pg *in vitro transcribed RNA*	PCR primer 3.2- (1/10)	> 35	0
0.00017pg *in vitro transcribed RNA*	PCR primer 3.2- (1/100)	> 35	0

Fig 3.9. Dilution of RT products 1/10 or 1/100 prevented residual RT primers from amplifying PCR products. Quantitation curves and calculated copy numbers are shown for RT real-time PCR of *in vitro* transcribed RNA **(A)** (+ ve) strand and **(B)** (- ve) strand. No products were detected in PCR reactions that only used DENV specific primers suggesting that dilution of RT products 1/10 or 1/100 is sufficient to prevent formation of any non specific products due to presence of residual primers.

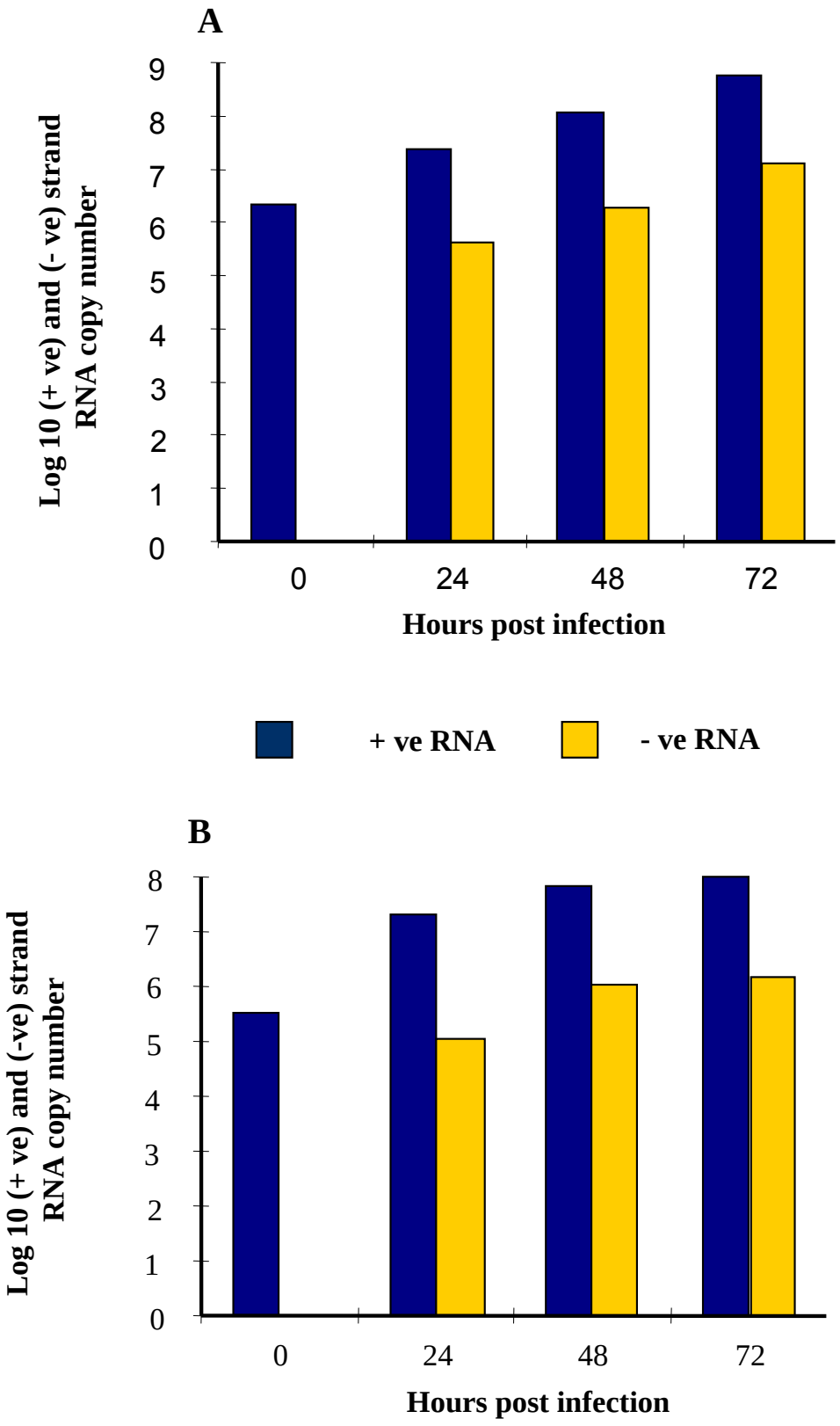


FIG 3.10. (+ ve) and (- ve) strand DENV RNA detected in infected (A) K562 and (B) HepG2 cells over 72 hrs, expressed as RNA copy number per 1 ng of input cyclophilin RNA. High levels of (+ ve) strand RNA could be detected at time 0 but no (- ve) strand was detected. Conversely high levels of both (+ ve) and (- ve) strands were detected at later time points post infection.

3.2.5.3 Sensitivity of DENV (- ve) and (+ ve) strand-specific tagged RT-PCR

To estimate the sensitivity of the tagged RT-PCR assay, known concentrations of (+ ve) or (- ve) strand *in vitro* transcribed RNA were serially diluted 10 fold and amplified using the above tagged RT real-time PCR system. Linear amplification was achieved with as little as 74 copies of (+ ve) strand RNA (Fig 3.11) and 63 copies of (- ve) strand RNA per reaction (Fig 3.12). Experiments in this study (chapter 4-5) used a MOI of 5 resulting in levels of cellular DENV RNA not less than 10^7 copies per 1 ng of cyclophilin RNA and thus lower sensitivity of RT real-time PCR assay was not required.

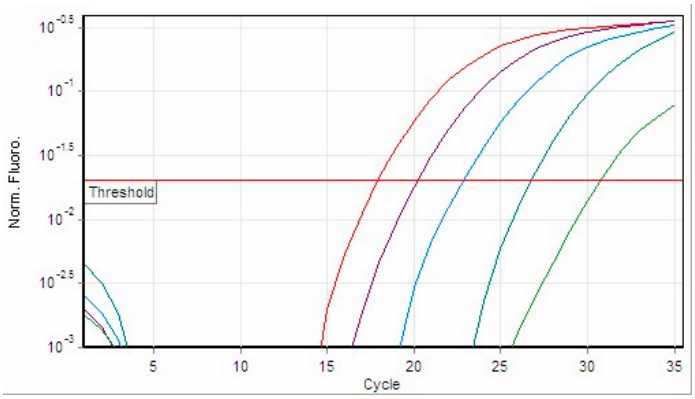
3.3 DISCUSSION

Later studies in this project aimed to determine the effect of cytokines on DENV replication, and thus a rigorous assay was needed to specifically quantitate DENV (- ve) strand RNA, as a marker of active replication without co-amplification of genomic (+ ve) strand RNA. We initially applied conventional RT-PCR methods to *in vitro* transcribed DENV RNA but observed significant PCR amplification of DENV specific products, even without addition of complementary primers. This is consistent with previous reports of non specific priming of *Flaviviridae* viruses RT reactions (Lanford *et al.*, 1994; Tolou, 1994; Lerat *et al.*, 1996; Mellor *et al.*, 1998; Peyrefitte *et al.*, 2003). As discussed in section 1.6.2.3 recent studies have shown that cyclisation of DENV RNA is a requirement for viral replication (Alvarez *et al.*, 2005; Filomatori *et al.*, 2006). These circularised structures, that are part of the inherent DENV replication strategy, may be responsible for confounding laboratory detection methods for DENV RNA, by inducing RNA self priming.

We examined if we could overcome this self priming by optimising the RT-PCR reaction. A number of strategies including different RT temperatures, enzymes and a range of primers at different concentrations (summarised in Table 3.1) were investigated. However, after extensive PCR optimisation, significant amplification of DENV specific products in the absence of specific priming of the RT reaction was still observed.

As the various optimisation methods outlined in Table 3.1 failed to stop non primer-mediated amplification completely, a Tagged RT real-time PCR was adapted to detect both (+ ve) and (- ve) strand RNA based on methods used by Peyrefitte *et al*., 2003. This method did not prevent non primer-mediated cDNA production during the RT step, but allowed amplification of those cDNA products that had incorporated the Tag-primer during strand-specific RT, thus

A



B

pg *in vitro* transcribed (+ ve) strand RNA	Ct	Input copy number	Calculated concentration (copies/reaction)
0.2	17.88	740 000	748 000
0.02	20.20	74 000	70 000
0.002	22.83	7 400	6 400
0.0002	26.72	740	574
0.00002	30.71	74	64
0.000002	> 35	7.4	0
No RNA control	> 35	0	0

C

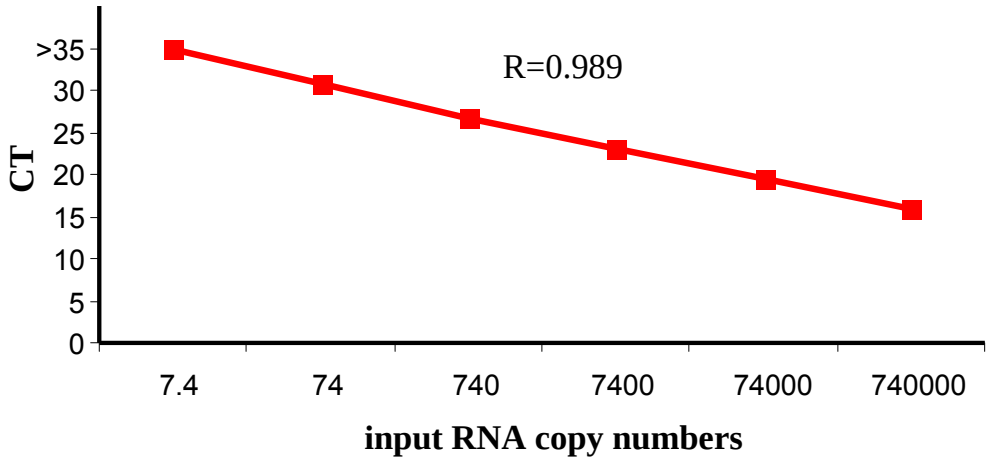
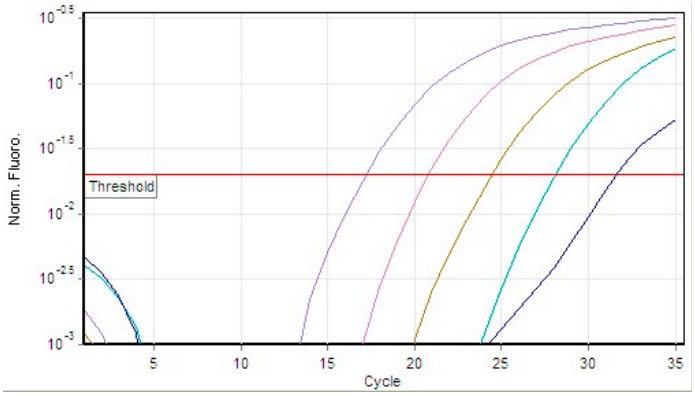


Fig 3.11. Sensitivity of RT real-time PCR was determined to be at least 7.4 -74 copies/reaction for (+ ve) strand RNA. RT real-time PCR was performed on 10 fold serially diluted 0.2 pg *in vitro* transcribed RNA. Quantitation curves **(A)** and input copy numbers (based on standard curve not shown) **(B)** are shown for RT real-time PCR of *in vitro* transcribed RNA. Relationship of known input RNA copies used in generating cDNA and the threshold cycle (CT) in the real time PCR assay are shown **(C).** R is the correlation coefficient.

A



B

pg RNA	Ct	Input copy number	Calculated concentration (copies/reaction)
0.17	17.19	630000	680000
0.017	20.80	63 000	59 460
0.0017	24.43	6 300	4 300
0.00017	28.09	630	530
0.000017	31.62	63	62
0.0000017	> 35	6.3	0
No RNA control	> 35	0	0

C

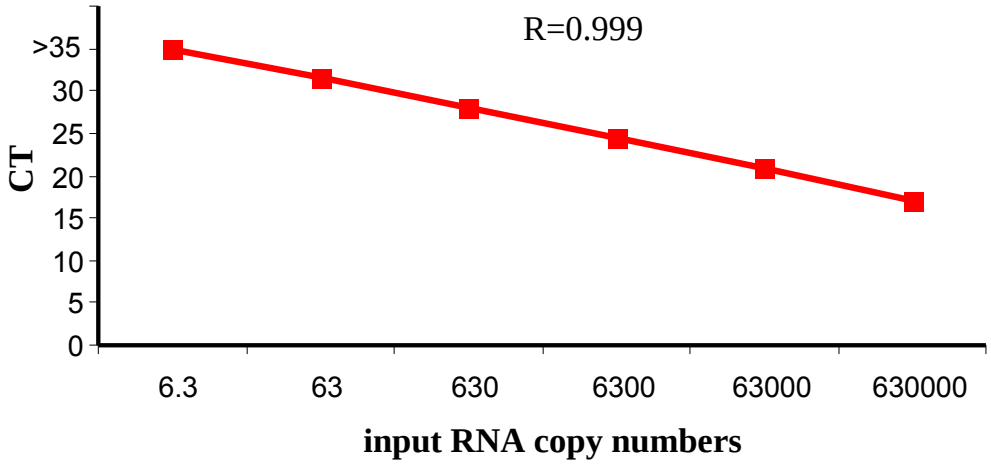


Fig 3.12. Sensitivity of RT real-time PCR was determined to be at 6.3 to 63 copies/reaction for (- ve) strand RNA. RT real-time PCR was performed on 10 fold serially diluted 0.17 pg *in vitro* transcribed RNA. Quantitation curves **(A)** and calculated copy numbers based on standard curves (not shown) **(B)** are shown for RT real-time PCR of *in vitro* transcribed RNA. Relationship of known input RNA copies used in generating cDNA and the threshold cycle (CT) in the real time PCR assay are shown **(C)**. R is the correlation coefficient.

	No RT enzyme	**Wrong primer**	**No primer**
Purpose	Detect DNA contamination	Show strand specificity	Specificity
Initial outcome	Positive signal	Positive signal	Positive signal
Implication	Contamination DNA	Contamination with opposite strand	Non specific priming
Approach	**(1) Further DNase treatment**	**(2)Further purification of plasmid and *in vitro* transcription product** **1.Treatment of template plasmid before *in vitro* transcription** (a) Complete digestion of plasmid (b) Gel purification of plasmid (c) Complete removal of opposite promoter **2.Treatment of *in vitro* transcription product** (d) Double RNeasy purification	**(3) Optimisation of RT conditions** (a) RT temperatures (b) Enzyme concentrations (c) Range of primer concentrations and combinations **(4) Tagged RT-PCR**
Outcome	**Successfully prevented signal in no RT enzyme control**	**No reduction in non specific DENV PCR amplification**	**Tagged RT-PCR prevented amplification of non specific priming**

Table 3.1. Summary of methods used to optimise RT real-time PCR products to prevent non specific priming of *in-vitro* transcribed RNA synthesised using pGEM-DENV-2CAP.

allowing specific quantitation of specific primer-generated (+ ve) or (- ve) RNA strands and was successfully adapted in our laboratory

We further simplified the published procedure of Peyrefitte *et al*., 2003 by dilution of RT products 1:10 or 1:100 before adding to real-time PCR to negate any PCR amplification from residual Tag-RT primer, rather than the cDNA purification step as used by Peyrefitte *et al*. 2003. The sensitivity of both (+ ve) and (- ve) strand tagged RT real-time PCR was estimated to be at least 74 and 63 copies per reaction for (+ ve) and (- ve) strand RNA respectively, much lower than the sensitivity needed to analyse DENV replication in cells in culture. DENV (- ve) strand RNA has been well established to be an active marker for DENV replication and studies on flavivirus replication using methods such as LiCl fractionation of RNA followed by Northern blot analysis and tagged RT-PCR (Liu *et al.*, 1997; Peyrefitte *et al.*, 2003) suggest that synthesis of DENV (- ve) strand occurs a few hours after infection. The results here using the tagged RT real-time PCR show that, in DENV-infected MDM, K562, and HepG2 cells at time 0 after infection, high levels of (+ ve) strand RNA but no (- ve) strand RNA could be detected, supporting the conclusion that the assays were strand-specific and can be successfully applied to quantitate DENV replication in infected cells. Thus, results from this chapter have shown strand-specific quantitation from *in vitro* transcribed RNA and during DENV-infection of cells. Such strand specific quantitation was not achievable through optimisation of standard RT-PCR protocols. The adaptation of this new Tag RT real-time PCR provides an accurate tool to allow study of intracellular DENV replication under different conditions and has been applied to the studies in chapter 4.

CHAPTER 4

DENV REPLICATION IN MDM IS NOT AFFECTED BY TNF-α, AND DENV-INFECTION INDUCES ALTERED RESPONSIVENESS TO TNF-α STIMULATION

4.1 INTRODUCTION

TNF-α is released from MDM after DENV-infection, and the peak of this release coincides with peak of virus production *in vitro* (Carr *et al.*, 2003; Espina *et al.*, 2003). Other cells of the immune system such as B and T cells that interact with monocytes and macrophages during viral infection can also release TNF-α when exposed to DENV (Lin *et al.*, 2002b; Mangada *et al.*, 2002; Mangada and Rothman, 2005). Thus TNF-α released from cells of the immune system may contribute to the elevated circulating (endocrine) levels of TNF-α that may in turn contribute to viral pathogenesis (discussed in section 1.8.2.4). In addition, TNF-α may act in an autocrine or paracrine manner to modulate viral replication in infected cells as seen with other viruses (discussed in section 1.8.2.4).

This chapter describes use of an *in vitro* primary cell culture system, to examine whether increased levels of TNF-α, as seen in DENV-infected patients and released from DENV-infected cultured cells, could affect DENV replication in macrophages, one of the main DENV target cell types *in vivo*. The first part of this chapter investigates the effect that addition of exogenous TNF-α, or inhibition of endogenously produced TNF-α, has on DENV replication. The second part of this chapter describes the cellular signalling response to TNF-α in DENV-infected cells.

4.2 RESULTS

4.2.1 Characterisation of a DENV MDM infection model

4.2.1.1 Characteristics of DENV replication in MDM

The tagged RT real-time PCR approach developed in chapter 3 was used to quantitate (+ ve) and (- ve) strand DENV RNA throughout DENV-infection of MDM. Our infection model utilised adherent MDM, 6 days post isolation and previously characterised to be 85-90% CD14 (+ ve), with adherent macrophage-like morphology (Pryor *et al.*, 2001). Cyclophilin A

mRNA was used to normalise all RT real-time PCR results, and therefore it was first necessary to validate whether cyclophilin A was consistently expressed during DENV-infection. MDM were infected with DENV and at day 0, 1, 2 and 3 post-infection, cells were harvested, RNA extracted and quantitated by spectrometry. For the same amount of input RNA, cyclophilin A mRNA did not vary over the 3 days in infected macrophages (Appendix III).

We next examined the time course of DENV replication over 72 hrs. MDM were DENV-infected and at different time points post-infection, the supernatants were collected to quantitate the release of infectious virus (pfu/ml) and intracellular RNA was extracted from cell lysates to measure levels of (+ ve) and (- ve) strand DENV RNA and normalised. Active replication of DENV in MDM was observed as indicated by the increasing production of infectious virus and increasing levels of (- ve) strand RNA. Maximum virus and RNA levels were seen at 48 hrs post-infection and levels of virus and RNA production continued at a lower level on day 3 (Fig 4.1A). The profile of (- ve) strand RNA coincided with infectious virus production (pfu/ml), whereas (+ ve) strand RNA was present in high levels at all time points (Fig 4.1A and B). Subsequent studies utilised this infection model and monitored infection by release of virus (plaque assay) and accumulation of (- ve) strand RNA (RT real-time PCR).

4.2.1.2 Characteristics of DENV-infected MDM

Having established a cell culture MDM model with active viral replication, the next set of experiments aimed to characterise the infected cell population. MDM were infected and cells stained for DENV antigens. Confocal analysis of stained cells showed 15-30% of MDM infected at 48 hrs post-infection, the time of peak virus production. Infection of MDMs by DENV was non cytopathic with no visual CPE or cell loss detected and DENV-infected MDM displayed diverse phenotype with variable shapes and sizes of DENV antigen positive MDM (Fig 4.2) consistent with previous reports that DENV-infects MDM regardless of its stage of differentiation (Chen and Wang, 2002). This indicates the low (15-30%) of infected MDM, is not due to infection of a subset of cells with specific morphology.

To assess changes in function of DENV-infected MDM, phagocytosis of opsonised SRBC by DENV-infected MDM was analysed. SRBC were opsonised by incubating in human sera at 37^0C, and added to MDM at 24 hrs post-infection at 37^0C for a further 24 hrs (section 2.3.4).

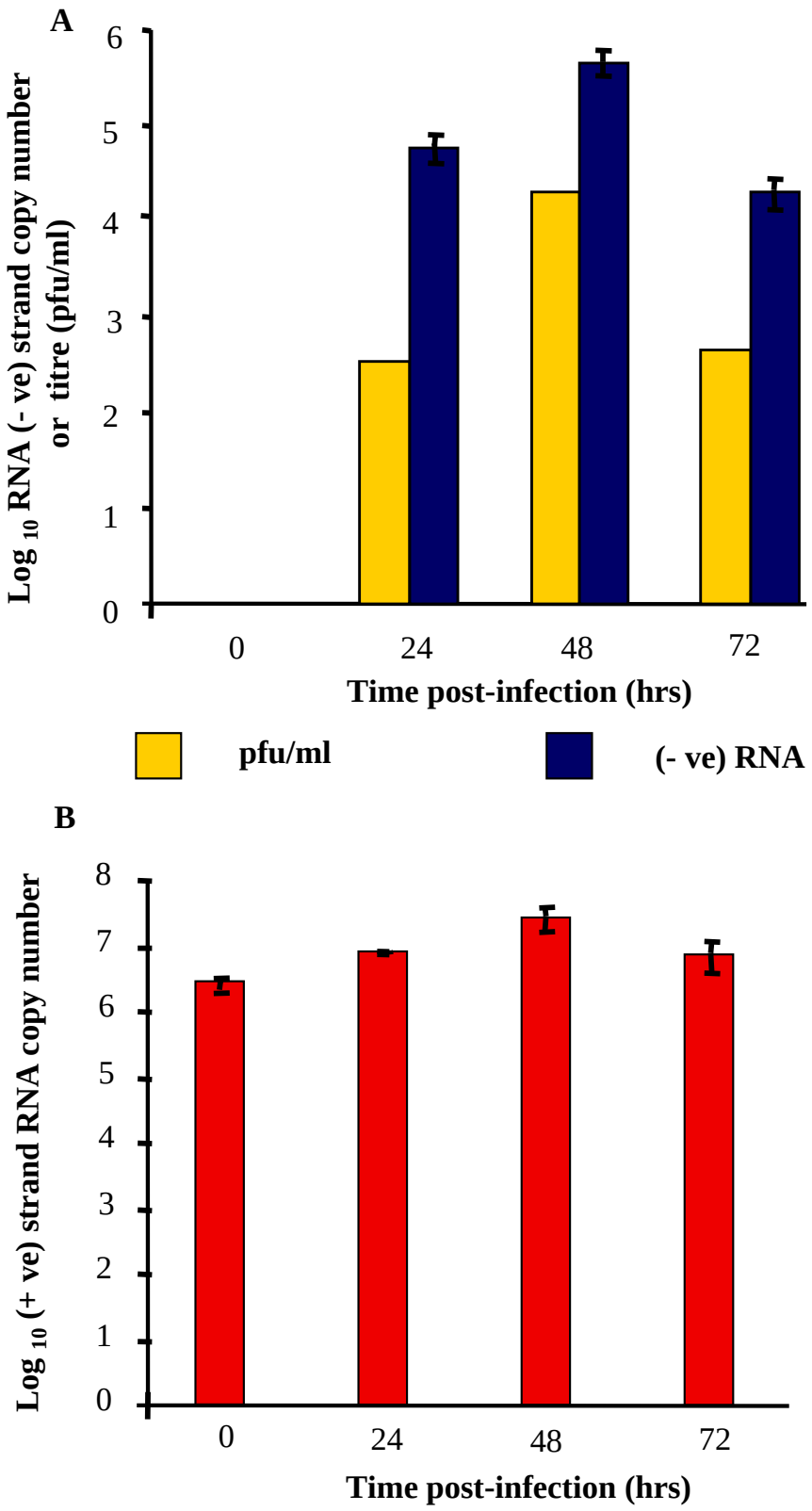


Fig 4.1. DENV replication in MDM. MDM were infected with DENV at MOI of 5. At 0, 24, 48 and 72 hrs post-infection supernatant was collected to determine infectious DENV released by plaque assay (pfu/ml), and RNA was extracted from cell lysates to determine levels of intracellular DENV RNA using a tagged RT real-time PCR. *In vitro* transcribed copy number standards from DENV2 capsid protein were used to quantitate DENV RNA. Tagged RT real-time PCR represent average ± SEM (n=2) and were normalised per 1 ng input cyclophilin RNA. Peak of infectious virus production and (- ve) strand RNA was seen at 48 hrs. (**A)** (- ve**)** strand RNA and pfu/ml **(B)** (+ ve) strand RNA

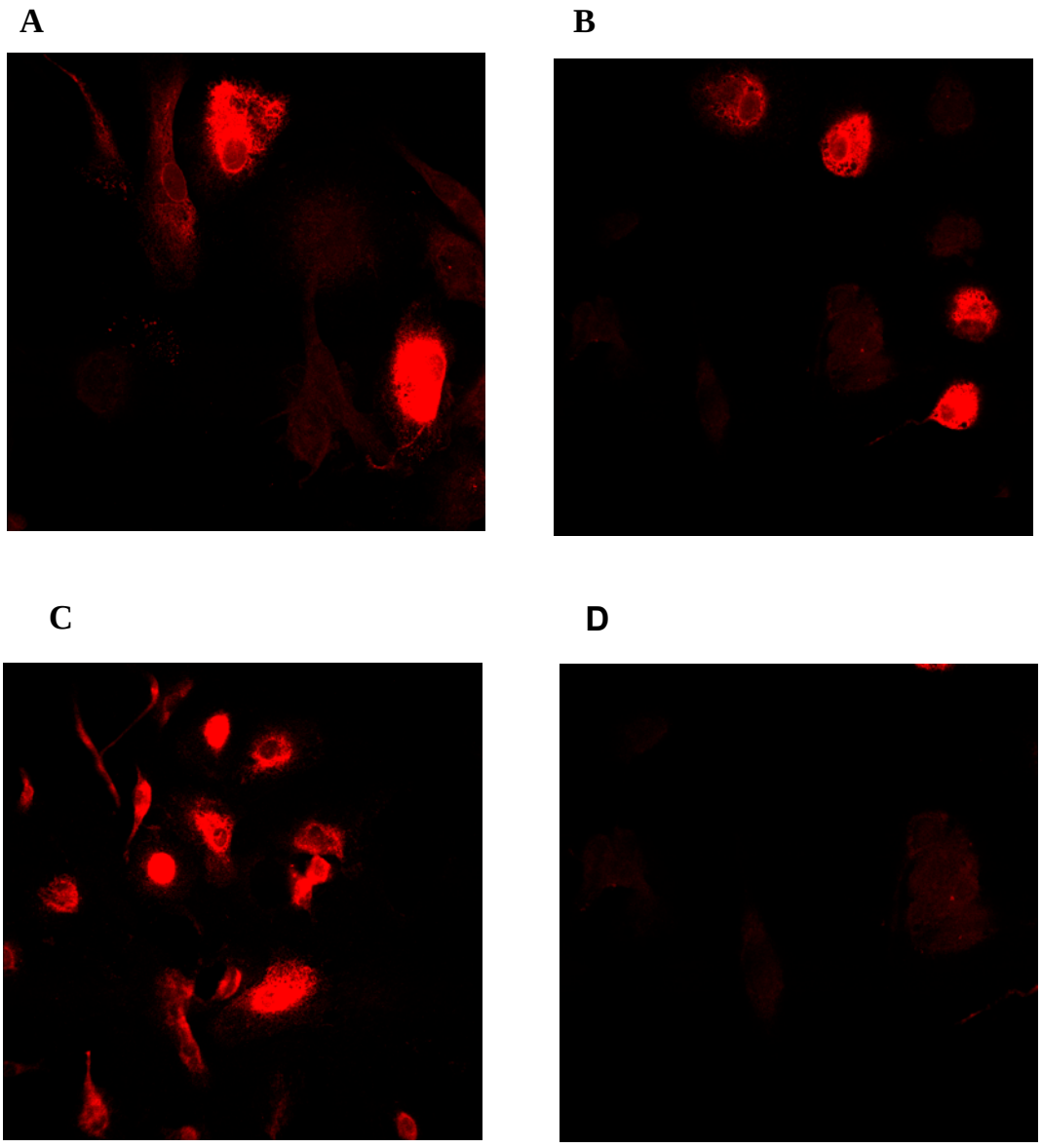


Fig 4.2. DENV infected MDM display diverse phenotype. MDM were infected with DENV at MOI of 5. After 48 hrs, infected cells were washed thoroughly fixed, permeabilised, stained with DENV anti-E mouse monoclonal antibodies and antigen/antibody complexes visualised with anti-mouse red Alexa Fluor 546 (conjugated secondary antibody) and confocal microscopy. Variable phenotype of DENV antigen positive MDM was observed. **A**, **B** and **C** shows different fields of infected macrophages, **D** shows DENV infected cells stained with control normal human sera.

After incubation cells were washed thoroughly, fixed, permeabilised and stained with DENV mouse monoclonal antibodies and Alexa Fluor 546 (conjugated anti-mouse IgG). Using direct light microscopy, uninfected MDM showed 80-85% of cells that successfully phagocytosed SRBC. The (10-15%) remaining cells had the same morphology but did not ingest SRBC (Fig 4.3A and B). This finding is consistent with other reports (Liao *et al.*, 1994) that MDM have a heterogeneous phagocytic ability. In contrast, to the DENV E - antigen (+ ve) cells in the DENV-infected population only 40% DENV (+ ve) MDM phagocytosed SRBC (Fig 4.3E and F, Table 4.1). However, some DENV positive cells stained densely with Alexa Fluor 546 and hindered visualisation of SRBC and therefore 40% SRBC phagocytosis may be an underestimate. Approximately 60% of uninfected MDM in the DENV-infected population showed presence of phagocytosed SRBC (Table 4.1). This finding again is consistent with literature (Chaturvedi *et al.*, 1983) where spleen and peritoneum macrophages isolated and cultured from DENV-infected mice showed a low phagocytic index for erythrocytes compared to uninfected controls. Thus DENV-infected MDM are heterogeneous with only a subpopulation (15-30%) of cells showing productive, non cytopathic infection. Although the number of phagocytic cells was reduced in the DENV-infection population, functional phagocytosis was retained in some DENV positive cells.

4.2.2 Addition of exogenous TNF-α prior to or after established DENV-infection does not dramatically affect DENV replication in MDM

We next assessed the ability of TNF-α to affect DENV replication in MDM. TNF-α is present at high levels in the circulation of DENV-infected patients and is released by DENV-infected macrophages and other cells normally present in the environment of macrophages. From the literature we know that pre-treatment of HepG2 cells with IFN-α, 4 hrs before infection inhibits subsequent DENV replication while pre-treatment with TNF-α for 24 hrs before infection has no effect on DENV replication in HepG2 cells (Diamond *et al.*, 2000b) and MDM (Chen *et al.*, 1999). We established conditions for DENV-infection in HepG2 cells to use as a comparative control. The replication profile of DENV in HepG2 cells is shown in Fig 4.4, showing increasing virus production from 0 - 72 hrs post-infection as previously reported (Marianneau *et al.*, 1998).

Prior studies in our lab have shown TNF-α is released by DENV-infected MDM, coinciding with the peak of DENV production at 36 hrs after established infection (Carr *et al.*, 2003). We therefore looked at the effect of TNF-α on DENV replication in MDM, with TNF-α

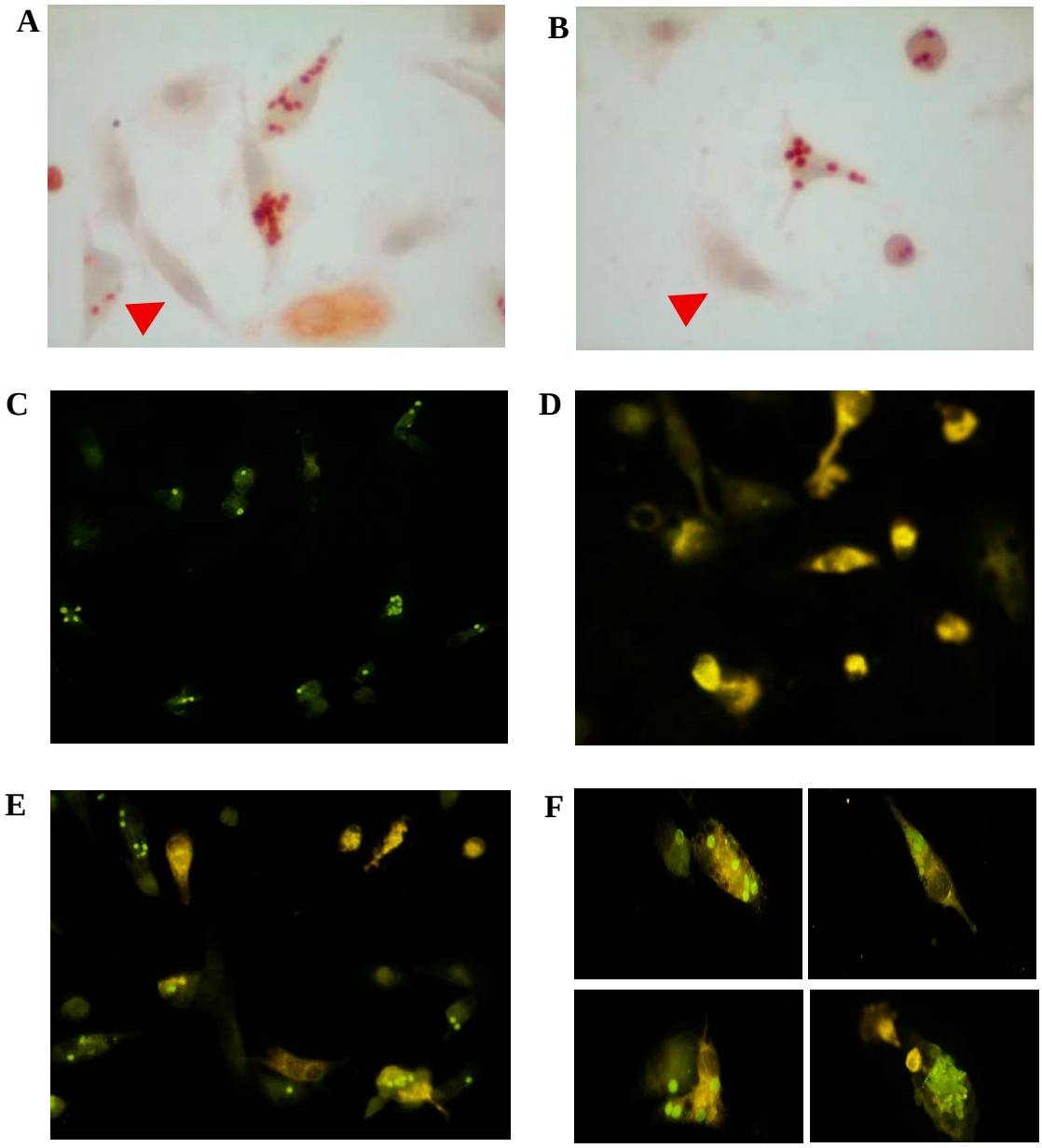


Fig 4.3. Phagocytosis of SRBC by MDM. (A-B) Uninfected MDM. 6 day old uninfected MDM were incubated with opsonised SRBC at 37^0C for 24 hrs. Cells were washed thoroughly fixed, permeabilised, stained with haematoxylin and observed using light microscopy. 80 to 85% of MDM successfully phagocytosed SRBC. The other 10-15% were morphologically similar to cells that phagocytosed SRBC (indicated via a red arrow) but did not ingest SRBC. **(C-F) Infected MDM.** MDM were infected with DENV at MOI of 5. 24 hrs after infection, opsonised SRBC were added to infected cells and left at 37^0C for 24 hrs. Infected cells were washed thoroughly fixed, permeabilised, stained with DENV anti E-mouse monoclonal antibodies and red Alexa Fluor 546 (conjugated secondary antibody). Presence of red blood cells were visualised using a fluorescent microscope with a green filter. SRBC appear green and infected cells appear yellow green in colour. **(C)** Negative control – DENV infected MDM + SRBC stained with normal mouse serum and Alexa Fluor 546 **(D)** DENV infected MDM - SRBC stained with DENV anti-E mouse antibody **(E)** and **(F)** DENV infected MDM + SRBC stained with DENV anti-E mouse antibody .

Condition	% MDM able to phagocytise opsonised SRBC
Uninfected MDM[a]	80-85%
DENV infected MDM population	
DENV infected cells[b]	40%
DENV uninfected cells[c]	60%

Table 4.1. Phagocytosis of SRBC by DENV infected and uninfected cells
Number cells counted a = 50, b = 65, c = 65

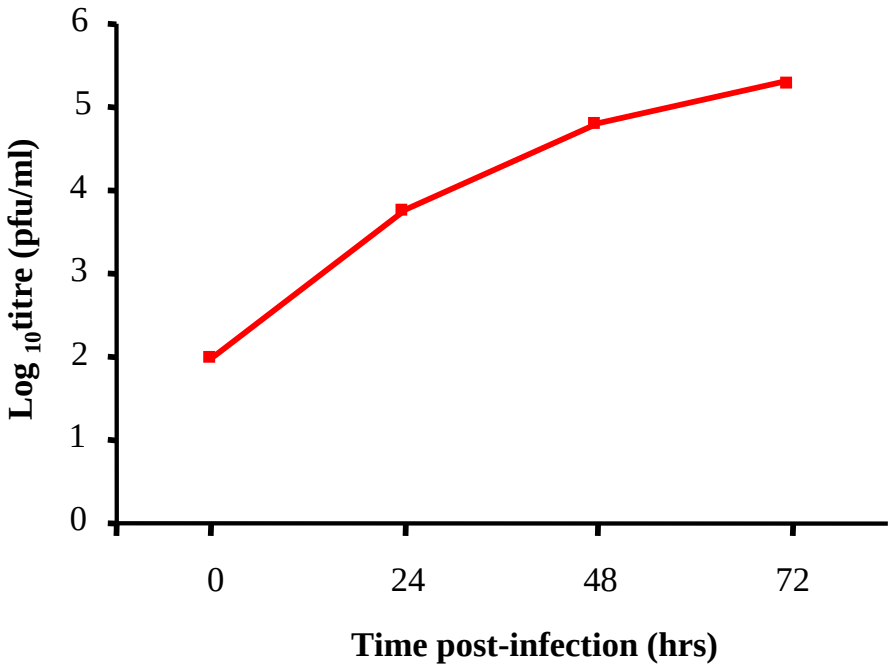


Fig 4.4. Replication profile of DENV in HepG2 cells. HepG2 cells were infected with DENV at a MOI of 5 and cell culture medium was collected and assayed for infectious virus released (pfu/ml).

added at 4 hrs prior to infection and at 24 hrs post-infection, a time point that represents established infection but is still prior to the peak of endogenous TNF-α release and progeny viral production.

The levels of TNF-α (500 ng/ml) used in this part of our study were higher than the pg/ml levels released from DENV-infected MDM (Carr *et al.*, 2003) consistent with levels known to induce TNF-α responses in the literature (Lane *et al.*, 1999; Minagawa *et al.*, 2004) and higher than the levels used later in this study to induce TNF-α signaling in uninfected cells and thus should be in the required range for TNF-α activity. Lower levels of TNF-α up to 0.3 ng/ml were also tested and similar results as that seen with 500 ng/ml were observed (data not shown). Addition of TNF-α at 24 hours post-infection showed no effect on subsequent DENV replication in MDM (Fig 4.5A) or HepG2 (Fig 4.6A) as measured by analysis of levels of DENV RNA and infectious virus at 48 hrs post-infection. Treatment with IFN-α at 24 hrs post-infection showed a small inhibitory effect on DENV (- ve) strand RNA accumulation and virus release from MDM (Fig 4.5B) and, as previously reported also for HepG2 cells (Fig 4.6B) (Diamond *et al.*, 2000b). These experiments were repeated four times in MDM and twice in HepG2.

In contrast, 4 hrs pre-treatment of MDM with TNF-α before DENV-infection had a small and reproducible inhibitory effect (1.4-4 fold) on release of infectious virus (pfu/ml) and RNA production from MDM (Fig 4.5A and Table 4.2). The paired Student t test from the results of four independent experiments measuring infectious virus release (pfu/ml) and two independent experiments that quantitated RNA levels showed that, although alteration in the release of infectious virus was not significant ($p = 0.28$), there was a significant reduction in RNA levels ($p = 0.03$), suggesting this to be a real and reproducible inhibition of DENV replication. However, pre-treatment of HepG2 cells for 4 hrs with TNF-α had no effect (< 2 fold) on DENV replication (Fig 4.6A). These moderate effects of pre-treatment with TNF-α contrast with the more significant effects seen with IFN-α where 4 hrs pre-treatment of MDM and HepG2 cells with IFN-α showed >10 fold inhibition of (- ve) strand RNA accumulation and virus release (Fig 4.5B and 4.6B, Table 4.2), consistent with the literature (Diamond *et al.*, 2000b). Finally, no effect of 24 hrs TNF-α pre-treatment on DENV replication in HepG2 cells (Fig 4.6B) and MDM (results not shown) were seen, again consistent with previous reports (Chen *et al.*, 1999; Diamond *et al.*, 2000b). To assess if this small but reproducible inhibitory effect of 4 hrs TNF-α pre-treatment was due to alterations in the levels of viral

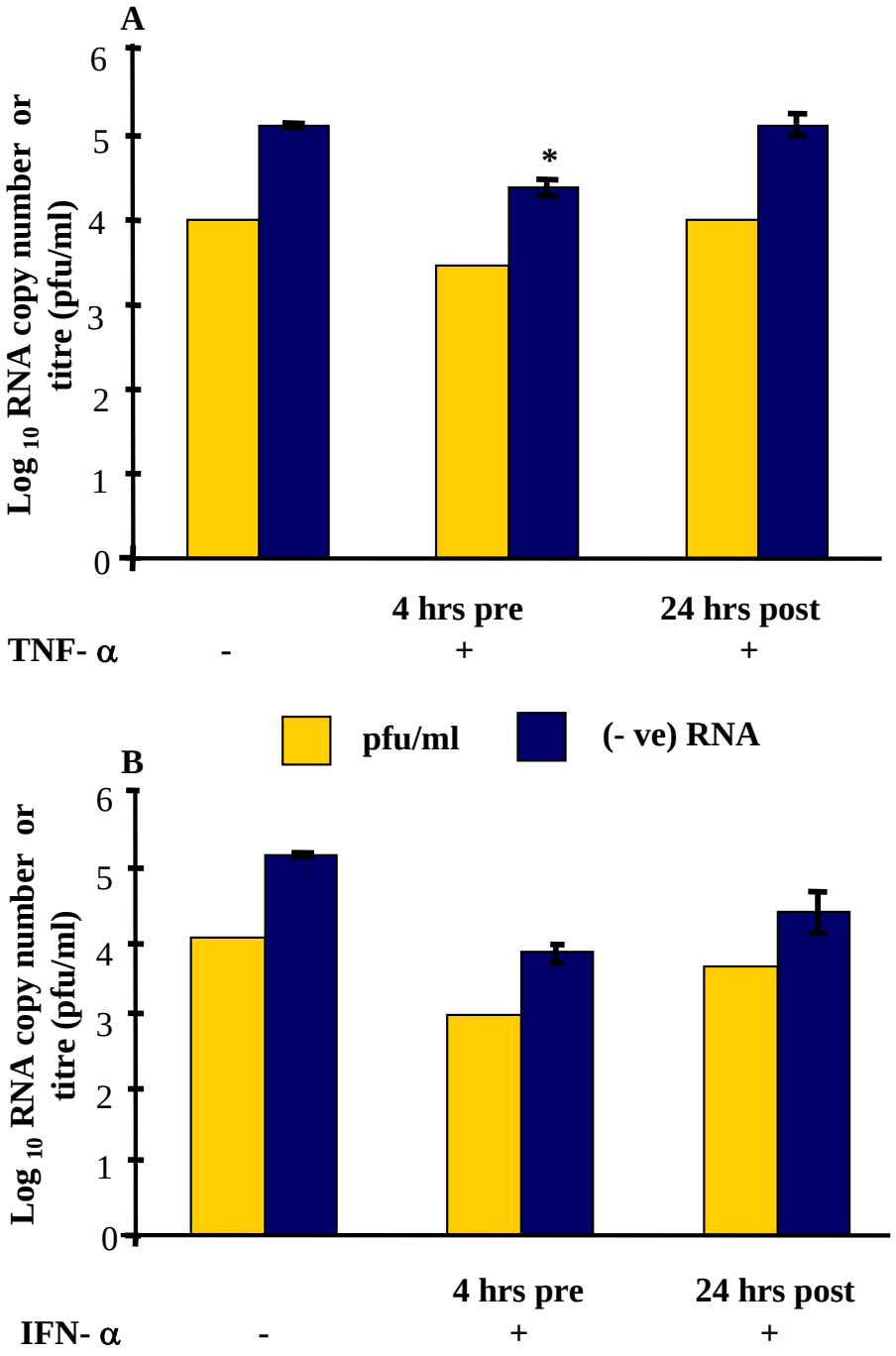


Fig 4.5. Exogenously added TNF-α shows minor inhibition of DENV replication in MDM when added 4 hrs pre but not 24 hrs post DENV infection. MDM cells were exposed to TNF-α or IFN-α for 4 hrs, infected with DENV MOI 5 or infected then cytokine treated 24 hrs post-infection. Cell culture supernatants were collected at 48 hrs after infection and assayed for virus release by plaque assays (pfu/ml). RNA was extracted from cell lysates and DENV RNA was quantitated by tagged RT real-time PCR and were normalised per 1 ng input cyclophilin mRNA. Values represent mean ± SEM from duplicate samples in RT real-time PCR of a representative experiment. Experiments were replicated (n=2, RNA; n=4, titre). Panels represent effects of **(A)** TNF-α **(B)** IFN-α (controls). *$p<0.05$ (students t-test).

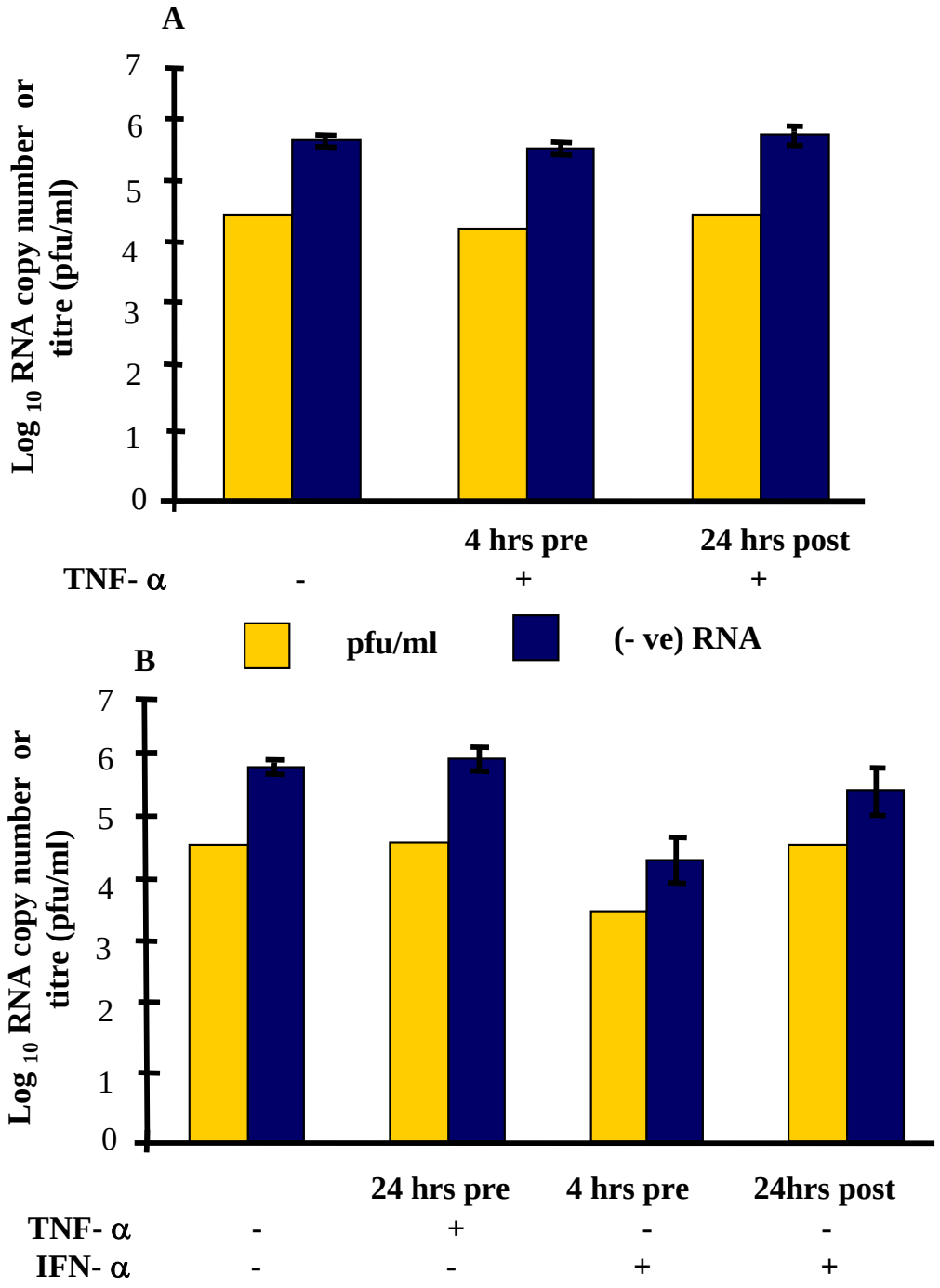


Fig 4.6. Exogenously added TNF-α shows no inhibition on DENV replication in HepG2 cells (used as controls) when added 4 hrs pre or 24 hrs post DENV infection. HepG2 cells were exposed to TNF-α or IFN-α for 4 or 24 hrs then infected with DENV MOI 5 or infected then cytokine treated 24 hrs post-infection. Cell culture supernatants were collected at 48 hrs after infection and assayed for virus release by plaque assays (pfu/ml). RNA was extracted from cell lysates and DENV RNA was quantitated by tagged RT real-time PCR and were normalised per 1 ng input cyclophilin mRNA. Values represent mean ± SEM from duplicate samples in RT real-time PCR of a representative experiment. Experiments were replicated (n=2). Panels represent effects of **(A)** TNF-α **(B)** TNF-α and IFN-α (controls).

Cell type and experiment number	Fold decrease in DENV replication (pfu/ml) with 4hrs TNF-α pre-treatment	Fold decrease in DENV replication (pfu/ml) with 4hrs IFN-α pre-treatment
MDM 1	4	12
MDM 2	4.5	Not done
MDM 3	1.4	10
MDM 4	3	15
HepG2 1	1.2	10
HepG2 2	1.5	10

Table 4.2. TNF-α pre-treatment 4 hrs before infection has a small but reproducible inhibitory effect on DENV replication compared to IFN-α pre-treatment. Difference in viral production pfu/ml are listed from multiple independent experiments.

attachment or entry, the levels of cell-associated input virus were quantitated by measurement of (+ ve) strand viral RNA immediately after infection in cells pre-treated for 4 hrs with TNF-α. Results show that levels of input RNA in TNF-α pre-treated cells were not different to untreated cells, indicating that pre-treatment with TNF-α did not affect virus binding or uptake (Table 4.3). Similar results with IFN-α pre-treatment (Table 4.3) were observed consistent with the literature (Diamond and Harris, 2001). Thus addition of high levels of exogenous TNF-α which were shown in subsequent studies to be biologically active (section 4.2.4.1-2) and were known to be stable in culture conditions (Aderka *et al.*, 1992) had a very small effect on DENV replication in MDM and only if added 4 hrs prior to infection.

4.2.3 Inhibition of endogenously produced TNF-α has no significant effect on DENV replication in MDM

Since DENV-infected macrophages can produce their own TNF-α it was possible that addition of exogenous TNF-α had no effect on DENV replication because responses were already saturated by endogenously released TNF-α. Thus siRNA was used to block production of endogenous TNF-α and TNF-α antibodies were used to block actions of TNF-α produced by infected cells, to further investigate the role of endogenous TNF-α on DENV replication.

4.2.3.1 Blocking TNF-α by siRNA knockdown has no effect on DENV replication

To achieve knockdown of production of endogenous TNF-α, 3 pre-designed stealth TNF-α siRNAs (Invitrogen) were used to transfect MDM. Stealth siRNAs were used to prevent stimulation of the dsRNA or IFN-α response that could complicate the interpretation of results.

4.2.3.1.1 Optimisation of TNF-α stealth siRNA to give maximum TNF-α knockdown

We first analysed siRNA uptake by MDM. A florescent siRNA was transfected into MDM under standard conditions recommended by the manufacturer (Invitrogen) and showed >90% uptake of the labelled oligonucleotides into MDM (Appendix V) demonstrating highly efficient uptake of siRNA by MDM. Functional TNF-α knockdown was assessed by stimulating TNF-α siRNA transfected cells with LPS and quantitating levels of TNF-α

Treatment 4 hrs before infection	Copy number (+ ve) strand RNA in DENV infected MDM at 0 hr post-infection	Copy number (+ ve) strand RNA in DENV infected HepG2 at 0 hr post-infection
TNF-α	8.9×10^6	2×10^5
IFN-α	9×10^6	4×10^5
Control – no treatment	9×10^6	3×10^5

Table 4.3. TNF-α or IFN-α pre-treatment 4 hrs before infection has no effect on the level of (+ ve) strand genomic DENV RNA associated with newly infected cells.

mRNA (by RT real-time PCR) and levels of TNF-α protein (by bioassay). Maximum TNF-α knockdown was achieved by using two rounds of transfection of 1.6 μM siRNA 24 hrs apart (data not shown) using Lipofectamine RNAiMAX, an agent found to be not as toxic to cells as conventional Lipofectamine.

To validate TNF-α protein knockdown, MDM were stimulated with 10 ng/ml of LPS 1-4 days after siRNA transfection, and TNF-α release was measured by bioassay. Results showed that all three siRNAs yielded 50-80% inhibition of bioactive TNF-α protein release that lasted over a 4-day period (Fig 4.7). This indicates rapid and persistent TNF-α knockdown in MDM from 1-4 days post-transfection using these siRNA transfection conditions.

4.2.3.1.2 siRNA reduction of TNF-α had no effect on DENV replication

To test if siRNA reduction of TNF-α in MDM had any effect on DENV replication, MDM were transfected with the same three stealth TNF-α siRNA's and a negative control (scramble siRNA), then infected with DENV (MOI of 5). Experiments were sampled at 32 hrs post-infection, since at the usual sampling point of 48 hrs, TNF-α mRNA could not be detected to confirm siRNA knockdown. We know, however, that DENV RNA and infectious virus levels are similar at these two time points. At 32 hrs post-infection the supernatant was collected and analysed for viral titer, DENV RNA and TNF-α protein production (by ELISA). Cellular RNA was extracted and analysed for DENV RNA and TNF-α mRNA production by RT real-time PCR. Two of the three stealth siRNA showed >75% inhibition of TNF-α mRNA and protein release from DENV-infected MDM (Fig 4.8A and B) showing efficient knockdown of endogenous TNF-α. However, analysis of DENV release and (- ve) strand RNA levels showed that even with this high level of TNF-α protein knockdown, no difference in virus replication was observed (Fig 4.9). The lack of effect of siRNA knock-down of TNF-α on DENV replication was also observed at 48 hrs post-infection, although concomitant siRNA knockdown of TNF-α could not be confirmed in these latter experiments.

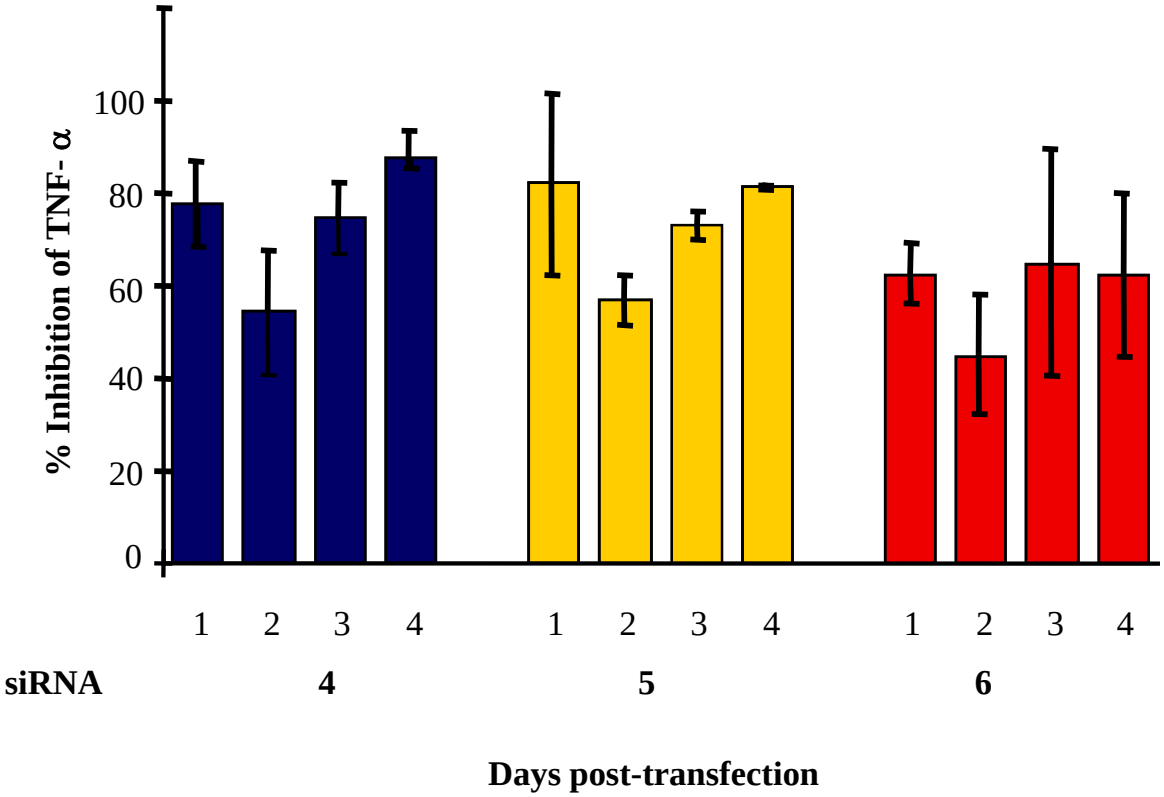


Fig 4.7. TNF-α siRNA inhibits LPS-stimulated release of TNF-α bioactivity. MDM were transfected with individual TNF-α siRNA's by two rounds of transfection with 1.6 μM TNF-α siRNA 4, 5 or 6. At 1-4 days post-transfection MDM were stimulated with 10 ng/ml LPS and TNF-α production in macrophages quantitated by TNF-α bioassay. Results are expressed as a % inhibition of TNF-α release following LPS-stimulation of mock transfected cells. Values are average of 2 wells and a representative experiment is shown. Experiments were replicated (n=2).

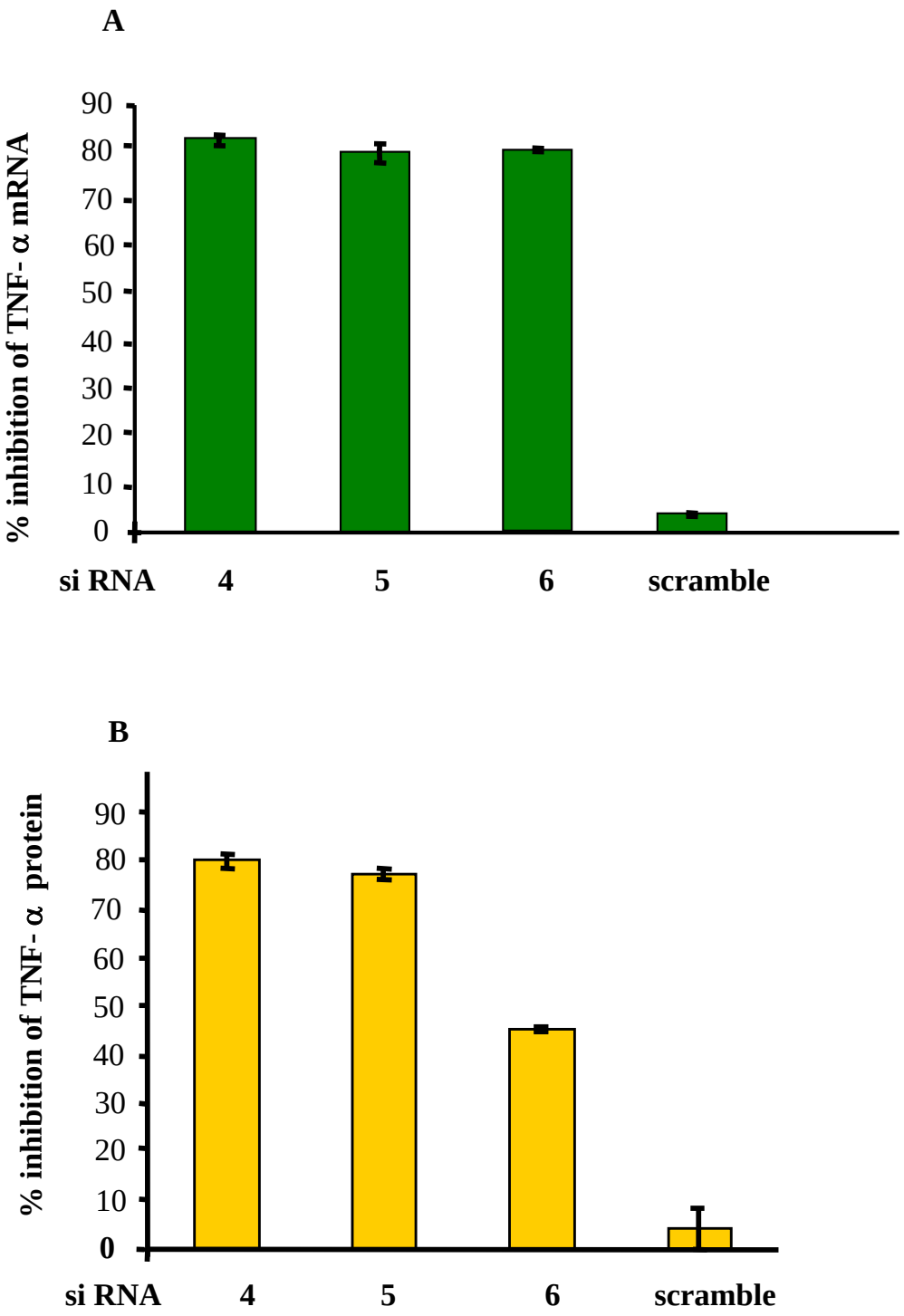


Fig 4.8. siRNA knock down of TNF-α in MDM infected with DENV. Cells were transfected with TNF-α siRNA, infected with DENV at MOI 5 and at 32 hrs after infection cell culture supernatants were assayed for **(A)** TNF-α mRNA levels in infected cells (by RT real-time PCR) normalised per 1 ng input cyclophilin mRNA **(B)** TNF-α production (by ELISA). Results were expressed as a percentage of TNF-α released from mock transfected MDM (2,027 pg/ml/24 hrs). Values are mean ± SEM of 2 wells and values represent mean ± SEM from duplicate samples in RT real-time PCR of a representative experiment. Experiments were replicated (n=2). siRNA's 4 and 5 showed > 75% inhibition of TNF-α protein and mRNA.

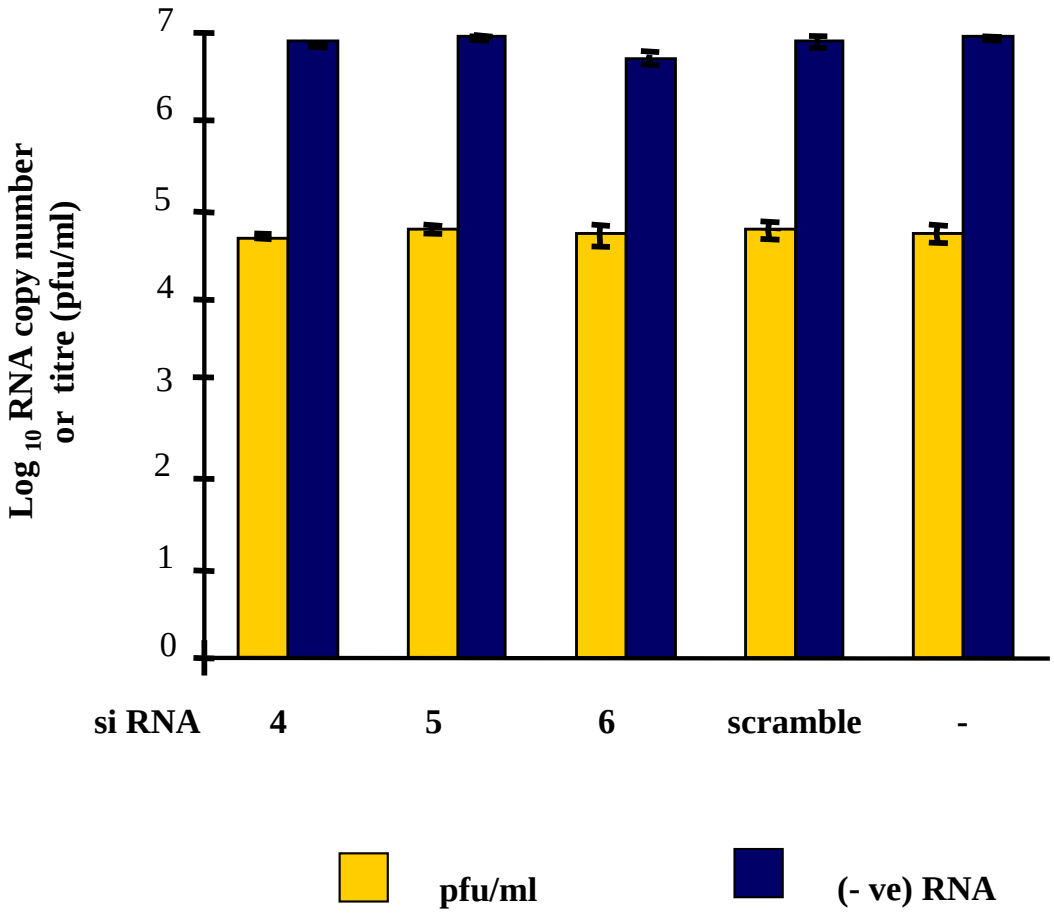


Fig 4.9. siRNA knock down of TNF-α in MDM had no effect on DENV replication. Cells were transfected with TNF-α siRNA infected with DENV at MOI 5 (same cells as in Fig 4.8) and at 32 hrs after infection cell culture supernatants were assayed for DENV replication by infectious virus released (pfu/ml) and (- ve) strand RNA levels in infected cells. *In vitro* transcribed copy number standards from DENV2 capsid protein were used to quantitate (- ve) viral RNA using a tagged RT real-time PCR normalised per 1 ng input cyclophilin mRNA. Values represent mean ± SEM from duplicate samples in RT real-time PCR of a representative experiment. Experiments were replicated (n=2). siRNA's 4 and 5 showed greater then 75% inhibition of TNF-α mRNA and protein release (Fig 4.8) but had no effect on DENV replication.

4.2.3.2 Blocking actions of endogenously released TNF-α had no effect on DENV replication

Although we achieved high level TNF-α mRNA and protein knockdown, TNF-α was not completely abolished. Therefore we next aimed to extend the siRNA knockdown studies by antibody blocking of endogenous TNF-α activity. MDM were isolated, infected with DENV and at 24 hrs post-infection, prior to the peak of TNF-α release (Carr *et al.*, 2003), neutralising TNF-α antibody or isotype matched control antibodies were added. The amount of TNF-α antibody used (2.5 μg/ml) is capable, in our laboratory, of neutralising > 50 000 pg/ml of TNF-α as tested in a bioassay (data not shown). In comparison, the cumulative level of TNF-α released from DENV-infected MDM in this study at 32-48 hrs post-infection were 1980-2185 pg/ml, as determined by TNF-α ELISA (data not shown). Cultures were supplemented with fresh medium and antibodies throughout the experiment. DENV replication profiles in the presence or absence of TNF-α antibodies showed little difference suggesting that endogenously released TNF-α has no effect on DENV replication in MDM (Fig 4.10A). Further cellular RNA extracted at 72 hrs showed no difference in levels of DENV RNA (Fig 4.10B).

4.2.4 DENV-infected cells do not respond normally to exogenously added TNF-α

Infection with HCV, which like DENV belongs to the Flaviviridae family, has been shown to confer resistance to TNF-α and prevents TNF-α signalling in infected cells (Marusawa *et al.*, 1999; Park *et al.*, 2002; Frese *et al.*, 2003; Choi *et al.*, 2006; Saito *et al.*, 2006). Since neither exogenously added nor blocking of endogenous TNF-α had any effect on DENV replication, we next aimed to investigate if DENV-infected cells could still respond to TNF-α by analysis of TNF-α-induced NF-kB responsive transcription and induction of NF-kB nuclear translocation.

4.2.4.1 DENV-infection inhibits TNF-α-stimulated, NF-kB mediated reporter gene transcription

In contrast to the efficient uptake of siRNA by MDM described in section 4.2.3.1.1, large DNA constructs can transfect these cells only with difficulty. Therefore, Huh7, hepatoma cells were used for these studies. These cells were in current use in an adjacent lab for analysis of IFN-α response in HCV (Dr Michael Beard, HCV lab, University of Adelaide)

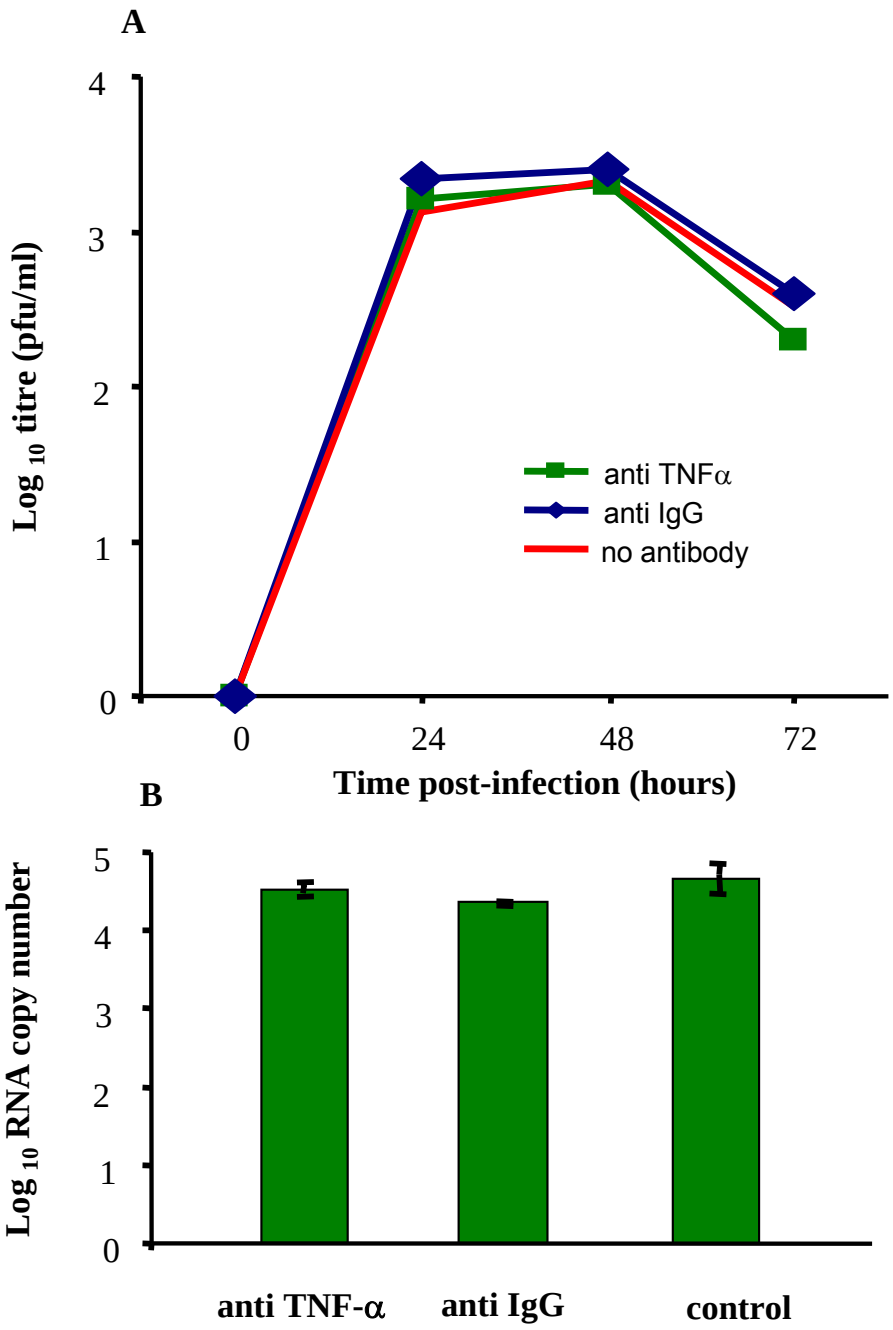
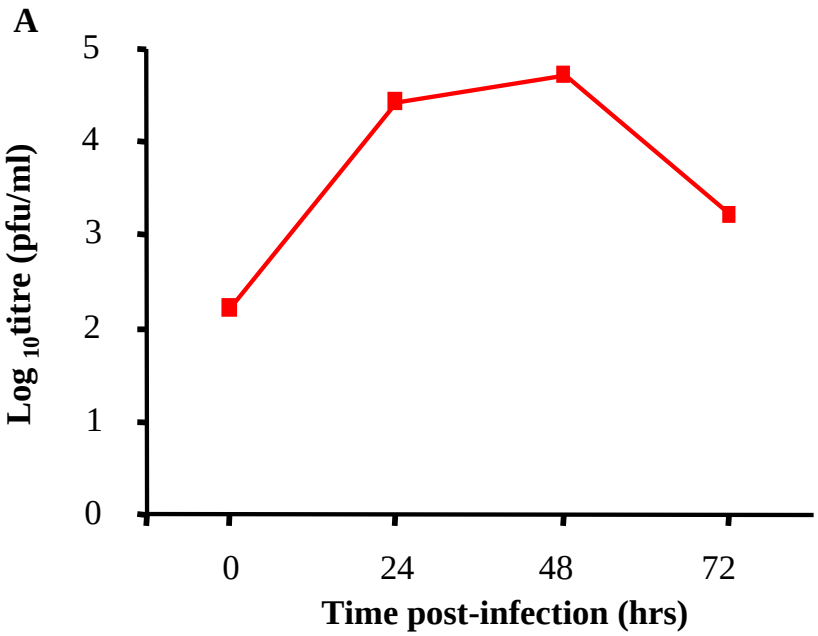


Fig 4.10. Blocking endogenous TNF-α with TNF-α antibodies had no effect on DENV replication. MDM were infected with DENV at MOI 5 and 24 hrs after infection cells were treated with either anti-TNF-α antibodies (2.5ug/ml), isotype matched anti-IgG control (2.5ug/ml) or fresh media. Supernatant was collected every 24 hrs and replaced with fresh antibody or media. **(A)** Infectious virus released was detected by plaque assay (pfu/ml). **(B)** Cell lysates at 72 hrs were used to extract RNA to determine DENV RNA levels in infected cells. *In vitro* transcribed copy number standards from DENV2 capsid protein were used to quantitate (- ve) viral RNA using a tagged RT real-time PCR and normalised per 1 ng input cyclophilin mRNA. Values represent mean ± SEM from duplicate samples in RT real-time PCR of a representative experiment. Results are of a representative experiment and were replicated (n=3). No significant differences were observed in any experiment.

and have been previously shown to be susceptible to DENV-infection (Lin *et al.*, 2000). Huh7 cells were shown to support active DENV replication with production of high titres of infectious virus (Fig 4.11A). DENV-infection in Huh7 cells was cytolytic, resulting in cell death by 48 to 72 hrs (data not shown), and thus all analyses were performed at 24 hrs post-infection when there is active replication and approximately 90% of cells were infected as shown by immuno-staining without a large degree of cell death (Fig 4.11B). Huh7 cells were co-transfected with two plasmids (i) pTK81NF-kBLUC which has NF-kB responsive promoter binding sites regulating the transcription of firefly LUC and (ii) pRL-TK (Appendix VII) which contains the renilla LUC gene under control of a constitutive TK promoter. Dually transfected cells were infected with DENV or mock-infected and 24 hrs after infection, cells were either left untreated or treated with TNF-α (10 ng/ml) and LUC activity in lysates was measured. Firefly LUC luminescence was normalised against total protein content of the lysate and renilla LUC activity. Both methods of normalisation gave similar results and only results from protein normalisation are shown (Fig 4.12A). Mock-infected, untreated cells showed basal levels of NF-kB mediated reporter gene transcription, and DENV-infection alone had no effect on this, with similar levels of LUC activity in mock and DENV-infected cells (Fig 4.12A). Addition of TNF-α to mock-infected cells saw a 5-fold increase in LUC activity, consistent with TNF-α stimulation of NF-kB mediated reporter gene transcription (Fig 4.12A). In contrast, TNF-α stimulation of DENV-infected cells resulted in only basal levels of LUC activity. As a control Huh7 cells were co-transfected with an IFN responsive LUC reporter construct (Appendix VIII) and renilla LUC. Cells were infected and stimulated with IFN-α, as above for TNF-α. DENV-infection prevented IFN stimulated reporter gene transcription (Fig 4.12B) consistent with the well described DENV inhibition of IFN signalling. Our novel observation of DENV inhibition of TNF-α signalling is similar in magnitude to this well described effect of IFN. Thus these results indicate that DENV-infected cells do not respond normally to TNF-α stimulation, as measured by NF-kB induction of gene expression.

4.2.4.2 DENV-infection inhibits TNF-α-stimulated translocation of NF-kB cellular proteins into the nucleus

Since the reporter assays shown in Fig 4.12 cannot be performed in primary MDM, TNF-α stimulation of DENV-infected MDM was investigated by analysis of TNF-α induced NF-kB nuclear translocation by immuno-staining for p65 NF-kB proteins and confocal microscopy. DENV-infected Huh7 at 24 hrs post-infection were used as controls. DENV-infected and mock-infected Huh7 cells that were not treated with TNF-α showed cytoplasmic staining of



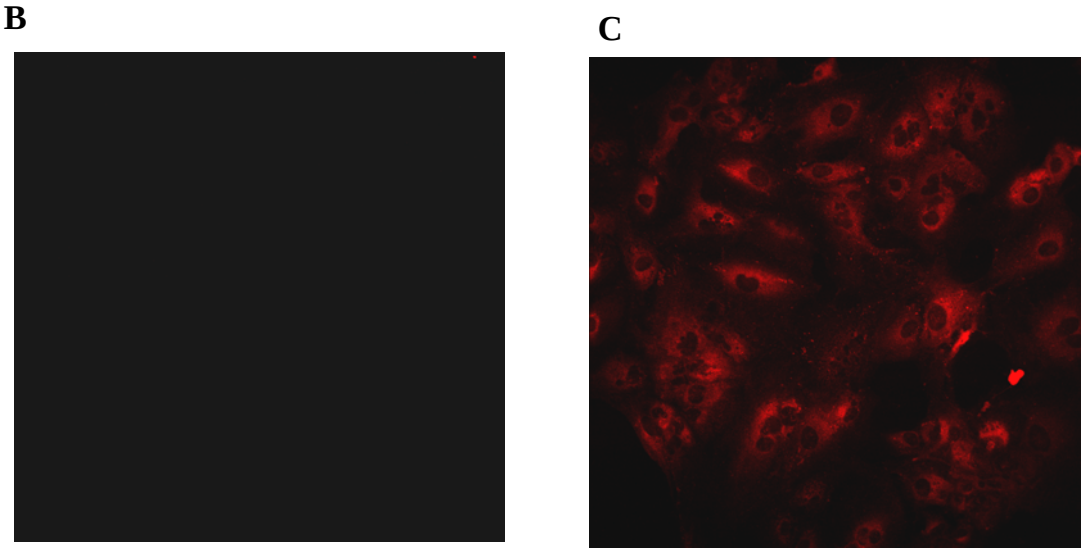


Fig 4.11. Replication profile of DENV in Huh7 cells. **(A)** Huh7 cells were infected with DENV at a MOI of 5 and cell culture medium was collected and assayed for infectious virus released (pfu/ml). Peak of virus production was seen at 48 hrs. After 24 hrs DENV infected Huh7 cells were washed thoroughly fixed, permeabilised, stained with **(B)** control normal serum and **(C)** DENV anti-E antibody and red Alexa Fluor 546 (conjugated secondary antibody) and visualised by confocal microscopy. Greater than 90% cells were positive for DENV antigen without visual cell death.

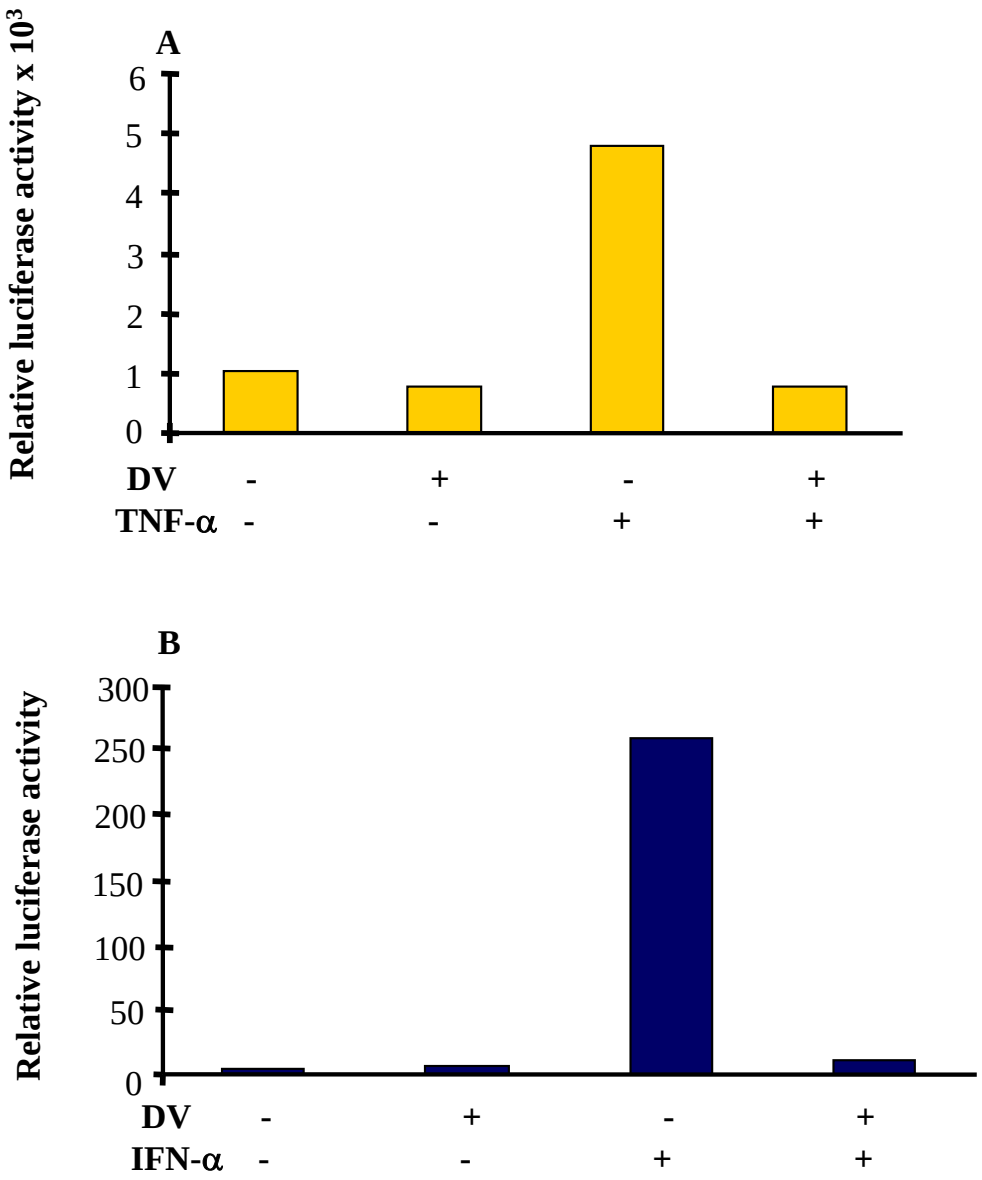


Fig 4.12. DENV infected Huh7 cells do not respond to TNF-α stimulation. **(A)** DENV inhibits TNF-α stimulated NF-kB responsive transcription. Huh7 cells were co-transfected with plasmids, containing the firefly LUC gene downstream of a NF-kB responsive promoter and a renilla LUC gene under control of a TK promoter. 24 hrs after infection cells were either treated or untreated with TNF-α (10ng/ml) for 6 hrs. Cells were lysed and reporter activity and total protein concentration was measured. Firefly LUC activity was normalized against total protein (shown here) or renilla LUC. Results shown are representative of three independent experiments. **(B)** DENV inhibits IFN-α stimulated ISRE responsive transcription. Huh7 cells were co-transfected with plasmids as in (A) except plasmid with ISRE promoter was used instead of NF-kB and IFN-α (100 IU/ml) was added instead of TNF-α. Cells were assayed as in (A). Results shown are representative of two independent experiments.

NF-kB p65 proteins as expected (4.13A). TNF-α stimulation of mock-infected Huh7 cells led to clear translocation of p65 NF-kB proteins to the nucleus (Fig 4.13B). However TNF-α stimulation of DENV-infected Huh7 cells failed to induce nuclear localisation of NF-kB (Fig 4.13(2a to 2c)) in cells that were confirmed to be DENV-infected (Fig 4.13(2b)). These data support the lack of normal TNF-α stimulation in DENV-infected cells seen in reporter assays shown in Fig 4.12A.

In contrast to the strong cytoplasmic staining for NF-kB in untreated Huh7 cells, mock-infected MDM that were not treated with TNF-α showed a variable distribution of intracellular NF-kB p65 proteins. Some uninfected MDM showed NF-kB proteins in both cytoplasm and nucleus, or either the nucleus or the cytoplasm exclusively (Fig 4.14(A) and Table 4.4). Similarly, DENV-infected cells without TNF-α treatment showed NF-kB proteins in both cytoplasm and nucleus, or in a few cells just in the nucleus (Fig 4.14(1a- 1c) and Table 4.4). However, after stimulation with TNF-α, mock-infected MDM clearly showed translocation of NF-kB p65 protein in 100% of the cells (Fig 4.14(B)). In contrast TNF-α-stimulated, DENV-infected MDM showed variable cellular localisation of NF-kB proteins, with no nuclear translocation in approx 50% of infected cells (Fig 4.14 (2a-2c)). Dual analysis for NF-kB and DENV antigens revealed that TNF-α stimulation of NF-kB nuclear translocation occurred in both DENV negative and DENV positive cells within the MDM population. Cytoplasmic localisation of NF-kB after TNF-α stimulation was only observed in DENV positive cells. These results suggest that the lack of movement of NF-kB in response to TNF-α stimulation occurs only in DENV-infected and not uninfected bystander cells, but that effective TNF-α induction of NF-kB nuclear translocation can still occur in some DENV-infected MDM.

Together, the observed lack of nuclear accumulation of NF-kB in response to TNF-α in DENV-infected Huh7 cells and MDM is consistent with reporter assays in Huh7 cells, and suggests that DENV-infected cells, including primary MDM are unable to respond normally to TNF-α stimulation.

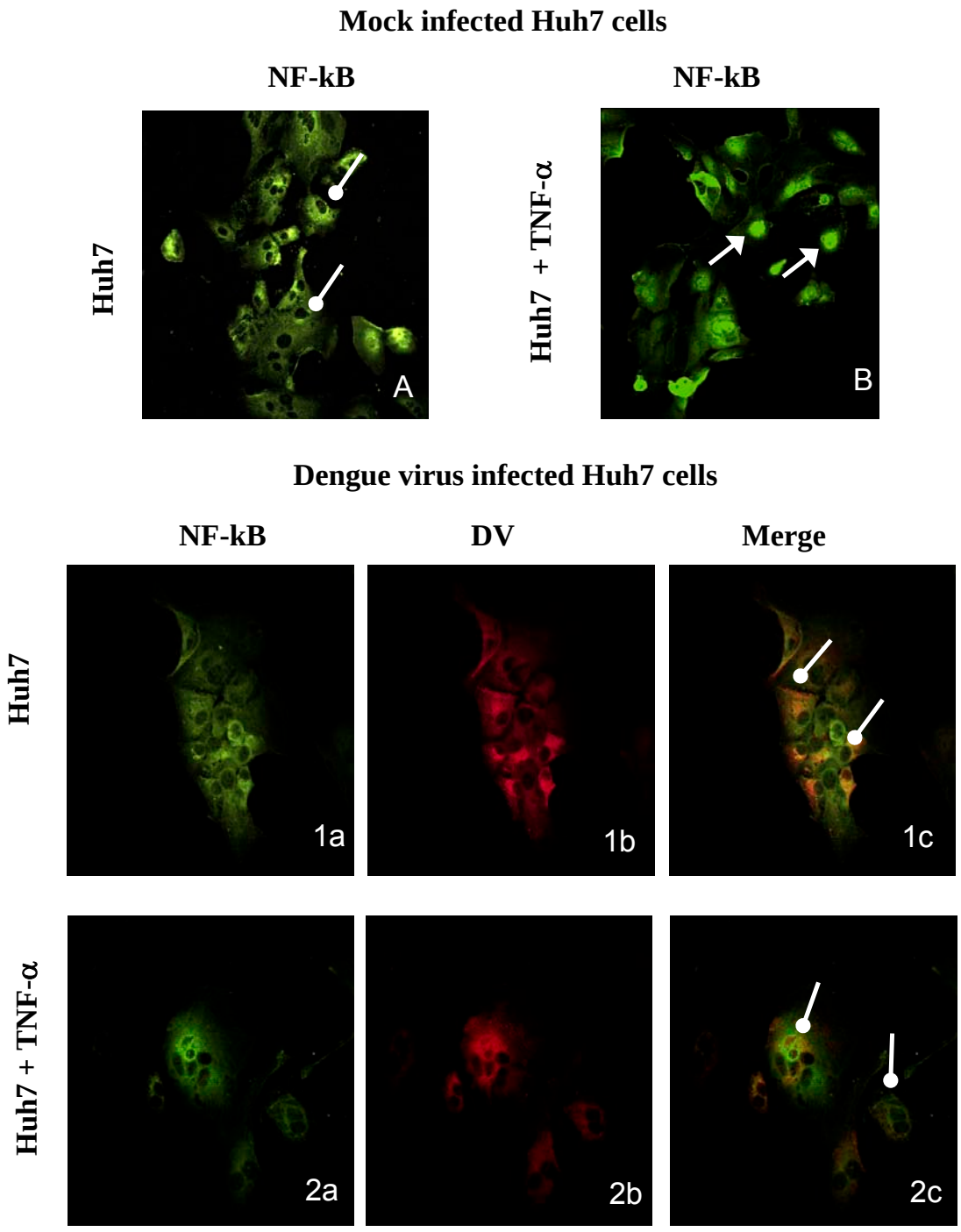


Fig 4.13. DENV inhibits TNF-α stimulated translocation of NF-kB proteins into the nucleus in Huh7 cells. Huh7 cells were either mock infected or infected with DENV and treated with or without TNF-α. After 30 mins of TNF-α treatment cells were fixed and stained with antibodies against DENV (red Alexa Fluor 546) and p65 NF-kB protein (green Alexa Fluor 488). Immunoreactivity was visualised by confocal microscopy. → indicates movement of NF-kB into nucleus —● shows absence of NF-kB proteins in nucleus. Mock infected Huh7 **(A)** = NF-kB protein localisation in cytoplasm. Mock infected Huh7 cells + TNF-α **(B)** = NF-kB proteins in the nucleus of all cells. Huh7 cells infected with DENV – TNF-α **(1a to 1c)** = NF-kB protein localisation in cytoplasm. Huh7 cells infected with DENV + TNF-α **(2a to 2c)** = 100% inhibition in nuclear movement of NF-kB proteins.

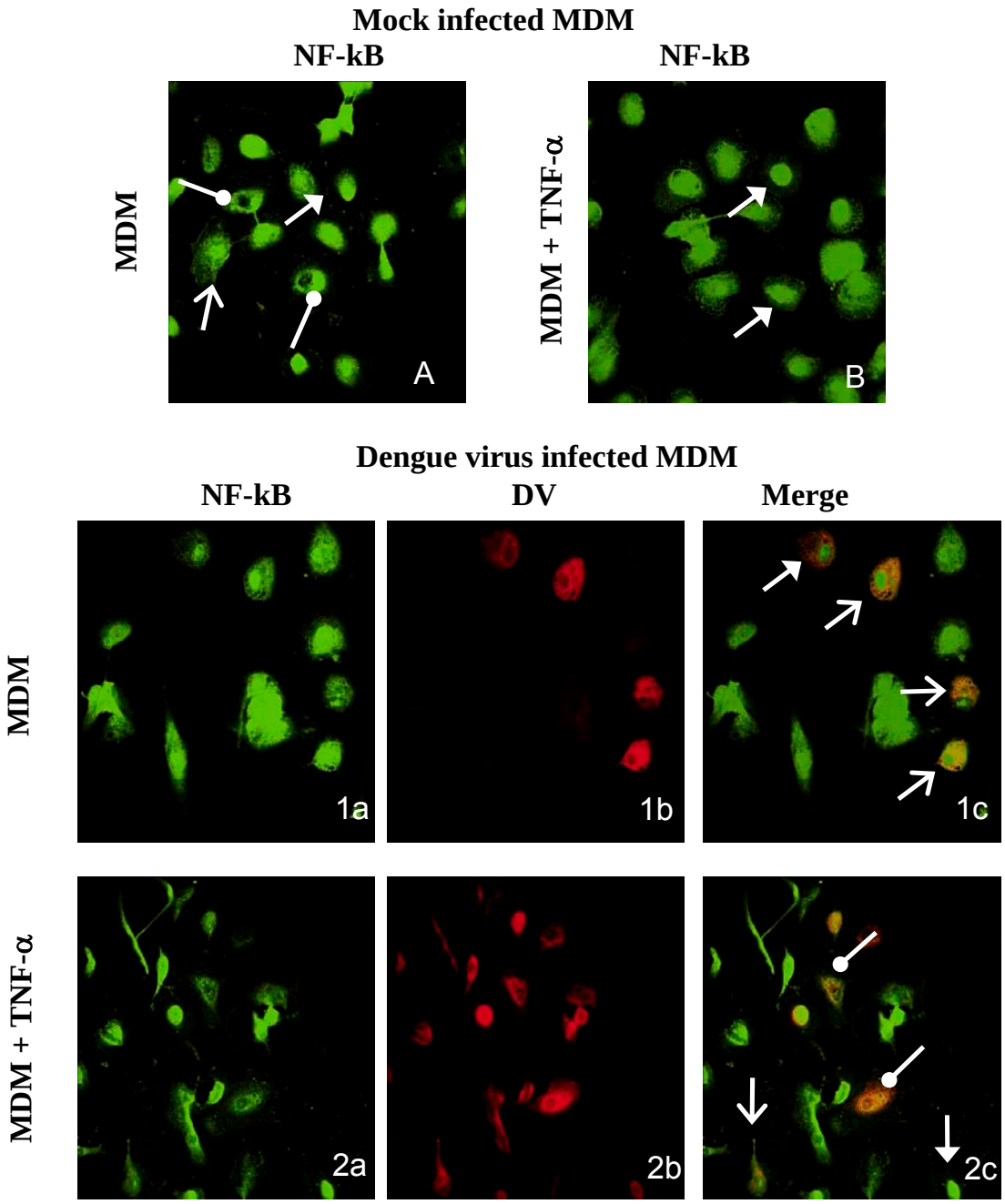


Fig 4.14. DENV inhibits TNF-α stimulated translocation of NF-kB proteins into the nucleus in MDM. Cells were either mock infected or infected with DENV and treated with or without TNF-α. After 30 mins of TNF-α treatment cells were fixed and stained with antibodies against DENV (red Alexa Fluor 546) and p65 NF-kB protein (green Alexa Fluor 488). Immunoreactivity was visualised by confocal microscopy. → indicates movement of NF-kB into nucleus, —● shows absence of NF-kB proteins in nucleus and → indicates NF-kB proteins present in both nucleus and cytoplasm. Mock infected MDM - TNF-α **(A)** = NF-kB protein localisation varying with proteins in nucleus or cytoplasm or both cytoplasm and nucleus. Mock infected MDM + TNF-α **(B)** = NF-kB proteins in the nucleus of all cells. DENV infected MDM **-** TNF-α **(1a to 1c)** = NF-kB proteins in nucleus or both nucleus and cytoplasm in infected cells. DENV infected MDM + TNF-α **(2a to 2c)** = inhibition of movement of NF-kB protein to the nucleus in 50% of DENV positive cells and in others NF-kB proteins distributed in both cytoplasm and nucleus.

Treatment and cell type	% of cells localised to:					
	Nucleus		Cytoplasm		Nucleus + Cytoplasm	
	+ TNF	- TNF	+ TNF	- TNF	+ TNF	- TNF
Mock Huh7	100	0	0	100		
DV Huh7	0	0	100	100		
Mock MDM	100	48	0	2	0	50
DV +ve MDM[a]	4	10	48	8	48	82
DV-ve MDM[a]	100	48	0	2	0	50

Table 4.4. Quantitation of NF-kB cellular localisation (% cells). Results for MDM are representative of 5 fields (average 10 cells per field) viewed from each of 3 independent experiments. [a] as assessed by DV antigen staining in the DV challenged MDM population.

4.2.5 Does DENV non-structural protein 5 (NS5) inhibit TNF-α signalling as occurs with HCV?

It has been established that HCV inhibition of TNF-α stimulation of the NF-kB signalling pathway is mediated by at least three HCV proteins (core, non structural 5A and 5B) (Marusawa *et al.*, 1999; Park *et al.*, 2002; Choi *et al.*, 2006; Saito *et al.*, 2006). We next aimed to investigate if DENV NS5 protein similarly inhibited TNF-α-stimulated signalling in DENV-infected cells using LUC reporter systems in Huh7 cells as in section 4.2.4.1. Plasmids expressing a GFP fusion of DENV NS5 (pEPI-GFP-NS5-1-900) were obtained from Dr David Jans and Steven Rawlinson (Pryor *et al.*, 2007) Monash Uni, Melbourne. Huh7 cells were co-transfected with pEPI-GFP-NS5-1-900 or pEPI-GFP alone in combination with pTK81NF-kBLUC (control) and pRL-TK, stimulated with TNF-α, and LUC activity measurement as in section 4.2.4.1. Results indicated no inhibition of TNF-α-stimulated NF-kB mediated reporter gene transcription (data not shown). However only 3% of Huh7 cells were visualized by confocal microscopy to express GFP-NS5 (data not shown). Thus these results were inconclusive due to low levels of NS5 expression, and further work is required to determine accurately if, similar to HCV, DENV NS5 is responsible for inhibiting TNF-α-stimulated signalling.

4.3 DISCUSSION

Results from this chapter show that DENV replication in a relevant primary cell system, MDM is unaffected by TNF-α, and further that DENV-infected cells do not respond normally to TNF-α stimulation.

DENV-infected MDM model

These studies were carried out in primary MDM, which represent a key target of DENV *in vivo* (Halstead *et al.*, 1977; Rothman and Ennis, 1999). Although the number of DENV-infected macrophages *in vitro* is relatively low (15-30%), DENV replicates to reasonably high levels releasing $>10^4$ pfu/ml of infectious virus, consistent with previous studies (Pryor *et al.*, 2001; Chen and Wang, 2002). To analyse DENV replication at the RNA level a tagged RT real-time PCR was developed (chapter 3). This showed that intracellular (- ve) strand DENV RNA coincided with infectious virus production, indicating it is a marker for active replication of DENV as suggested by others (Diamond *et al.*, 2000b; Wang *et al.*, 2002c). Visually DENV-infected MDM did not differ from uninfected cells and retained the ability

for phagocytosis. Thus, in our model, infected MDM produce DENV RNA co-incident with release of infectious virus, are not killed by DENV-infection and still retain at least some important macrophage functions.

Effect of TNF-α on DENV replication

Studies in our lab showed that the peak of TNF-α released from DENV-infected MDM coincided with peak of DENV release (Carr *et al.*, 2003). Therefore in this study the effect of addition of *exogenous* TNF-α in regulating DENV replication was investigated. When high concentrations of TNF-α were added at 24 hrs after established DENV-infection but prior to the peak of virus production, no change was observed in the amount of virus released or viral RNA detected in infected cells. To test whether this observation was due to a lack of responsiveness to cytokines in our system we performed a positive control: inhibition of DENV replication 4 hrs following IFN-α pre-treatment of cells prior to infection (Diamond *et al.*, 2000b). This inhibitory effect of IFN-α was reproduced in our studies, while in contrast we observed a very small inhibition of DENV replication in macrophages following 4 hrs pre-treatment with TNF-α. Analysis of the amount of input RNA suggested this was not due to a reduction of the initial infection but a minor effect of TNF-α pre-treatment in inhibiting subsequent DENV-replication was not pursued any further. Since it is known that DENV-infected MDM produce TNF-α (Carr *et al.*, 2003; Espina *et al.*, 2003) the role of *endogenous* TNF-α in DENV replication was investigated. Firstly TNF-α production by MDM was blocked using TNF-α siRNA. TNF-α siRNA was shown to inhibit LPS-stimulated release of TNF-α protein from one to four days after siRNA transfection, showing successful and stable siRNA knockdown of its target in MDM for at least 4 days. This TNF-α siRNA transfection protocol also successfully down regulated 80% of the TNF-α mRNA and protein production in DENV-infected MDM. However TNF-α siRNA knockdown had no effect on either infectious DENV production or DENV RNA levels. Secondly the activity of endogenously produced TNF-α was blocked by incubating MDM with TNF-α antibodies prior to and during the known peak of TNF-α and DENV production. The level of TNF-α antibody used blocked the activity of > 50 000 pg/ml of TNF-α (validated using L929 bioassay), a much higher level than the approx 2000 pg/ml of total TNF-α released by DENV-infected MDM over three days (Carr *et al.*, 2003). Again however, no effect on DENV replication was observed. Together these results conclusively show that DENV replication is not affected by either the addition of *exogenous* or the blocking of *endogenous* TNF-α in the natural target cell the macrophage. In studies with WNV, another *Flavivirus*, addition of TNF-α 1 hr post-infection significantly

reduces the number of infected cells from TNF-α deficient mice but has a small but not significant effect in cells from wild type animals (Cheng *et al.*, 2004). These results of WNV infection in wild type cells show some similarities to our results for DENV, with lack of effect of exogenously added TNF-α on DENV replication when added at later stages of infection. However, the lack of an anti-viral action of TNF-α contrasts sharply to the significant anti-viral actions of TNF-α seen in other viral systems such as CMV and HSV1 (Rossol-Voth *et al.*, 1991; Pavic *et al.*, 1993).

Sera from patients with DHF/DSS contain greater levels of several pro-inflammatory cytokines such as interleukin-6 (IL-6), interleukin-10 (IL-10), interleukin-8 (IL-8) and TNF-α than sera from individuals with uncomplicated dengue fever (Hober *et al.*, 1993; Raghupathy *et al.*, 1998; Green *et al.*, 1999a; Green *et al.*, 1999b; Kittigul *et al.*, 2000). Anti-TNF-α treatment of mice infected with DENV has been shown to significantly decrease fatality (Atrasheuskaya *et al.*, 2003; Shresta *et al.*, 2006) suggesting that TNF-α is at least in part responsible for more severe DENV disease in this model. However anti-TNF-α antibodies do not affect DENV viremia *in vivo*, consistent with the findings in this chapter that blocking TNF-α did not affect DENV replication in cells *in vitro*. Culture fluids from PBMC infected with DENV by antibody enhancement triggered activation of endothelial cells *in vitro*, which could be abolished by treatment of monocyte culture fluids with TNF-α antibody (Anderson *et al.*, 1997a; Cardier *et al.*, 2005). Thus, measures to block TNF-α actions might be of value for the improved clinical management of dengue complications. Results in this chapter suggest that such approaches should not compromise any ongoing anti-viral role of TNF-α, as TNF-α does not have any effect on DENV replication, unlike the case in other virus infections such as CMV and HSV1 (Rossol-Voth *et al.*, 1991; Pavic *et al.*, 1993).

Blockade of TNF-α signalling in DENV-infected cells

There are precedents in the literature of viral infections blocking cytokine signalling e.g. HCV blockade of TNF-α signalling (Marusawa *et al.*, 1999; Park *et al.*, 2002; Choi *et al.*, 2006; Saito *et al.*, 2006) and DENV blockade of IFN signalling (Munoz-Jordan *et al.*, 2003; Munoz-Jordan *et al.*, 2005). Since in this study it was found that TNF-α did not affect DENV replication in infected cells, it was further investigated if DENV-infected cells were capable of responding to TNF-α. TNF-α responsiveness was assessed by a NF-kB LUC reporter assay, and analysis of NF-kB cellular localisation. TNF-α treatment of uninfected Huh7

cells-stimulated NF-kB transcription as shown previously (Hilgard *et al.*, 2004) but TNF-α treatment of DENV-infected Huh7 cells failed to stimulate NF-kB mediated transcription. This clearly suggests that DENV-infection inhibits TNF-α-stimulated NF-kB promoter responsive transcription in Huh7 cells.

As this reporter system could not be used to determine TNF-α responsiveness in DENV-infected MDM, TNF-α signalling in DENV-infected and uninfected macrophages was examined by assessing NF-kB nuclear translocation by immuno-staining and confocal microscopy. Again, Huh7 cells clearly showed that DENV-infection inhibited TNF-α-stimulated nuclear translocation of NF-kB and thereby supported the reporter studies above. Results in MDM were not as clear as in Huh7 cells, as TNF-α stimulation of NF-kB nuclear translocation was inhibited in some (approximately 50%) but not all DENV-infected cells within the MDM population. However, this is in stark contrast to the 100% stimulation of NF-kB nuclear translocation by TNF-α seen in uninfected MDM which again clearly suggests that DENV infection alters TNF-α-stimulated responses. Primary MDM are a heterogeneous cell population, compromising macrophages in different maturational states from monocytes to macrophages. The cell-to-cell variability in TNF-α-stimulated nuclear translocation of NF-kB proteins could be attributed to a number of factors, including differences in the stage of macrophage maturation or the stage of DENV replication. For example, inhibition of NF-kB translocation by CMV infection is seen only at late stages after infection (Jarvis *et al.*, 2006). Additionally, DENV-infected MDM can release many cytokines and chemokines (Chen and Wang, 2002) that may mediate NF-kB nuclear translocation by paracrine activity on a subpopulation of cells, independent of TNF-α signalling.

In contrast to our observed DENV blockade of TNF-α-stimulated NF-kB nuclear translocation, other studies have shown DENV activation of NF-kB (Marianneau *et al.*, 1997; Avirutnan *et al.*, 1998; Chang *et al.*, 2006), including a study showing DENV-induced nuclear movement of NF-kB in 60% of DENV-infected A549 cells (Chang *et al.*, 2006). In these studies, including studies in HepG2 hepatoma cells, similar to those used in this chapter, the infection models are immortalised cell lines and investigations were performed at a time when these cells undergo DENV induced apoptosis, a cell death pathway well documented to involve NF-kB activation. We have performed some limited DENV-infection studies in A549 cells and observed DENV-induced nuclear movement of NF-kB in a small percentage of cells (10%) compared with Chang et al., 2006, but within 48 hrs these DENV-infected cells

are killed (data not shown). In these extensive studies reported in this chapter, using *in vitro* cell models representing DENV-infection of macrophages and liver cells, two important cell targets for DENV *in vivo*, no DENV induction of NF-kB activation was observed, but instead we observed DENV-blockade of TNF-α-stimulated NF-kB activation, at a time of established DENV-infection but with little (3-10%) cell death (Espina *et al.*, 2003; Mosquera *et al.*, 2005). Thus, while high level DENV-infection of cell lines may activate NF-kB and induce apoptosis, during active DENV replication in primary MDM or hepatoma cell lines DENV does not activate NF-kB and in fact has the capacity to prevent its activation by TNF-α.

This is the first study to show that DENV-infected MDM do not respond to TNF-α stimulation, but a similar observation has been made in DENV-infected dendritic cells (Palmer *et al.*, 2005). TNF-α plays a role in differentiating dendritic cells to a mature phenotype, by increasing expression of co stimulatory and activation molecules, and recent experiments have shown that DENV-infected dendritic cells do not up regulate co-stimulatory molecules CD80 and CD86 in response to TNF-α (Palmer *et al.*, 2005). Thus, DENV blocks responses to TNF-α in infected dendritic cells, as well as in macrophages as demonstrated in the current study. This may alter the stimulation of many genes normally involved in immune responses via disruption of the NF-kB signalling pathway. This may complement the previously described DENV-induced inhibition of IFN-α signalling and lead to multiple mechanisms for subversion of the host immune response. Future work needs to determine the mechanism of DENV inhibition of responsiveness to TNF-α. Alteration to expression of IFN-stimulated genes by DENV NS4B, NS4A, NS2A and blockade of STAT-1 and ISRE promoter activation of NS4B (Munoz-Jordan *et al.*, 2003; Munoz-Jordan *et al.*, 2005) suggest a role for these DENV proteins in interfering with IFN signalling. Inhibition of TNF-α activation of NF-kB in HCV infection can be mediated by three HCV proteins (core, NS5A and NS5B) (Marusawa *et al.*, 1999; Park *et al.*, 2002; Choi *et al.*, 2006; Saito *et al.*, 2006). Simarily we assessed if DENV NS5 was also capable of inhibiting TNF-α-stimulated NF-kB transcription. However poor transfection or expression levels of GFP-NS5 in Huh7 cells prevented further investigation of this issue within the time constraints of this PhD. Thus future work should pursue methods to achieve better transfection of GFP-NS5 or trial alternative cell lines and investigate other DENV proteins to determine which DENV proteins can mediate inhibition of TNF-α responsiveness in DENV-infected cells.

In conclusion, results presented in this chapter have demonstrated that DENV replication in macrophages is not affected by addition of high levels of TNF-α, or by blocking production of the endogenous TNF-α released by macrophages. Analyses of transcription from a NF-kB responsive promoter and NF-kB nuclear translocation has also shown that DENV-infected cells do not respond normally to TNF-α stimulation. These findings suggest that the excessive levels of TNF-α seen in DENV-infected patients do not contribute to DENV clearance by reducing viral replication in DENV-infected macrophages, but that the virus sets up a cellular state of non-responsiveness to TNF-α which may represent a viral mechanism for subverting host anti-viral responses.

CHAPTER 5

HOST CELLS RESPOND TO DENV-INFECTION BY UP-REGULATION OF GRP78 AND HSP70

5.1 INTRODUCTION

Virus replication can alter normal host cell proteins, and analysis of the proteins present in a host cell can be used to determine the cellular responses to infection. To date most DENV studies have focussed on viral pathogenesis, virulence and the host immunological response. A more limited number of studies have looked at host *intracellular* responses to viral infections but at the level of gene expression in endothelial cells and liver cells (Warke *et al.*, 2003; Liew and Chow, 2006; Ekkapongpisit *et al.*, 2007) or by analysis of cytokine and chemokine release from infected cells such as MDM (Chen and Wang, 2002). Since changes in gene expression do not always correlate with changes in protein levels (for example as observed when comparing mRNA and protein abundances in human liver (Anderson and Seilhamer, 1997) we chose to examine changes in host protein expression in DENV-infected cells.

As discussed earlier, macrophages are the main target cell for DENV replication *in vivo* but using our *in vitro* MDM model only up to 30% of cells can be infected with DENV. This low level of infection may mean that any DENV induced responses could be masked in the background of uninfected cells and thus we felt it was not feasible to examine the proteomic profile of DENV-infected MDM. We thus sought a "macrophage-like cell line DENV-infection model" for proteomic studies and two alternatives were investigated (i) U937 cells and (ii) K562 cells. The promonocytic cell line U937, derived from a human diffuse histiocytic lymphoma (Sundstrom and Nilsson, 1976) has been widely used as a model for human monocytes. These cells can be differentiated to become more macrophage-like by the addition of phorbal myristate acetate (PMA), which makes them susceptible to DENV-infection without requiring antibody enhancement (O'Sullivan and Killen, 1994). However the literature suggests that in the U937 DENV-infection model, again the level of cells infected is relatively low (0.6 – 17.4%) (Kurane *et al.*, 1990).

K562 cells were first isolated from a pleural effusion from a patient with chronic myelogenous leukaemia in blast crisis (Lozzio and Lozzio, 1975) and were found to be strongly positive for immunoglobulin Fc receptors and initially described as macrophage-like. However the Fc receptors on K562 cells are not functional in terms of phagocytosis or mediating antibody dependent phagocytosis (Klein *et al.*, 1976), and these cells were later characterised as a erythroleukemia cell line (Andersson *et al.*, 1979). K562 cells can be infected with DENV yielding infection rates as high as 99% on third day post-infection (Kurane *et al.*, 1990) which was also confirmed in our studies herein.

This chapter describes characterisation of DENV-infection in these two cell lines to establish a model for proteomic analysis of whole cell lysates of DENV-infected and uninfected macrophage-like cells by two dimensional (2D) gel electrophoresis: (1) the first dimension (isoelectric focussing) allowed separation of proteins based on their charge and (2) the second dimension (SDS PAGE) allowed separation based on the mass of the proteins. Separated proteins were visualised by Coomassie blue G250 staining. Triplicate gels from three independent DENV-infections of K562 cells showed consistent and reliable up-regulation of one protein spot. This was identified by mass spectrometry as GRP78, a member of the heat shock protein 70 (HSP70) family and up-regulation of GRP78 and the prototype family member HSP70 was further confirmed by immuno-staining and confocal microscopy in DENV-infected K562 and MDM cells.

5.2 RESULTS

5.2.1 Identification of a good macrophage model for proteome studies

5.2.1.1 The monocytic cell line U937 was not highly susceptible to DENV-infection and did not represent a good macrophage proteome model

Since primary macrophages are difficult to culture in high numbers and only a minor proportion of cells become DENV-infected, the differentiated macrophage-like cell line U937 was first investigated, as a macrophage-like DENV-infection model, for proteome analysis. Differentiation of U937 suspension cells to strongly adherent macrophage phenotype was achieved by addition of PMA for 48 hrs as described (O'Sullivan and Killen, 1994), and cells were infected with DENV at a high MOI of 10. DENV released in supernatant over a three-day period was assessed by plaque assay (Fig 5.1A). The replication profile of DENV in PMA treated U937 cells showed peak virus production at day 2 and a decrease in virus release

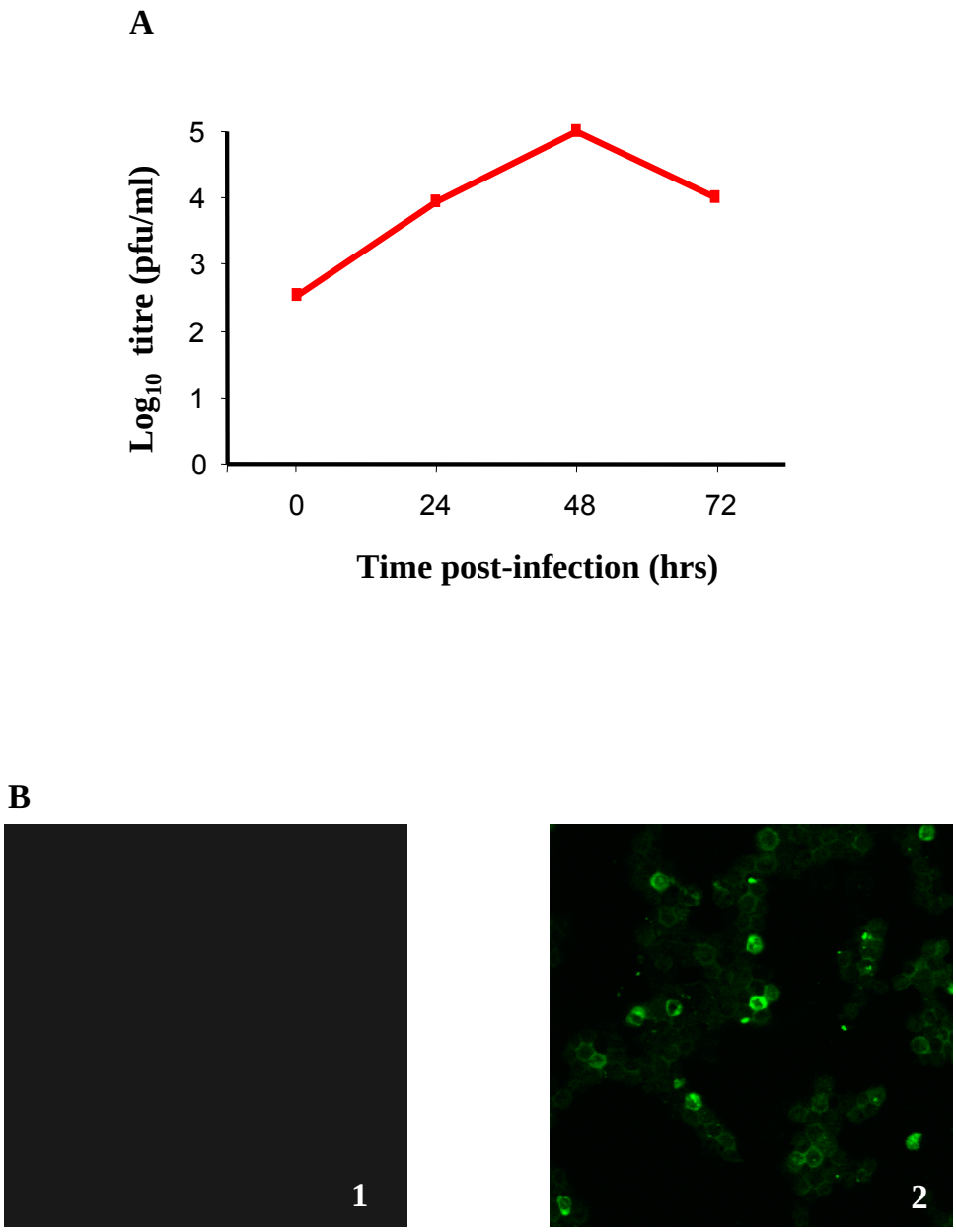


Fig 5.1. Low levels of DENV-infection in PMA treated U937 cells. U937 cells were differentiated with PMA, infected with DENV (MOI 10) and **(A)** at the indicated time post-infection supernatant was collected and infectious DENV release was quantitated by plaque assay (pfu/ml) (**B)** At 48 hrs post-infection DENV-infected cells were fixed and stained with **(1)** control normal human sera **(2)** DENV positive patient sera and complexes were detected with Alexa Fluor 488 conjugated secondary antibody and visualised by confocal microscopy.

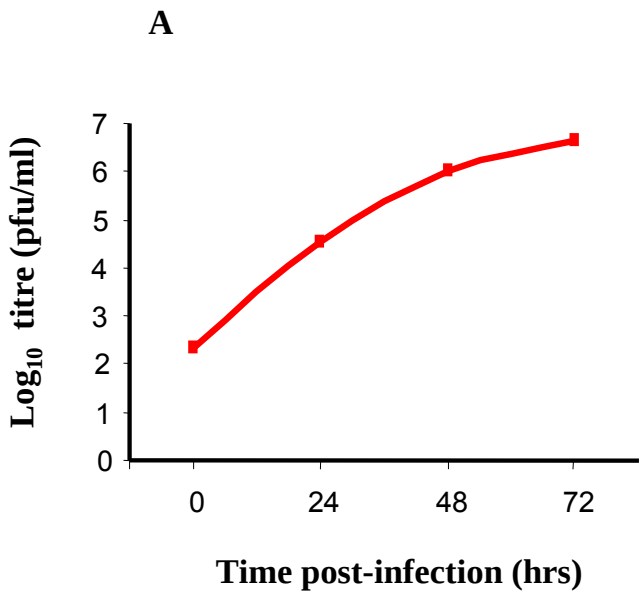
at day 3 similar to MDM (chapter 4, Fig 4.1A) and as previously reported (O'Sullivan and Killen, 1994). To determine the percentage of infected cells, at 48 hrs post-infection, cells were stained for DENV antigens and visualised by confocal microscopy (Fig 5.1B). The number of DENV positive cells was enumerated by counting total cells using light microscopy and infected cells using confocal microscopy. Only 6% of cells were found to be expressing DENV antigens even when infected at this high MOI of 10. This low level of DENV-infection made the U937 cell line unsuitable for proteome studies, and an alternate DENV susceptible macrophage-like cell line was sought.

5.2.1.2 K562 cells were highly susceptible to DENV-infection and represent a good MDM proteome model

K562 cells have been previously described as a human mononuclear cell line (Kurane *et al.*, 1990) although they also have erthrocytic properties (Andersson *et al.*, 1979), K562 cells are highly susceptible to DENV-infections showing 99% cells infected at 3 days post-infection (Kurane *et al.*, 1990) and have been reported to establish persistent DENV-infection and maintain a high percentage (more than 70%) of DENV antigen positive cells for at least 25 weeks (Kurane *et al.*, 1990). To assess DENV replication over a time course of 72 hrs, K562 cells were infected with DENV at a MOI of 5 in four separate wells. At different time points after infection, all supernatant was collected from respective wells and the release of infectious virus measured (pfu/ml). Active replication of DENV was observed in K562 cells (Fig 5.2A) with continued increasing virus production up to 72 hrs post-infection as reported previously (Kurane *et al.*, 1990). At 72 hrs post-infection, cells were stained for DENV antigens showing 100% of cells infected (Fig 5.2B). Cells were visually healthy, without obvious CPE and thus at this time point post-infection, proteomic responses should not be predominated by uninfected cells or a cell death response, as such K562 cells were used in all subsequent proteomic analyses.

5.2.2 Proteomic analysis of DENV-infected and uninfected K562 cells

Three different DENV-infections were performed in K562 cells. At 72 hrs post-infection all 3 replicate infections showed 100% of DENV positive cells by immunofluorescence staining of DENV E antigens and visualisation by confocal microscopy and similar high levels of infectious virus release in the supernatant by plaque assay (Fig 5.3). The whole cell lysates of the three replicate DENV-infected cultures and three replicate mock-infected cultures were examined separately by 2D gel electrophoresis and staining with Coomassie blue stain G 250



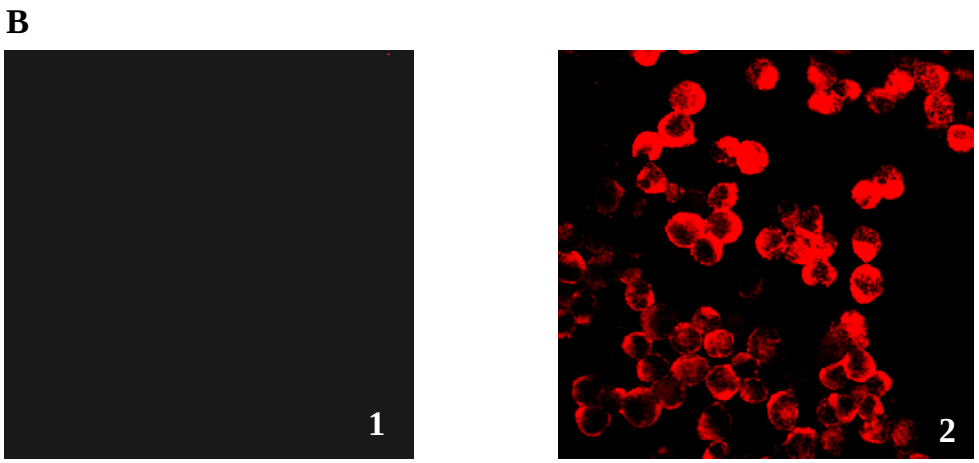


Fig 5.2. High levels of DENV-infection in K562. K562 cells were infected with DENV (MOI 5) and **(A)** at the indicated time post-infection supernatant was collected and infectious DENV release was quantitated by plaque assay (pfu/ml) **(B)** At 72 hrs post-infection DENV-infected cells were fixed and stained with **(1)** control normal mouse sera **(2)** DENV anti-E monoclonal antibodies and complexes detected with red Alexa Fluor 546 conjugated secondary antibody and visualised by confocal microscopy.

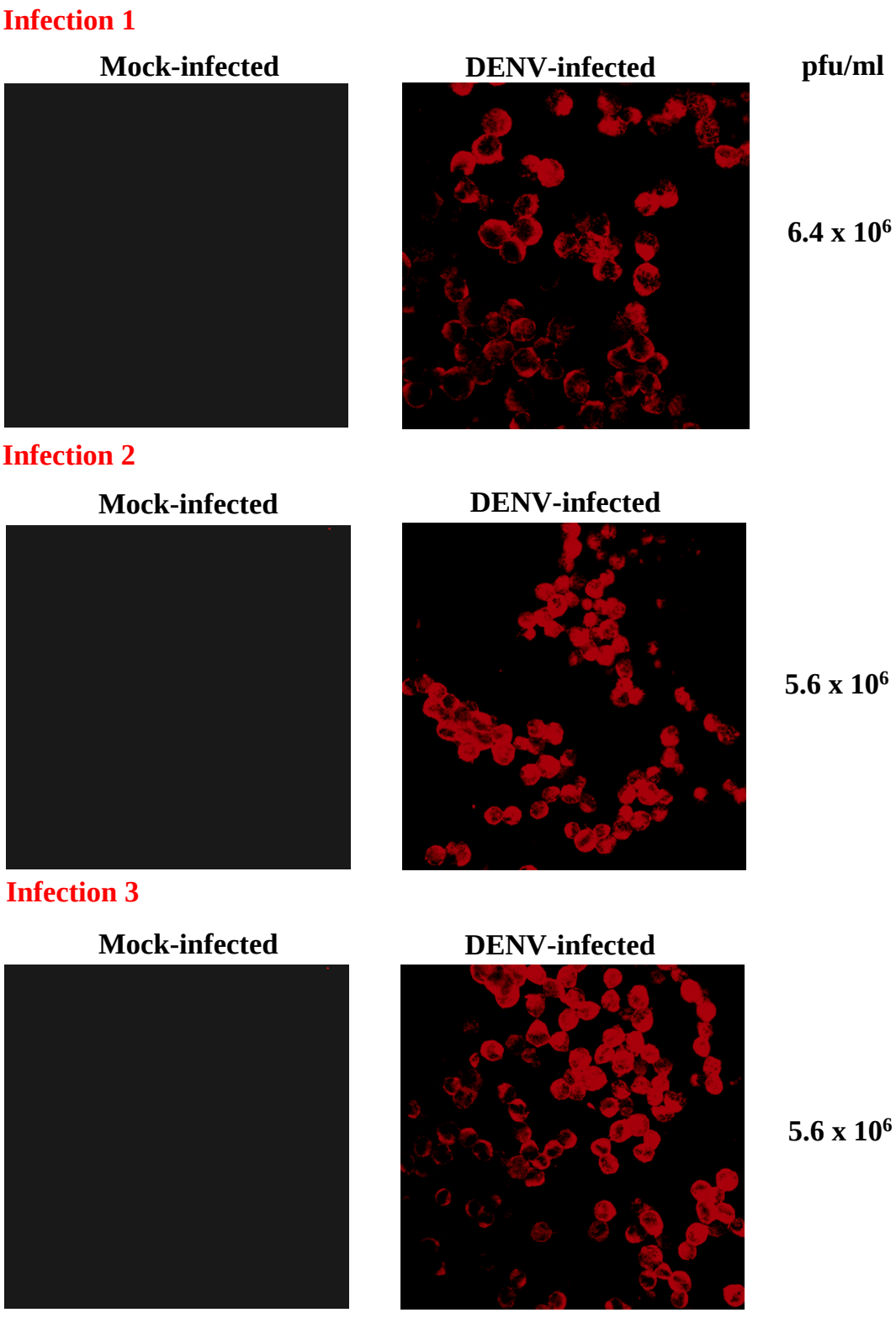


Fig 5.3. Efficient infection of K562 cells. K562 suspension cells were either infected with DENV at MOI of 5 or mock-infected in three replicate biological infections. At 72 hrs post-infection, supernatant was used to determine infectious DENV released by plaque assay (pfu/ml) and cells fixed and stained using DENV anti-E monoclonal antibodies and complexes detected with red Alexa Fluor 546 conjugated secondary antibody and were visualised by confocal microscopy.

(Fig 5.4). Results from a total of 6 gels were scanned individually, and a match set of consistently detected proteins were constructed using the PDQuest image analysis software (Biorad) (Fig 5.5). This digital manipulation program lines up key protein spots and digitally skews images to account for small gel to gel migration patterns to allow accurate identification and comparison of protein spots between gels. The mean density of each individual protein spot was then calculated using PDQuest and compared between infected and mock-infected cell lysates. A total of 344 distinct protein spots were identified in both infected and uninfected cell lysates. Using a cut-off value of > 2 and Student's t-test ($P < 0.05$) only 1 protein spot (approx 72 kDa, pI ~6) out of the 344 spots was found to have a mean spot density significantly different in DENV compared with mock-infected cell lysates. This protein was 2-3 fold higher in DENV-infected than mock-infected control cell lysates and this difference was reproducibly detected in each of the three DENV-infected 2D gels (Table 5.1). No unique protein or down regulated protein was observed in any of the DENV-infected cell lysates.

5.2.2.1 Identification of the up-regulated protein as GRP78 by mass spectrometry and Mascot database-search

The 72 kDa protein spot identified above as up-regulated in DENV-infected cell lysates was excised from the gel and subjected to in gel digestion and mass spectrometry (MS) analysis (using MALDI TOF/TOF MS). Mascot database search identified homology to a human protein GRP78 with a highly significant Mowse scores (Fig 5.6). The top 20 data base matches for the MS search, MS Spectrum and peptide molecular weight matches are shown in Appendix IX to XI respectively. The MS identified peptides mapped to 52% of the GRP78 sequence (Fig 5.7). The predicted MW of the observed protein was 72 kDa, which matches the observed MW in comparison to SDS PAGE protein standards in our 2D gels. This data is summarised in Table 5.2 and together conclusively identifies the 72 kDa protein as GRP78.

GRP78 is a member of the HSP70 family of proteins and both GRP78 and HSP70 have been implicated as part of a receptor complex for DENV (Jindadamrongwech *et al.*, 2004; Reyes-Del Valle *et al.*, 2005; Cabrera-Hernandez *et al.*, 2007). We next aimed to confirm up-regulation of GRP78 in DENV-infected K562 and MDM cells and also, although not identified by proteomic analysis, determine if HSP70 was similarly up-regulated in these cells.

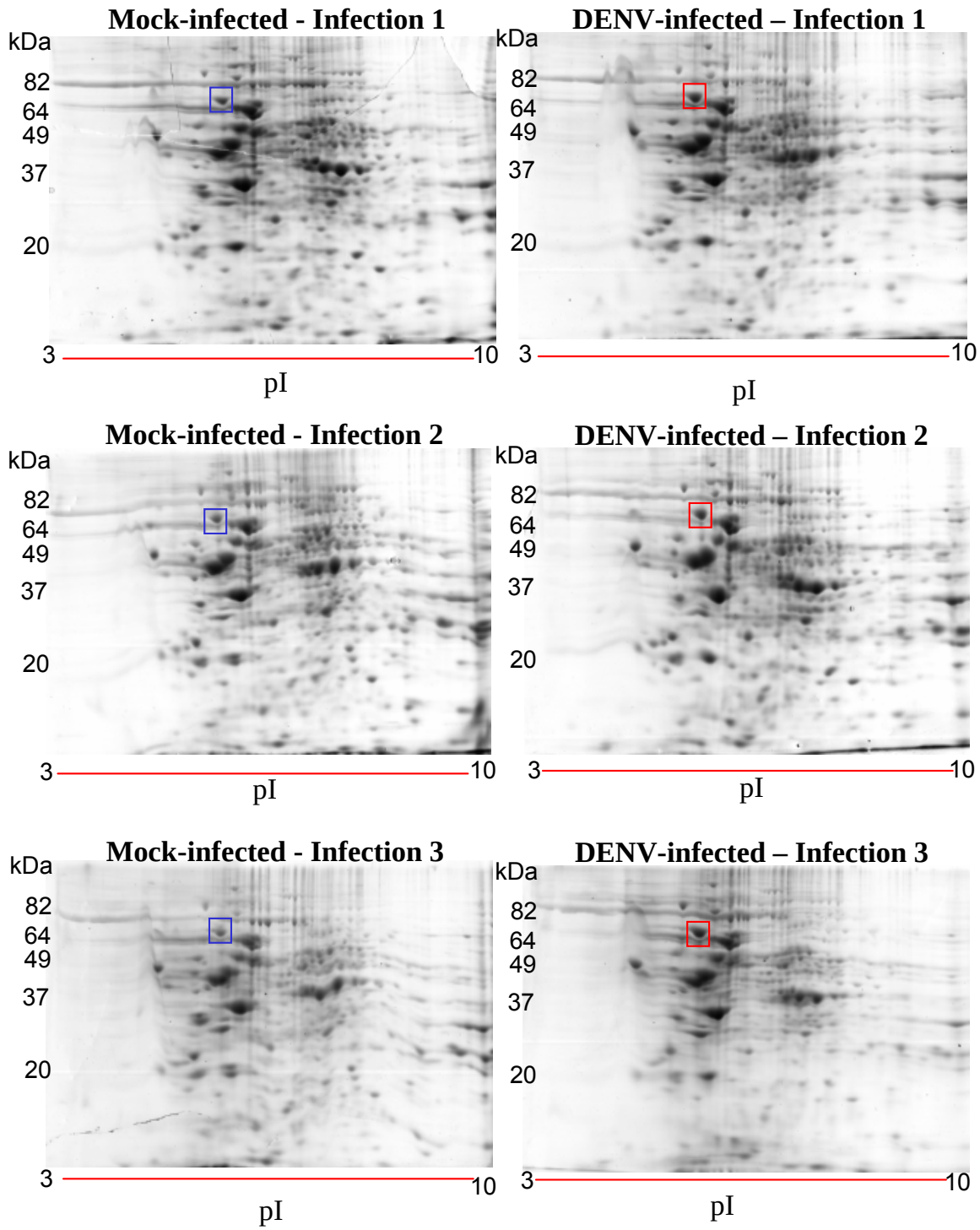


Fig 5.4. Proteins expressed by DENV-infected and mock-infected K562 cells. K562 cells were either DENV-infected or mock-infected in three replicate experiments as shown in Fig 5.3. At 72 hrs post-infection whole cell lysates were obtained and subjected to 2D gel electrophoresis and protein spots were stained with Coomassie blue G250. The horizontal axes represent the iso-electric focussing gradient (pI) and the vertical axes represent molecular mass (kDa). A 72 kDa protein spot was identified by image analysis (Table 5.1) as up-regulated in infected cell lysates and is highlighted in the red box compared to mock-infected gels where the same protein is shown in a blue box.

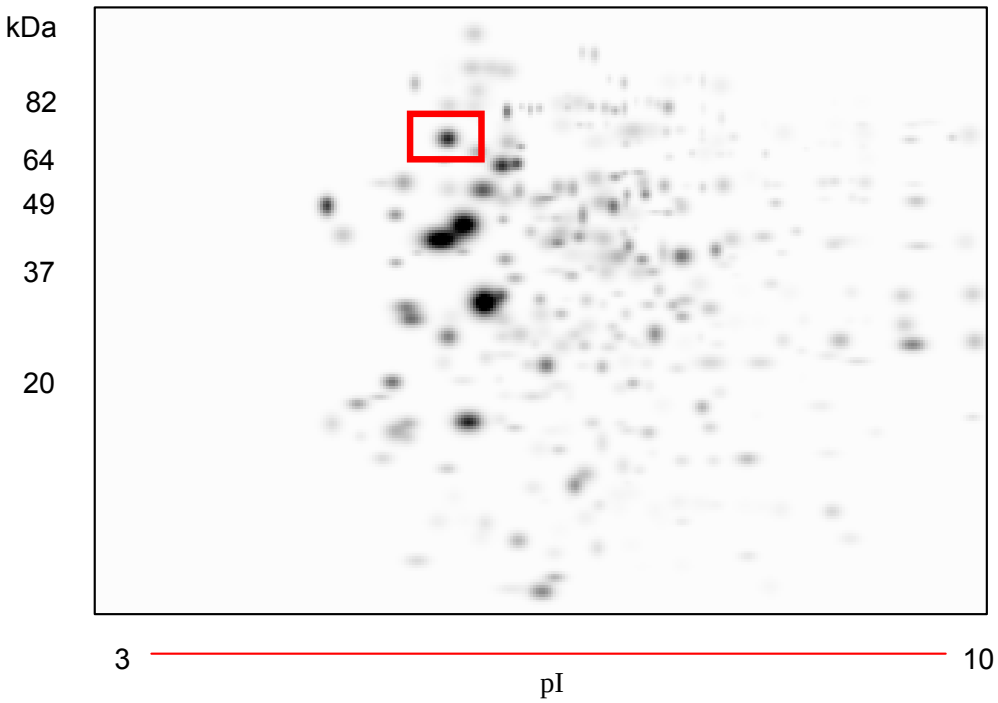


Fig 5.5. Matched set of protein spots detected following 2D gel electrophoresis. A matched set representing proteins consistently detected in the 6 gels shown in Fig 5.4 was constructed using PDQuest image analysis software. A 72 kDa protein spot was identified by image analysis (Table 5.1) as up-regulated in infected cell lysates and is highlighted in the red box.

Infection	Mock-infected gel	DENV-infected gel	Ratio
1	7605	17228.9	1:2.2
2	7424	14930	1:2
3	7731	24840	1:3.2

Table 5.1. Comparison of mean spot density of the 72kDA protein in DENV-infected and mock-infected gels. Quantitation of protein spots by PDQuest showed a statistically significant and reproducible 2-3 fold increase in mean spot density in all three infected gels when compared to mock-infected as indicated by using Student's t-test ($P<0.05$).

Database : **SwissProt 51.3 (250296 sequences; 91444238 residues)**
Taxonomy : **Homo sapiens (human) (15347 sequences)**
Top Score :**242 for GRP78_HUMAN,**
78 kDa glucose-regulated protein precursor (GRP 78)
(Heat shock 70 kDa protein 5)
(Immunoglobulin heavy chain-binding protein)
(BiP) (Endoplasmic reticulum)

Probability Based Mowse Score
Protein score is -10*Log(P), where P is the probability that the observed match is a random event.
Protein scores greater than 54 are significant (p<0.05).

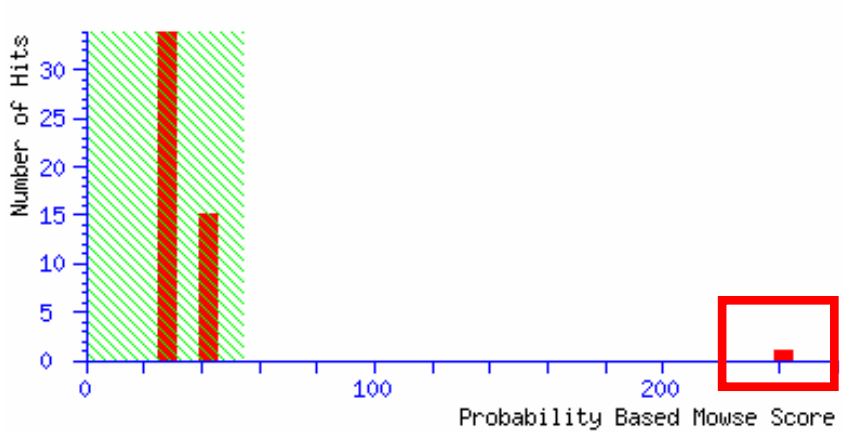


Fig 5.6. Mascot search results. Mascot search resulted in a single significant hit of greater than the cut off of 54 (highlighted in green) with a highly significant Mowse score of 242 (highlighted in red box). The single hit was for the human protein GRP78.

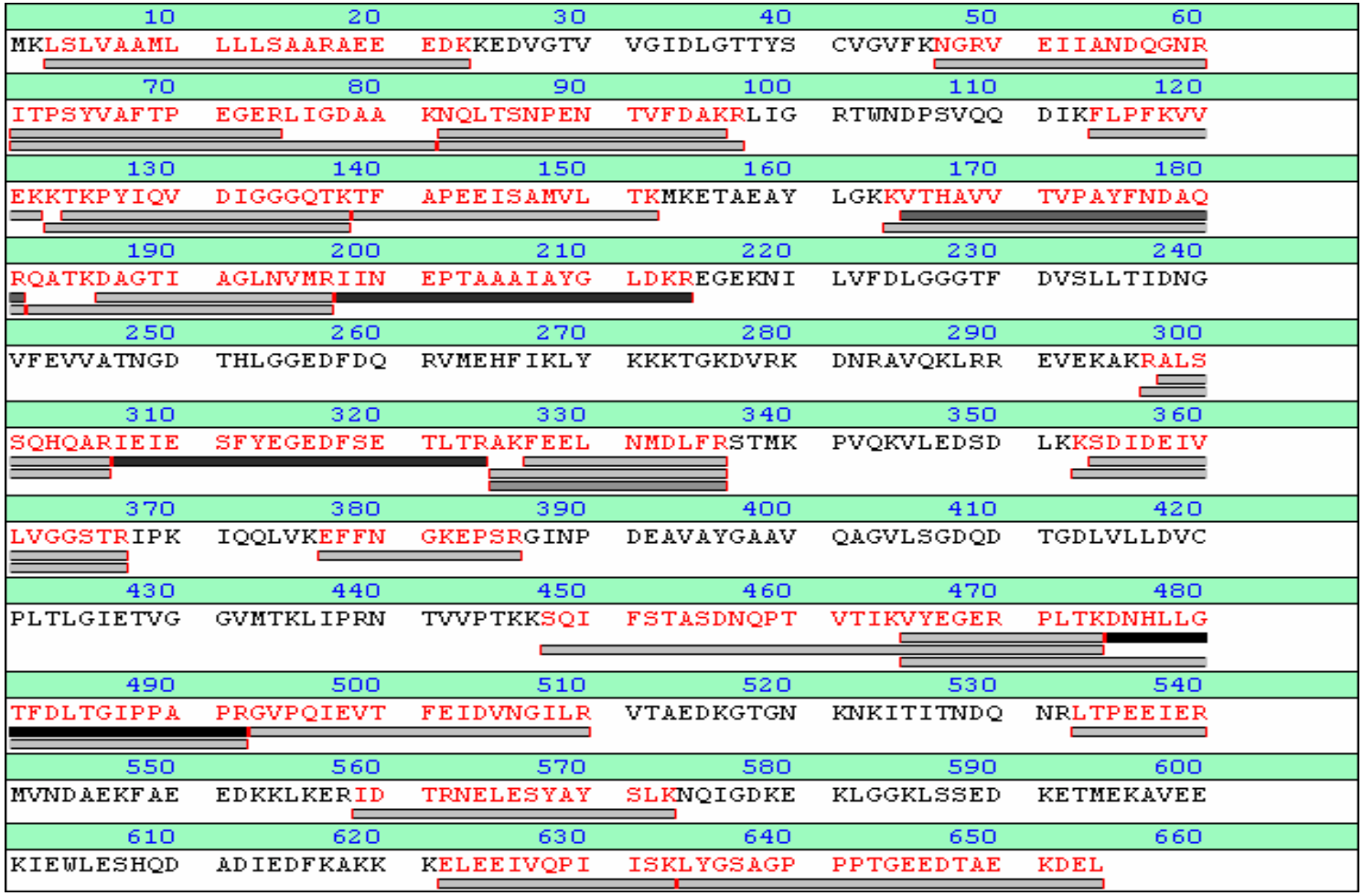


Fig 5.7. Percentage sequence coverage diagram as determined by MS/MS Mascot search results. Sequences of peptides identified in Fig 5.6 were mapped to the GRP78 protein sequence and are highlighted by grey bars. 52% of the GRP78 protein sequence was identified by MS analysis as summarised in Table 5.2.

Sample	Protein identification	Organism	Accession no.	MOWSE score/cut	% seq. MS	Observed MW	Predicted MW
SP1074	GRP78	*Homo sapiens*	GRP78_HUMAN	242/54	52%		72402

Table 5.2. The up-regulated protein seen in DENV-infected cells by 2D gel electrophoresis is GRP78. Presented data summarises MALDI TOF/TOF MS and mascot database search results.

5.2.3 Confirmation of up-regulation of GRP78 in DENV-infected K562 and MDM cells

K562 cells at 72 hrs post DENV and mock-infection (time point used in proteomic analysis in section 5.2.2 where 100% cells are infected), were fixed, permeabilised and analysed for changes in GRP78 by immuno-staining and confocal microscopy (Fig 5.8). GRP78 staining was only seen in the cell cytoplasm and was up-regulated in DENV-infected (Fig 5.8 A,B) compared to mock-infected cells (Fig 5.8 C,D). K562 cells were 100% DENV-infected, as seen previously.

We further confirmed the observed up-regulation of GRP78 in our primary MDM-DENV-infection model. MDM were isolated and either DENV or mock-infected (as described in section 2.3.3). At 48 hrs post-infection (peak of infection) only 15-30% of MDM were DENV-infected (Fig 5.9 B,C). GRP78 staining was seen in distinct puncate pattern in the cytoplasm. The intensity of GRP78 immuno-staining was similar in DENV-infected and neighbouring uninfected cells in the DENV-infected population. However the intensity of GRP78 staining was clearly up-regulated in comparison to GRP78 staining in mock-infected cells (Fig 5.9). This suggests up-regulation of GRP78 in both DENV-infected and uninfected bystander cells in a DENV-infected MDM population.

Demonstration of up-regulation of GRP78 by western blot was attempted using GRP78 specific antibodies (C-20, Santa Cruz Biotechnology catalogue number sc-1051) but was unsuccessful in detecting any GRP78. Due to time constraints establishment of a successful western detection system for GRP78 was not pursued.

5.2.4 Up-regulation of HSP70

HSP70 is up-regulated in many viral infections, has a chaperone and stress-response function, is reported as part of DENV receptor complex in MDM and is closely related to GRP78 identified in our proteomic analysis. Thus we also determined if HSP70 was up-regulated in DENV-infected cells by western blot analysis and confocal microscopy.

5.2.4.1 Up-regulation of HSP70 in DENV-infected K562 cells

The infected and uninfected K562 cell lysates used for 2D gel analysis were subjected to one dimensional SDS PAGE, proteins transferred to nitrocellulose and probed by western blot using HSP70 antibody (Chemicon catalogue number AB3470) (Fig 5.10). Two bands were

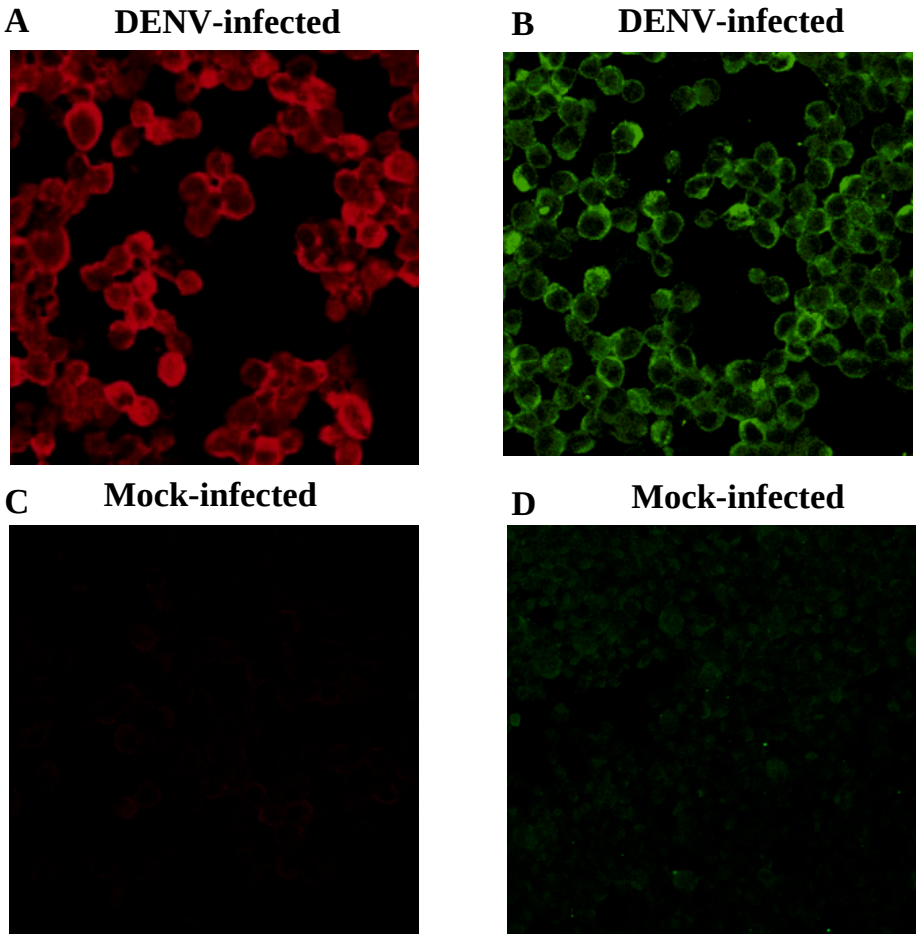


Fig 5.8. Up-regulation of GRP 78 in DENV-infected K562 cells. K562 cells were infected with DENV and 72 hrs post-infection cells were fixed permeabilised and subjected to immuno-staining for either **(A and C)** DENV (using DENV anti- E monoclonal antibodies and complexes detected with red Alexa Fluor 546 conjugated secondary antibody) or **(B and D)** GRP78 (using GRP78 (C-20) and complexes detected with green Cy 2 conjugated secondary antibody) separately.

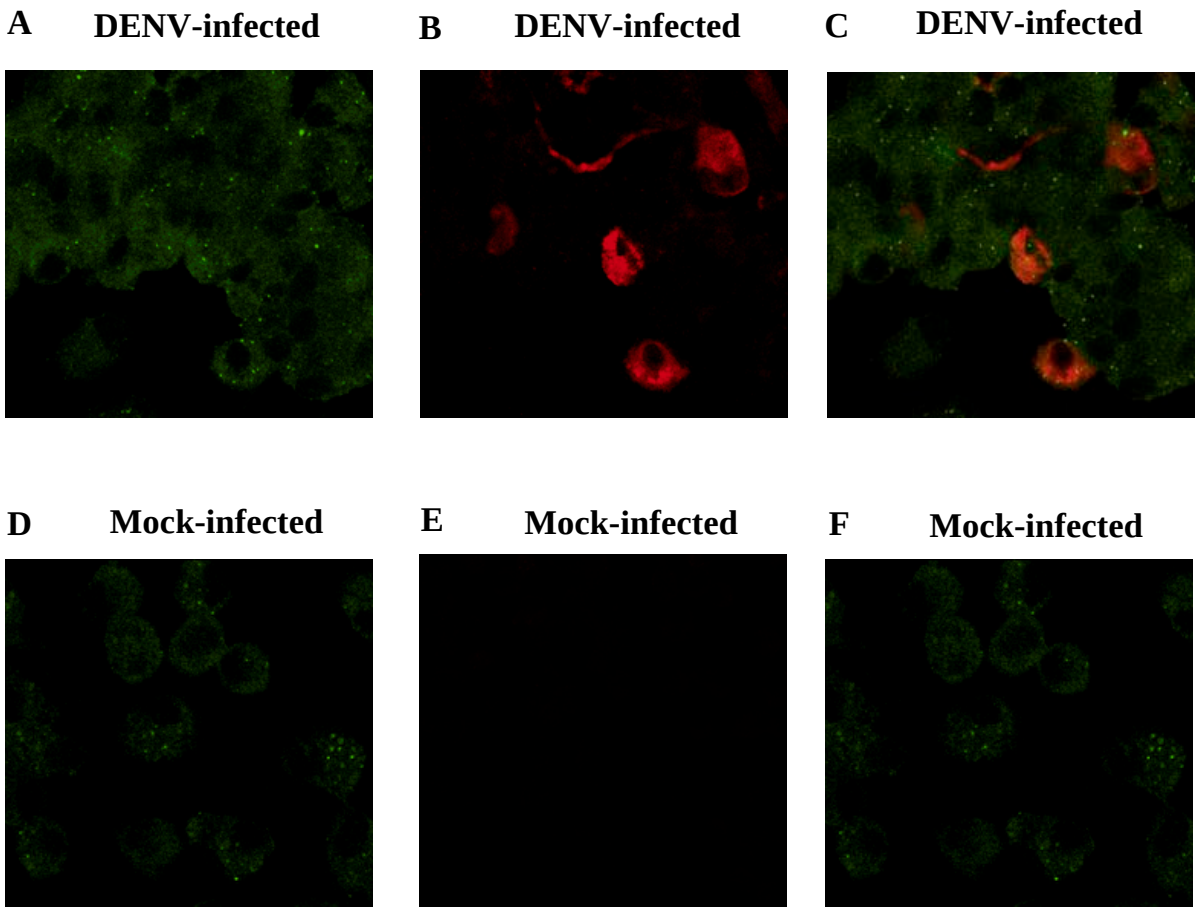


Fig 5.9. Up-regulation of GRP78 in DENV-infected MDM and bystander cells. MDM were infected with DENV and 48 hrs post-infection cells were fixed permeabilised and subjected to dual immuno-staining for both DENV (using DENV anti- E monoclonal antibodies and complexes detected with red Cy 5 conjugated secondary antibody) or GRP78 (using GRP78 (C-20) antibodies and complexes detected with green Cy 2 conjugated secondary antibody). **A.** DENV-infected cells stained for GRP78 (green). **B.** DENV-infected cells stained for DENV (red). **C.** Merged image. **D.** Mocked-infected cells stained for GRP78 (green). **E.** Mock-infected cells stained for DENV antigens (red). **F.** Merged image.

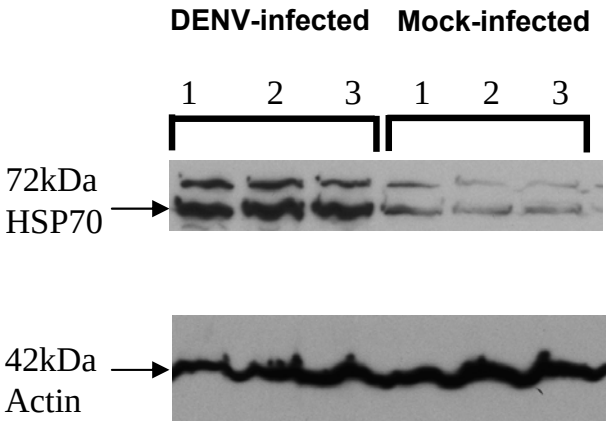


Fig 5.10. Up-regulation of HSP 70 in DENV-infected K562 cells. K562 cells were DENV or mock-infected and cell lysates obtained at 72 hrs post-infection as described in Fig 5.4. The same cell lysates used for 2D gel electrophoresis were run on 1D SDS PAGE gels and immunoblotted using HSP70 antibodies. Lanes (1-3) represent replicate experiments from either DENV or mock-infected cells. The filters were stripped and re-probed for actin to normalise protein loading.

detected of approximate MW of 72 and 78 kDA, consistent with potential cross-reactivity of the HSP70 antibody raised against *Ecoli* DNAK HSP70 homologue with other HSP70 family members, possibly also GRP78. Protein load was normalised by actin staining. Results show that HSP70 is up-regulated in DENV-infected cells compared to mock-infected cells.

5.2.4.2 Both intracellular and membrane associated HSP70 is up-regulated in DENV-infection

HSP70 is present both intra-cellularly and on the cell surface (Schmitt *et al.*, 2007). It has been previously shown that blocking cell membrane associated HSP70 in monocyte/macrophages by HSP70 antibodies blocks DENV entry into cells (Reyes-Del Valle *et al.*, 2005). Thus the cellular location of the up-regulated HSP70 in DENV-infected K562 cells was assessed. DENV-infected and mock-infected K562 cells at 72 hrs post-infection were either detergent permeabilised (to allow intracellular staining) or not permeabilised (to allow staining only of the outer membrane). Comparison of DENV and mock-infected cells show up-regulation of both intracellular and cell membrane associated HSP70 (Fig 5.11).

Immuno-staining for HSP70 was also performed in MDM at 48 hrs after DENV or mock-infection. Results showed slight up-regulation of membrane bound HSP70 in cells co-stained for DENV E antigen (Fig 5.12 (1)). When cells were permeabilised to stain for intracellular HSP70, up-regulation was observed not only in DENV antigen positive cells, but also in uninfected neighbouring cells (Fig 5.12 (2)). Thus similarly to K562, both intracellular and membrane-associated HSP70 was up-regulated in DENV-infected cells. Unlike the cytoplasmic staining observed for GRP78 (Fig 5.8-5.9) immuno-staining indicated HSP70 proteins in cytoplasm, and cell surface. Similar to GRP78, HSP70 was also up-regulated in uninfected bystander cells.

5.3 DISCUSSION

This chapter describes the first study using proteomic approaches to analyse the changes in host cell proteins induced by DENV-infection and has identified an ER molecular chaperone BiP (Immunoglobulin heavy chain-binding protein), also called glucose-regulated protein 78 (GRP78), a member of the HSP70 family as up-regulated during DENV-infection.

For proteomic profiling a high percentage of DENV-infected cells were needed, as protein changes in a small number of infected cells could be masked by uninfected cells. Therefore

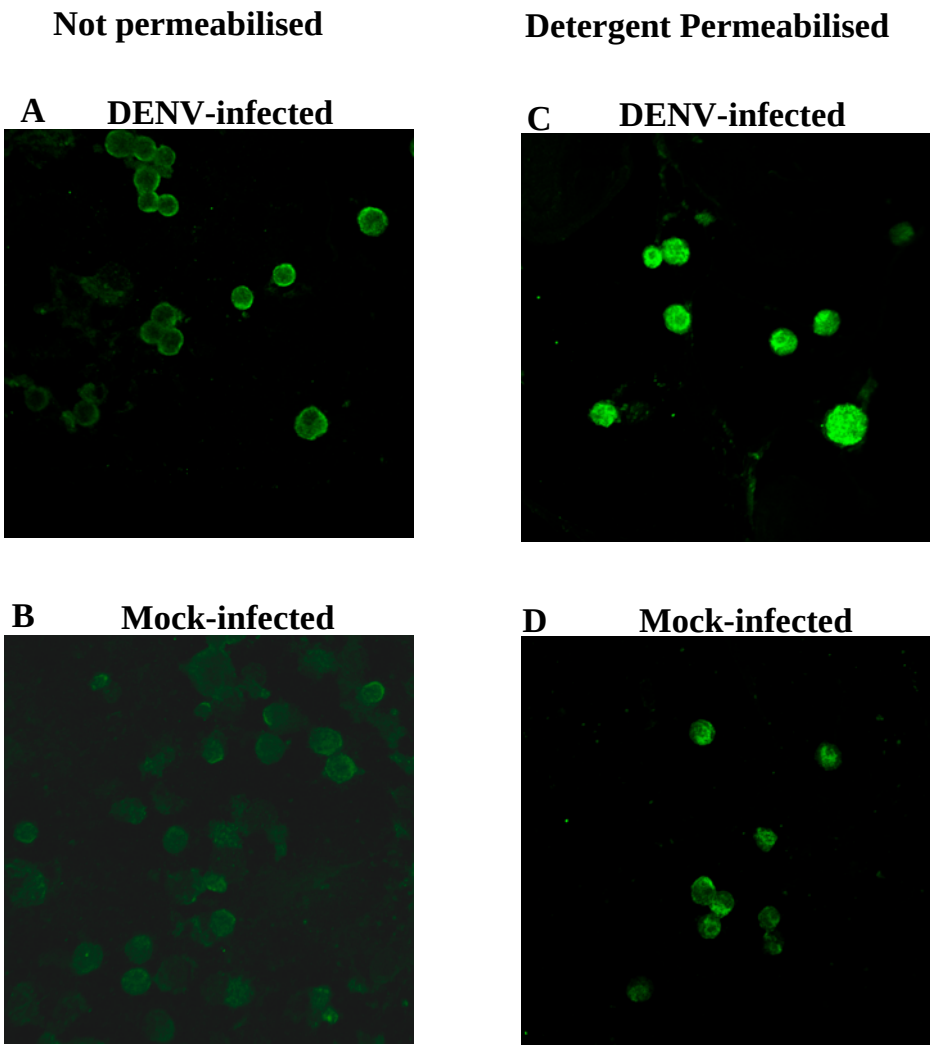


Fig 5.11. Up-regulation of intracellular and membrane bound HSP70 in DENV-infected K562 cells. K562 cells were DENV or mock-infected and at 72 hrs post-infection cells were fixed and subjected to immuno-staining for HSP70 and complexes detected with green Alexa Fluor 488 conjugated secondary antibody in either non permeabilised cells (**A** and **B**) or permeabilised cells (**C** and **D**), allowing detection of both external membrane and intracellular HSP70 respectively.

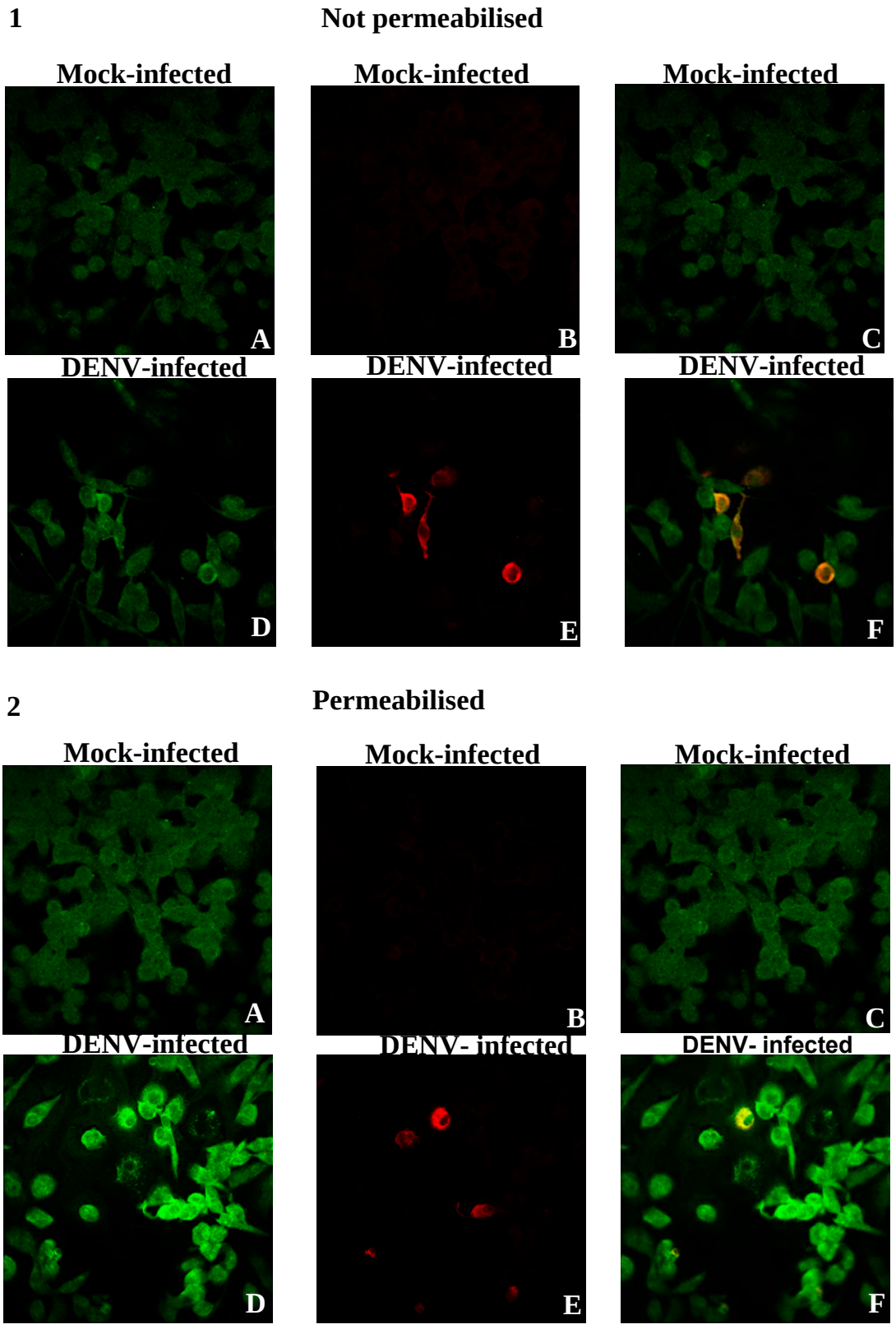


Fig 5.12. Up-regulation of intracellular and membrane bound HSP70 in DENV-infected MDM and bystander cells. MDM were infected with DENV and 48 hrs post-infection cells were fixed and either **(1)** not permeabilised or **(2)** permeabilised and subjected to dual immuno-staining for both DENV (using DENV anti-E monoclonal antibodies and complexes detected with red Cy 5 conjugated secondary antibody) or HSP70 (using HSP70 and complexes detected with and green Alexa Fluor 488 conjugated secondary antibody). **A.** Mock-infected cells stained for HSP70 (green). **B.** Mock-infected cells stained for DENV antigens (red). **C.** Merged image. **D.** DENV-infected cells stained for HSP70 (green). **E.** DENV-infected cells stained for DENV (red). **F.** Merged image.

our *in vitro* MDM model, that only yields a maximum of 15-30% of infected cells even with high MOI's could not be used. Similarly, U937 cells which are widely used as a model for human monocytes and can be differentiated to become macrophage-like by the addition of PMA (O'Sullivan and Killen, 1994) also showed < 15% DENV-infected cells at a MOI of 10 and thus were not suitable. K562 cells, a human erythroleukemic cell with some macrophage-like properties, could be highly infected with 100% DENV-infected cells at 72 hrs post-infection without cell death as a result of DENV-infection and was suitable for initial proteomic analysis, although any protein change identified would need to be confirmed in MDM.

Using mock and DENV-infected K562 cells, 344 distinct and reproducible protein spots were visualised following 2D gel electrophoresis and Coomassie blue G250 staining. Only one protein was found to differ significantly between mock and DENV-infected cell lysates. This 72 kDa protein was up-regulated > 2-3 fold with a mean spot density significantly different from mock-infection ($p < 0.5$) and was observed reproducibly in all 3 independent infections. There are no absolute criteria for setting the cut-off to determine genuine change (i.e. up or down-regulation) in protein level, but less than a two fold change in protein expression has been suggested to be unreliable (Zilm *et al.*, 2007) and > 2 fold change was thus used as a cut-off point in this study.

Mass spectrometry and mascot database search identified the up-regulated protein in DENV-infected cells as an ER molecular chaperone BiP (Immunoglobulin heavy change-binding protein), also called glucose-regulated protein 78 (GRP78). Immuno-staining confirmed up-regulation of GRP78, with GRP78 protein localised to the cytoplasm, consistent with reported localisation of this protein in cells (Benali-Furet *et al.*, 2005; Luo *et al.*, 2006). In addition to up-regulation in DENV-infected cells, we also observed up-regulation in uninfected neighbouring cells within the DENV-infected cell population. DENV-infected MDM and other cell types are well documented to release enhanced levels of inflammatory mediators such as TNF-α and NO (discussed in section 1.8.2.2). Such stimuli are also reported to induce GRP78 levels (Xu *et al.*, 2004) and thus up-regulation of GRP78 in DENV-infected cells may involve both virus-specific intracellular response and/or a secondary response to DENV induced release of inflammatory mediators. GRP78 is a member of the heat shock 70 protein family. GRP78 has a specific role in the cell in preventing ER stress and in the unfolded protein response (UPR). GRP78 works as a master control of ER stress and the UPR by interacting with three factors: PKR-like endoplasmic kinase (PERK), activating

transcription factor 6 (ATF6) and the ER trans-membrane protein kinase/endoribonuclease (IRE1) reviewed in (Kaufman *et al.*, 2002; He, 2006). GRP78 is normally bound to these three mediators, however under stress conditions, high levels of unfolded proteins sequesters GRP78 away, releasing PERK, ATF6 and IRE1. On release PERK is activated to start protein synthesis, IRE1 leads to transcription induction of a subset of gene encoding protein degradation and ATF6 stimulates transcription of responsive genes that refold misfolded proteins leading to a response that promotes cell recovery from stress. While induction of GRP78 is required to maintain cellular homeostasis in response to ER stress and over expression of GRP78 protects cells from ER stress induced apoptosis, prolonged ER stress has been shown to induce apoptosis or programmed cell death in JE, BVDV and HCV infections (all viruses of the *Flaviviridae* family).

ER stress (via the PKR mechanism) is a normal host cell response to many viral infections inducing cell death. GRP78 is up-regulated in viral infections such as HIV, Rabies, Influenza, HCV and Rotavirus (Hurtley *et al.*, 1989; Earl *et al.*, 1991; Gaudin, 1997; Choukhi *et al.*, 1998; Xu *et al.*, 1998). Further, GRP78 has been shown to be associated transiently with folding intermediates of HIV viral membrane proteins, misfolded forms of influenza virus hemagglutinin, to be involved in folding of HCV glycoprotein and can interact with rotavirus structural proteins suggesting active involvement in the control of virus replication or viral protein and virion assembly (Hurtley *et al.*, 1989; Earl *et al.*, 1991; Choukhi *et al.*, 1998; Xu *et al.*, 1998). Recent studies have shown up-regulation of GRP78 in HCMV infection that correlates with production of virion structural proteins and glycoprotein synthesis/processing and virion assembly (Buchkovich *et al.*, 2007). These authors hypothesised that this up-regulation of GRP78 provides the needed chaperone for viral assembly. In the study by Buchkovich et al., 2007 depletion of GRP78 during HCMV infection using GRP78 siRNA and subAB toxin which cleaves GRP78 had little effect on HCMV protein expression within infected cells but dramatically reduced virion production. Additionally subAB toxin was highly cytotoxic for uninfected cells but not HCMV infected cells suggesting that virus up-regulation of GRP78 potentially alters cellular stress signalling and the cell death response.

Flaviviruses use the ER of host cells as the primary site of viral replication and assembly and ER stress mediated signalling has been implicated in apoptosis induced by some *flaviviruses* such as JE and BVDV (Jordan *et al.*, 2002; Su *et al.*, 2002). Potentially viruses, such as *flaviviruses* that use the ER as an integral part of their replication strategy, may need to overcome the ER stress response in order to successfully replicate or may utilise GRP78 for

viral assembly, as is highlighted above for HCMV. In this study K562 cells infected with DENV replicated similarly to mock-infected cells with no visible CPE indicating absence of apoptosis. Up-regulation of GRP78 in this cell line may play a role in cell survival allowing successful virus replication.

Although our proteomic analysis identified GRP78 as the only up-regulated host cell protein, the literature suggests particular importance of the HSP70 in DENV-infected cells in Fc receptor cell lines (Reyes-Del Valle *et al.*, 2005). Additionally since some HSP70 antibodies can cross-react with GRP78 and vice versa there is some uncertainty as to the exclusive specific roles of GRP78 or HSP70 previously reported in the literature and we thus chose to also analyse changes in HSP70 in DENV and mock-infected cells. Even though HSP70 mainly functions as a cytoplasmic chaperone, it has also been observed at the cell surface in DENV-infected cells and proposed to play a role in a DENV receptor complex (Reyes-Del Valle *et al.*, 2005). Therefore cells were analysed for HSP70 with or without permeabilising the cells to investigate cytoplasmic and membrane bound HSP70 respectively. In both K562 and MDM cells we observed up-regulation of both membrane-associated and intracellular HSP70 in DENV-infected cells. Similar to that seen for GRP78, HSP70 was up-regulated in the cytoplasm of both DENV-infected MDM and neighbouring uninfected cells, again suggesting up-regulation of HSP70 via bystander effects, such as release of stimulus such as TNF-α and NO.

HSP70 is present in all cell types, and increased expression of HSP's has been seen in cells subject to stress including heat or physiological and environmental conditions such as nutrient supply, infection and cytokine production. HSP70 alters proteins or genes known to be involved in inflammation by inhibition of NO synthase generation, nuclear translocation of the transcription factor, NF-kB (Feinstein *et al.*, 1997; Guzhova *et al.*, 1997) and also protects cells from the cytoxicity mediated by TNF-α, by inhibiting TNF-α induced activation of phospholipase A2 (Jaattela, 1993; Jaattela and Wissing, 1993; Wissing *et al.*, 1997). Of particular interest are reports of roles for HSP70 in the replication of some RNA and DNA viruses. Up-regulation of HSP70 is not a general response to the stress of viral infection, but rather a highly specific response depending on cell type and virus interaction (Phillips *et al.*, 1991). Some DNA viruses such as HSV1 and adenovirus type 5 have been shown to induce HSP70, whereas infection with other DNA viruses such as simian virus 40 and vaccinia virus do not (Phillips *et al.*, 1991). In replication of a number of viruses including adenovirus, cytomegalovirus, HSV 1 and 2 (extensively reviewed in (Mayer, 2005)) heat shock proteins

assist in viral replication (Table 5.3). In contrast HSP70 may also be involved in a host protective response and resist virus replication. In rotavirus infection of caco-2 cells, HSP70 is rapidly induced, and blocking of HSP70 production using siRNA results in an increase in rotavirus replication, thus suggesting that HSP70 negatively controls rotavirus replication (Broquet *et al.*, 2007). Similarly HIV-1 infection induces HSP70 and siRNA knockdown of HSP70 in MAGGI cells shows an increase in HIV-1 production and also an increase in cell cycle arrest and apoptosis (Iordanskiy *et al.*, 2004).

In DENV-infection HSP70 has been previously identified as part of a DENV receptor complex in monocytes/macrophages and cell lines that have Fc receptors (eg neuroblastoma and U937) (Reyes-Del Valle *et al.*, 2005) but not in the liver cell line (HepG2) (Cabrera-Hernandez *et al.*, 2007). In the study of Cabrera-Hernandez *et al*., 2007, blocking of HSP70 extra-cellularly with antibody, did not reduce DENV-infection. Given that GRP78 is also reportedly part of DENV receptor complex in HepG2 cells, it is possible that HSP70 family members may play specific roles in DENV entry in different cell types. Alternatively, these two reports may be identifying the same receptor complex with discrepancies in the identity of GRP78 and HSP70 due to cross reactivity of antibodies against these homologous proteins. Recent studies by cDNA-amplified fragment length polymorphism (AFLP) have shown up-regulation of HSP70 mRNA in DENV-infected HepG2 cells from day 1 to 4 post infection, although this study did not look at HSP70 protein (Ekkapongpisit *et al.*, 2007). The authors suggest HSP70 is a stress protein and this up-regulation of HSP70 is most likely to be associated with the innate cellular response of cells to virus infection.

The present study herein is the first to report induction of GRP78 and HSP70 in DENV-infected K562 cells and MDM, but its functional role has not been characterised. Future work should include (i) a time course study to determine up-regulation of GRP78 and HSP70 in relation to cell death or viral replication, as done in HCMV studies (Buchkovich *et al.*, 2007) (ii) knockdown studies of GRP78 and HSP70 using GRP78 or HSP70 siRNA which we have found to be a particularly effective technique in MDM (chapter 4) or subAB toxin knockdown of GRP78, as done in HCMV studies (Buchkovich *et al.*, 2007) and subsequent analysis of viral replication. Together (i) and (ii) will clarify whether GRP78 and HSP70 play a role in enhancing or counteracting viral replication, and help understand cellular protective and cytopathic responses to DENV-infection. (iii) Extension of our initial proteomic analysis using more advanced proteomic techniques such as passive rehydration of IEF strips (Barry *et al.*, 2003), cup loading of protein samples onto IEF strips, use of smaller pH range

Virus	Functional interaction of HSP70 during viral replication
Adenovirus	Transcription, mRNA export
Hepatitis B virus	Reverse transcription
Herpes Simplex virus 1	Genome replication
Herpes Simplex virus 2, Varicella Zoster	Nuclear localisation
Measles virus	Genome replication
Poliovirus	Virion assembly?
Reovirus	Capsid protein folding

Table 5.3. Some viruses that require HSP70 for replication (adapted from Mayer *et al.*, 2005).

(Campostrini *et al.*, 2005), use of more sensitive stains such as SYPRO Ruby and use of bigger gels. All these technical advances in proteomics have developed since the beginning of this study and could enhance the visualisation of more proteins and thus increase the probability of identification of other novel host factors that may regulate DENV-infection as shown with SARS virus where a total of 344 unique proteins have been identified (Jiang *et al.*, 2005). Identifying novel proteins involved in a host cell response to DENV-infection may further assist in our understanding of DENV pathogenesis at the protein level.

CHAPTER 6

GENERAL DISCUSSION

6.1 DISCUSSION AND SUMMARY

DENV-infection of humans results in symptomatic infection ranging from self limited febrile illness (DF) to more severe fatal diseases (DHF/DSS). DHF is characterised by increased capillary permeability resulting in decreased plasma volume, which may be accompanied by hemorrhagic manifestations (WHO, 1997). The pathogenesis of DENV-infection is not fully understood. A number of factors such as T cell cross-reactivity, increased viral load, antibody dependent enhancement of infection and importantly, induction of a range of cytokines and chemokines, have been reported in severe forms of illness and have been implicated as playing a role in pathogenesis of DENV-infection (as discussed in chapter 1). Cytokines, in particular TNF-α are elevated in the sera of DENV-infected patients and even higher levels are associated with more serious forms of the DENV disease (Hober *et al.*, 1993; Green *et al.*, 1999a; Kittigul *et al.*, 2000). *In vitro,* TNF-α release coincides with the peak of DENV production from infected MDM (Carr *et al.*, 2003) and high levels of TNF-α are released from other cells of the immune system such as B and T cells when exposed to DENV (Lin *et al.*, 2002b; Mangada *et al.*, 2002; Mangada and Rothman, 2005). TNF-α is a powerful pro-inflammatory cytokine with pleiotropic properties and can also have direct anti-viral effects on several viruses (reviewed in (Herbein and O'Brien, 2000)). In relation to DENV, TNF-α may have anti-viral or regulatory effects on DENV replication but these are poorly understood. Additionally, the overall host response to DENV-infection is clearly important in mediating DENV disease and we need to understand how the DENV-infected host responds to and regulates DENV-infection.

Aims of this study:-

1. To determine the role of TNF-α in regulating DENV replication in an important target cell type; the macrophage.
2. To determine changes in host cell protein expression within cells infected with DENV.

6.2 MAIN CONCLUSIONS OF THIS THESIS

1. In contrast to the known effects of TNF-α on replication of some other viruses, this thesis shows that TNF-α does not have any autocrine or paracrine effect on DENV replication.
2. Cells supporting DENV replication show reduced activation of NF-kB mediated reporter gene transcription and NF-kB nuclear translocation in response to TNF-α, while neighbouring uninfected cells respond normally to TNF-α.
3. DENV-infection up-regulates the host proteins GRP78 and HSP70 in both DENV-positive and DENV-uninfected bystander cells

These findings are schematically illustrated in Fig 6.1.

6.3 IMPLICATIONS OF THESE FINDINGS

6.3.1 Altered TNF-α responses may allow DENV to evade the immune response

These results show that TNF-α does not affect DENV replication, and that DENV infected cells do not respond normally to TNF-α, as NF-kB nuclear translocation and NF-kB-mediated reporter gene transcription following TNF-α-stimulation are both reduced compared to uninfected cells (chapter 4). NF-kB is a central mediator of the immune response and also cell death discussed in section 6.3.4. Thus, the reduced activation of NF-kB following TNF-α stimulation seen in DENV-infected cells, may reduce the expression of hundreds of target genes (including growth factors, cytokines and stress inducing agents) that are involved in the *intracellular* defence against DENV and inhibit processes such immune cell activation, thereby allowing viral replication in the absence or reduced anti-viral responses (Fig 6.1 [4]).

6.3.2 Future identification of the DENV-specific protein that mediates DENV inhibition of TNF-α-stimulated NF-kB activation

Inhibition of TNF-α-stimulated activation of NF-kB in HCV infection (another member of the *Flaviviridae*) can be mediated by three HCV proteins, core, NS5A, or NS5B (Marusawa *et al.*, 1999; Park *et al.*, 2002; Choi *et al.*, 2006; Saito *et al.*, 2006). HCV NS5A inhibits the TNF-α signalling cascade by binding to the TNF-α receptor (TNFR1) signalling complex, and through its interaction with TNF-α receptor associated factor 2 (TRAF2) it inhibits NF-kB activation (Park et al., 2002). NS5B has been shown to interact with both TRAF2 and IkB kinase complex (IKK) (Choi et al., 2006). In the same way, DENV NS5 may be responsible

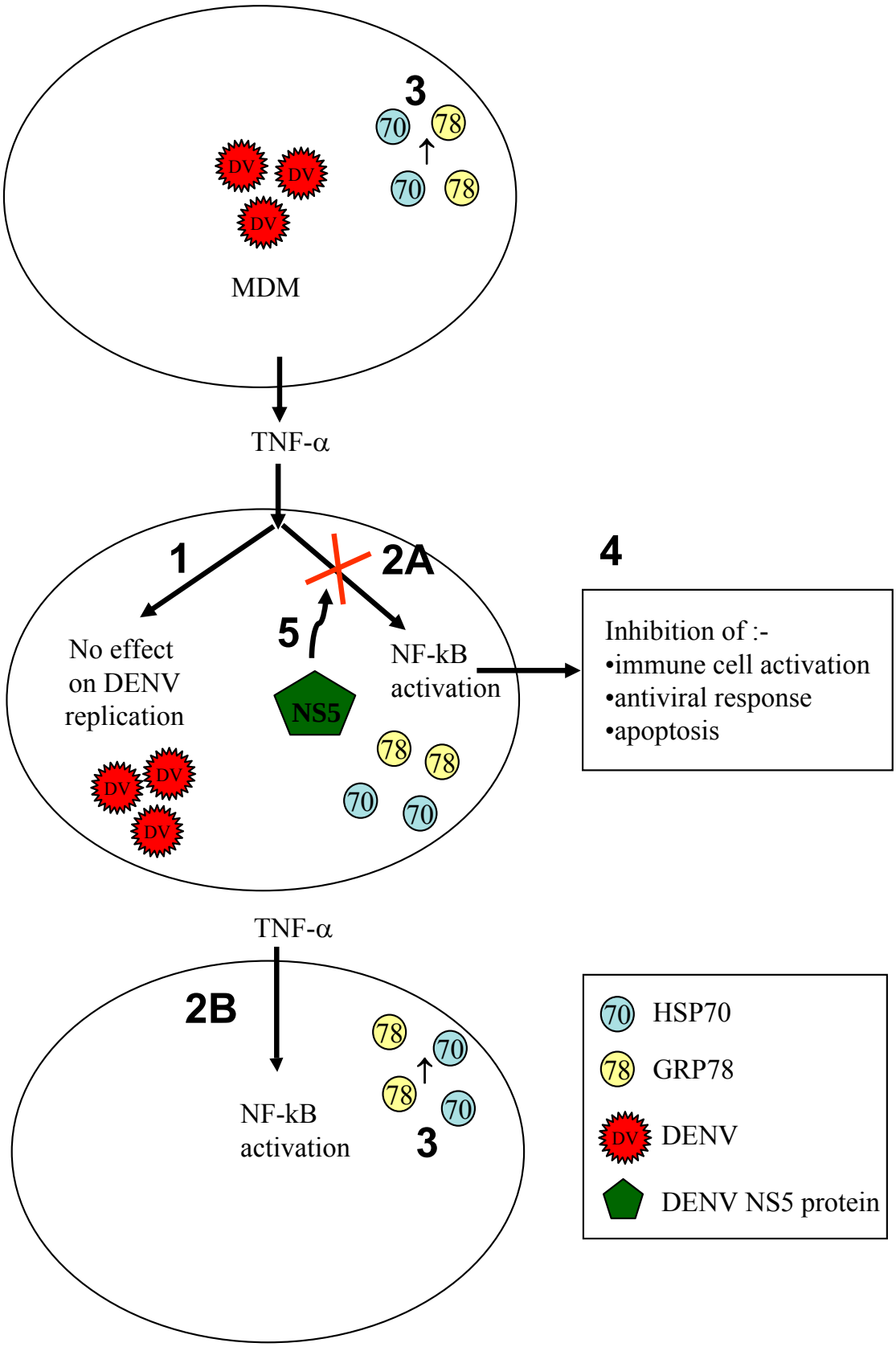


Fig 6.1. Summary of the main conclusions from this thesis. TNF-α is released from DENV infected cells and acts on DENV-infected cells but **(1)** does not affect DENV replication and **(2A)** does not activate NF-kB. However **(2B)** activates NF-kB normally in neighbouring uninfected cells of a DENV positive population. **(3)** DENV up-regulates GRP78 and HSP70 in DENV infected and uninfected cells. We hypothesise DENV inhibition of NF-kB activation may **(4)** alter cell function and **(5)** be mediated by the DENV NS-5 protein.

for blocking TNF-α-stimulated NF-kB activation (Fig 6.1 [5]). This possibility should be formally examined in the future.

6.3.3 Up-regulation of GRP78 and HSP70 in DENV-infected cells may assist viral replication and viral spread

In DENV-infected cells GRP78 and HSP70 are up-regulated (discussed in chapter 5) (Fig 6.2A). This may promote viral production by eliciting ER stress and the UPR, seen with other viruses (and discussed in chapter 5), and thereby assisting viral protein folding, virion assembly and virus production (Fig 6.2B). Additionally ER stress and DENV-infection induces the release of elevated levels of inflammatory mediators such as TNF-α and NO (discussed in chapter 1) (Fig 6.2C) and has been shown to up-regulate GRP78 and HSP70 (Heimbach *et al.*, 2001; Xu *et al.*, 2004) (Fig 6.2D). Since GRP78 has been described as a DENV-2 receptor complex in liver cells (Jindadamrongwech *et al.*, 2004) and HSP70 itself is part of a receptor complex of Fc receptor-positive cells such as U937 cells and MDM (Reyes-Del Valle *et al.*, 2005) (chapter 5), the increased levels of GRP78 and HSP70 may function as receptors to enhance virus entry and spread (Fig 6.2D). Such an effect would not be seen in the one-step viral culture described in this thesis, but may be important *in vivo*. Thus the up-regulation of GRP78 and HSP70 seen in DENV-infected culture in this study may have important consequences for both infected and uninfected cells. This is summarised in Fig 6.2.

6.3.4 Proposed host cell - DENV interactions and cell death

In contrast to the above DENV-cell interactions that may aid DENV replication (section 6.3.1 and 6.3.3) other processes occurring concurrently may increase the susceptibility of DENV-infected cells to death.

By blocking NF-kB activation, DENV might prevent NF-kB-mediated cell death (Fig 6.3-1A). However NF-kB activation stimulated by TNF-α can also be a pro-survival signal (reviewed in (Hiscott *et al.*, 2001)). In this situation, it is well described that blocking of NF-kB activation enhances the alternative TNF-α-stimulated NF-kB-independent cell death pathway (Fig 6.3-1B) and thus TNF-α stimulation of DENV-infected cells may induce NF-kB independent cell death.

Similarly DENV-induced up-regulation of GRP78 and HSP70 may have contrasting effects on cell survival. Up-regulation of GRP78 may mediate UPR and promote cell survival (Fig

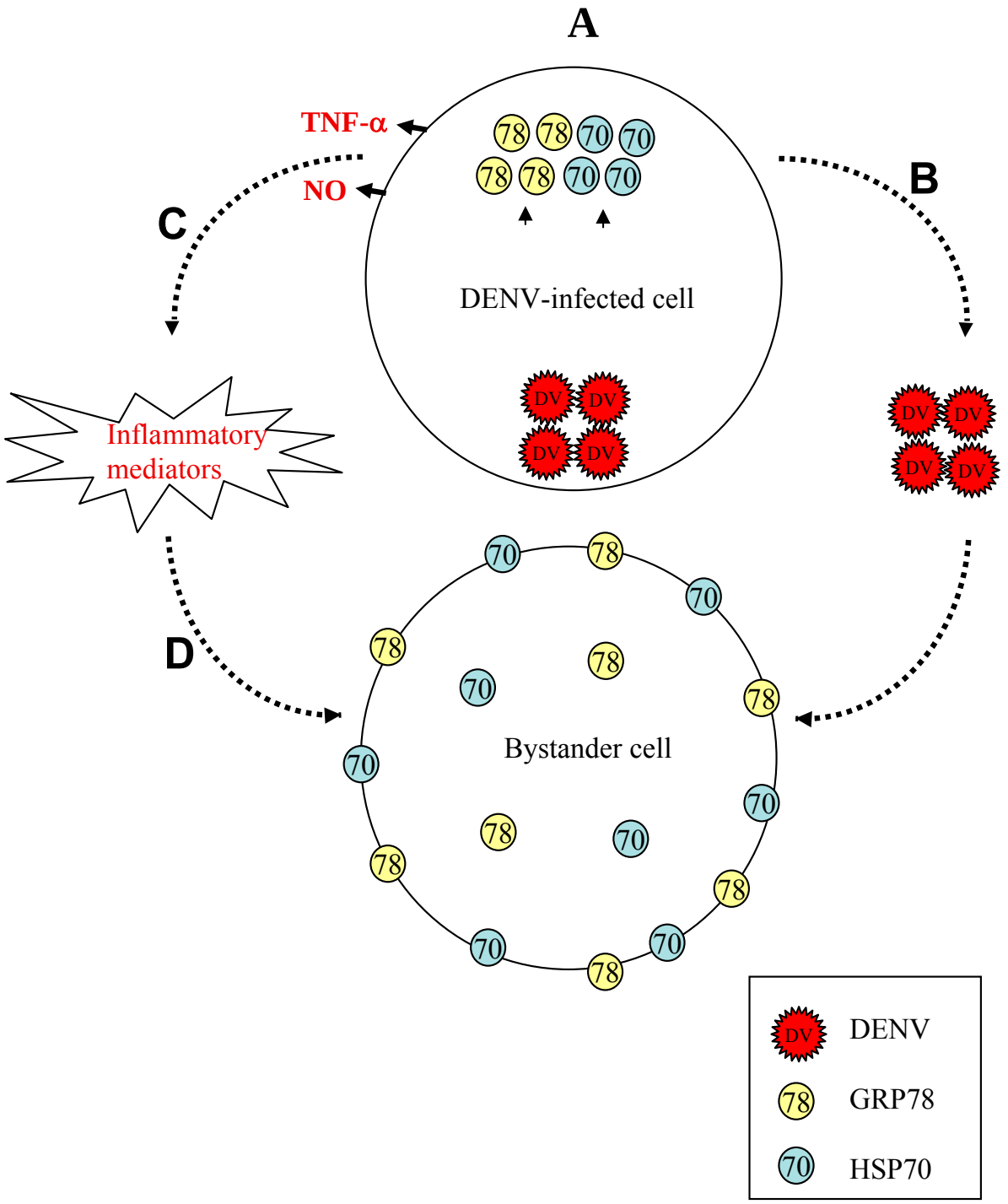


Fig 6.2. Up-regulation of GRP78 and HSP70 during DENV replication. **(A)** In DENV-infected cells at the peak of viral replication, GRP78 and HSP70 are increased **(B)** which we hypothesise may assist in viral production. **(C)** DENV infection is also known to induce release of inflammatory mediators that we propose may **(D)** up-regulate GRP78 and HSP70 in uninfected bystander cells which would aid in spread of DENV infection.

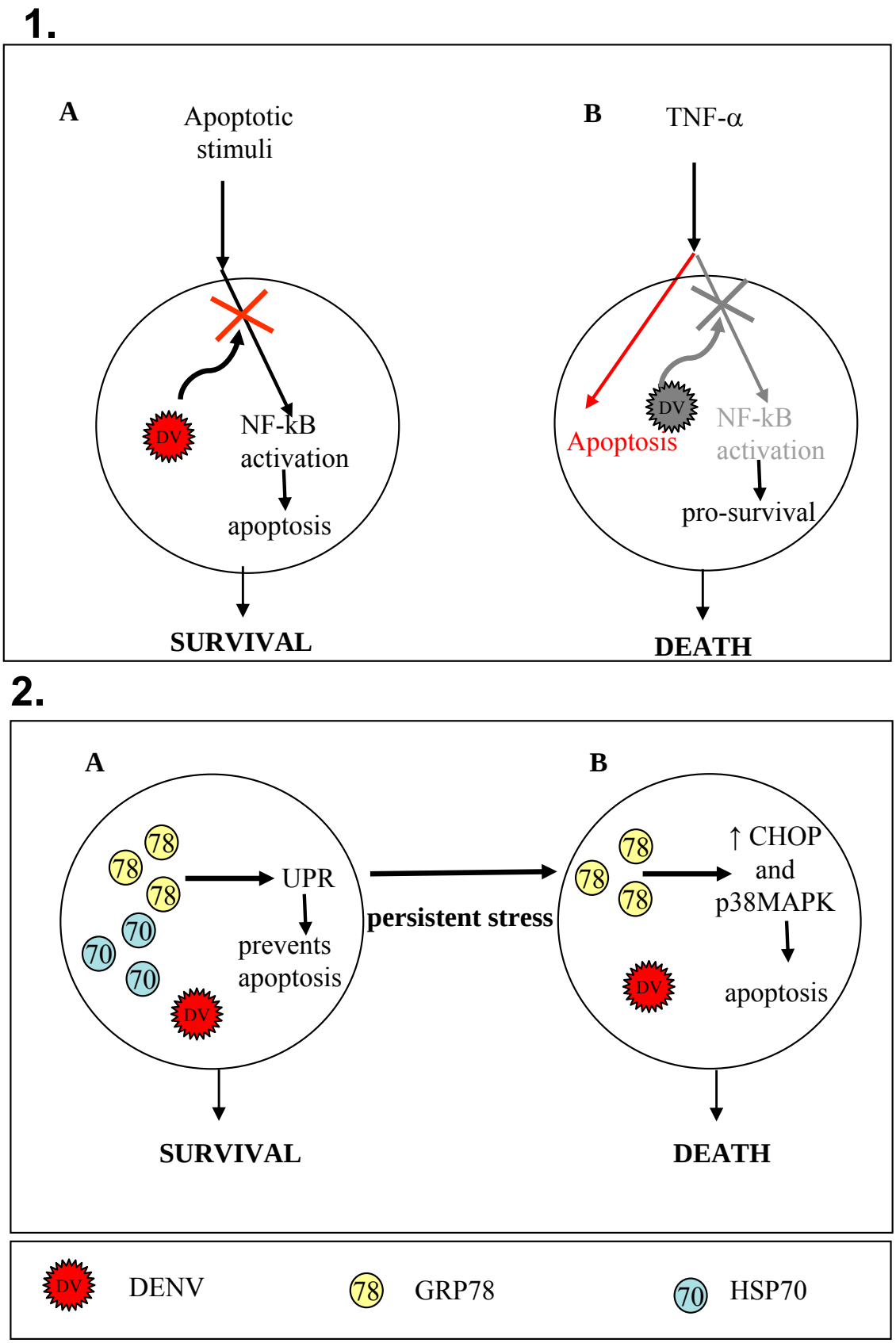


Fig 6.3. Host-DENV interactions and cell death. 1. DENV blockade of TNF-α stimulated NF-kB activation may (**A)** prevent actions of NF-kB mediated apoptotic stimuli or **(B)** promote TNF-α stimulated NF-kB independent apoptosis? **2.** DENV up-regulation of GRP78 and HSP70 may **(A)** induce UPR and protect against apoptosis, or **(B)** during persistent stress may promote CHOP-mediated cell death.

6.3-2A). However up-regulation of GRP78 in the presence of persistent stress, such as viral infection, may result in stress induced apoptosis mediated by the cell death factors CHOP and p38 MAPK as has been reported for JE infection (another *Flavivirus*) (Bartelma and Padmanabhan, 2002)) (Fig 6.3-2B).

Thus the relationship between DENV-infection and cell death may be dependent on the stage of viral infection, accumulation of particular viral proteins, presence of TNF-α and other apoptotic stimuli and levels of cell stress. Further work is needed to clarify these interactions.

6.4 CONCLUSION

This study has described perturbations to normal cellular signalling networks during DENV infection, including TNF-α signalling pathways and intracellular levels of GRP78 and HSP70 that are likely to have important consequences for anti-viral responses, cell death, virus production and spread. The final balance between these observed effects is likely to impact on the clinical severity of DENV infection and the occurrence of DHF/DSS. In particular, future work should determine whether the altered TNF-α signalling in DENV-infected cells and up-regulation of GRP78 and HSP70 in infected and neighbouring cells culminate to (1) assist the virus by decreasing pro-inflammatory anti-viral cytokines and increasing viral assembly production and spread, or do they (2) assist the host by promoting death of infected cells. Further work should explore the significance of these findings in terms of the regulation of virus replication and the development of disease.

Appendix I. Sequence of DENV-2, New Guinea C strain from which pMON601 was constructed. The DENV capsid region cloned into pGEM-DENV-2CAP is highlighted in yellow

```
LOCUS       AF038403               10724 bp    RNA     linear   VRL 23-
MAR-1998
DEFINITION  Dengue virus type 2 strain New Guinea C polyprotein mRNA,
complete
            cds.
ACCESSION   AF038403
VERSION     AF038403.1  GI:2723944
KEYWORDS    .
SOURCE      Dengue virus type 2
  ORGANISM  Dengue virus type 2
            Viruses; ssRNA positive-strand viruses, no DNA stage;
Flaviviridae;
            Flavivirus; Dengue virus group.
REFERENCE   1  (bases 1 to 10724)
  AUTHORS   Gruenberg,A., Woo,W.S., Biedrzycka,A. and Wright,P.J.
  TITLE     Partial nucleotide sequence and deduced amino acid sequence
of the
            structural proteins of dengue virus type 2, New Guinea C and
            PUO-218 strains
  JOURNAL   J. Gen. Virol. 69 (Pt 6), 1391-1398 (1988)
   PUBMED   3385407
REFERENCE   2  (bases 1 to 10724)
  AUTHORS   Gualano,R.C., Pryor,M.J., Cauchi,M.R., Wright,P.J. and
            Davidson,A.D.
  TITLE     Identification of a major determinant of mouse neurovirulence
of
            dengue virus type 2 using stably cloned genomic-length cDNA
  JOURNAL   J. Gen. Virol. 79 (Pt 3), 437-446 (1998)
   PUBMED   9519821
REFERENCE   3  (bases 1 to 10724)
  AUTHORS   Gualano,R.C., Pryor,M.J., Cauchi,M.R., Wright,P.J. and
            Davidson,A.D.
  TITLE     Direct Submission
  JOURNAL   Submitted (11-DEC-1997) Department of Microbiology, Monash
            University, Wellington Rd, Clayton, Melbourne, VIC 3123,
Australia
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121 agaaataccc ctttcaatat gctgaaacgc gagagaaacc gcgtgtcgac tgtacaacag
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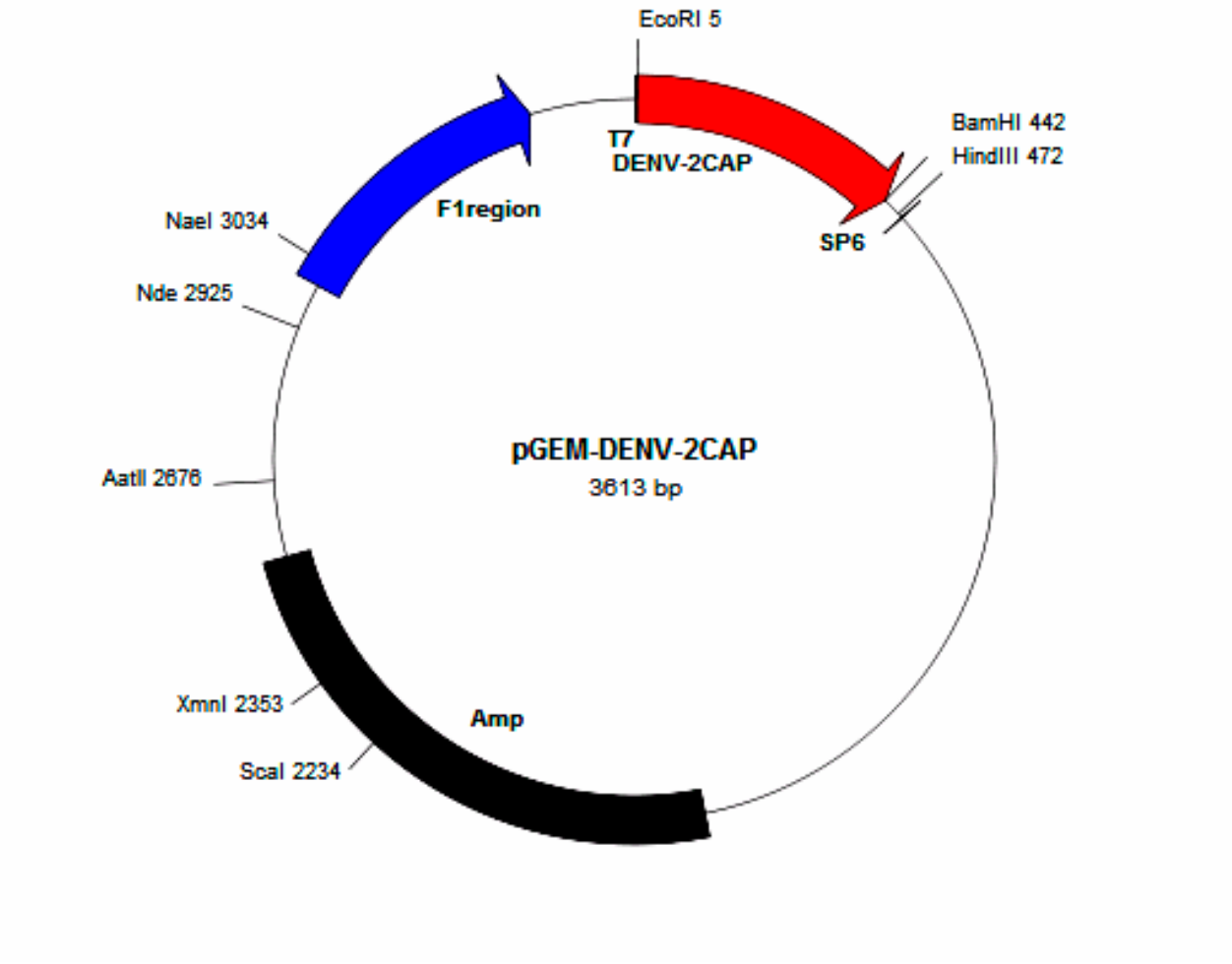
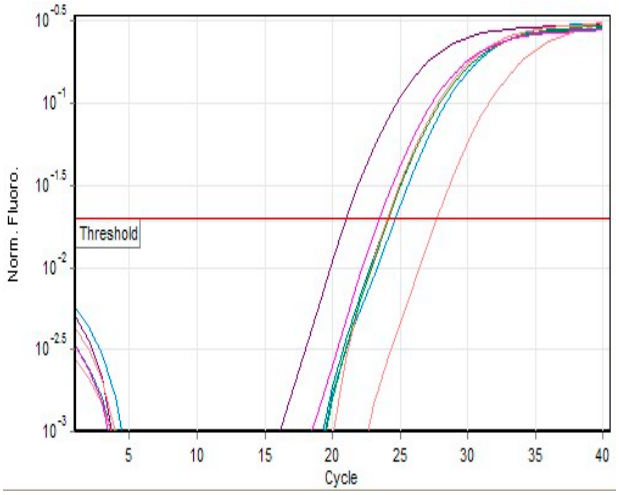
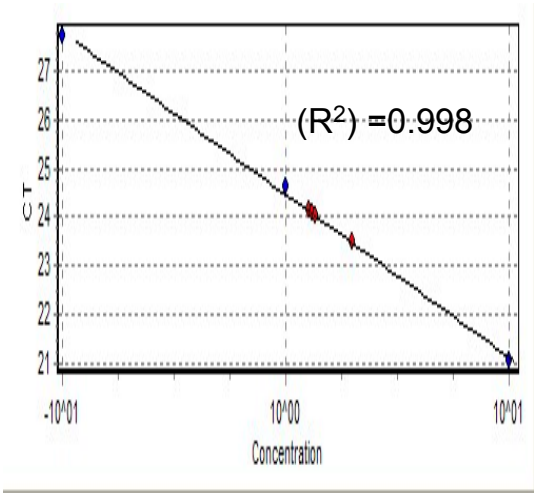


Appendix II. pGEM-DENV-2CAP

A



B

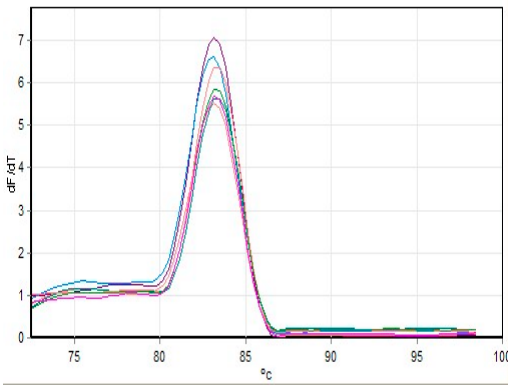


C

	RNA	Ct	Calculated concentration (ng/reaction)
Standards in ng	10	21.04	10.553208
	1	24.61	.897906
	0.1	27.71	.105532
1 ng DENV infected MDM RNA	day 0	24.06	1.316502
	day 1	24.02	1.351594
	day 2	24.11	1.267649
	day 3	23.46	1.987405

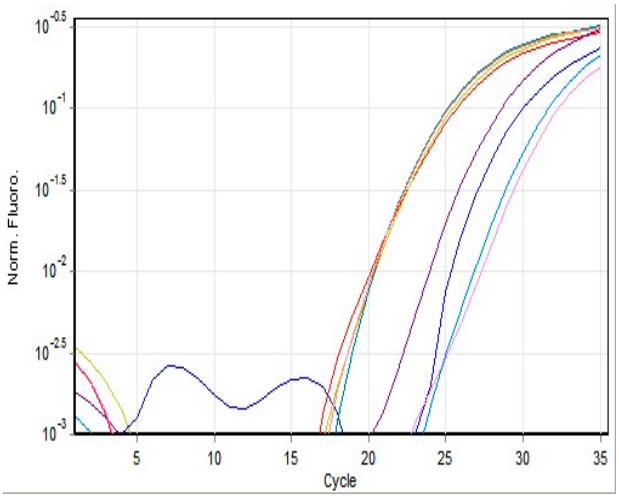
Standards = MDM total RNA

D

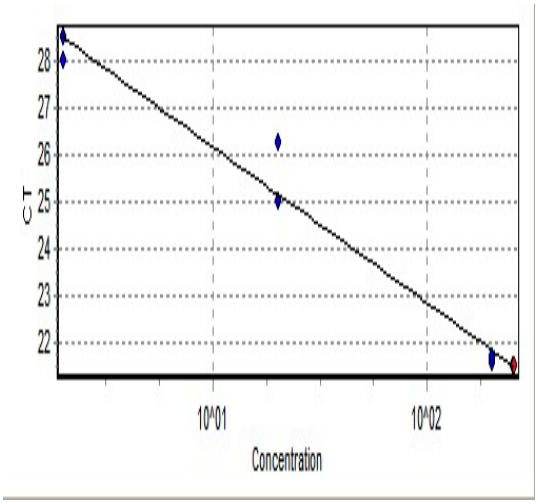


Appendix III. Cyclophilin A mRNA does not vary in DENV infected MDM over 3 days. Quantitation curves **(A)** and calculated copy numbers **(C)** based on standard curve **(B)** are shown for RT real-time PCR of 1 ng total RNA extracted from DENV infected MDM over three days . Melting curves **(D)** show one major peak at 82^0C.

A

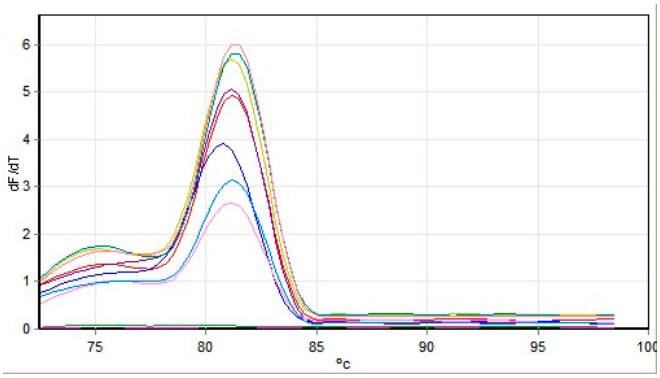


B



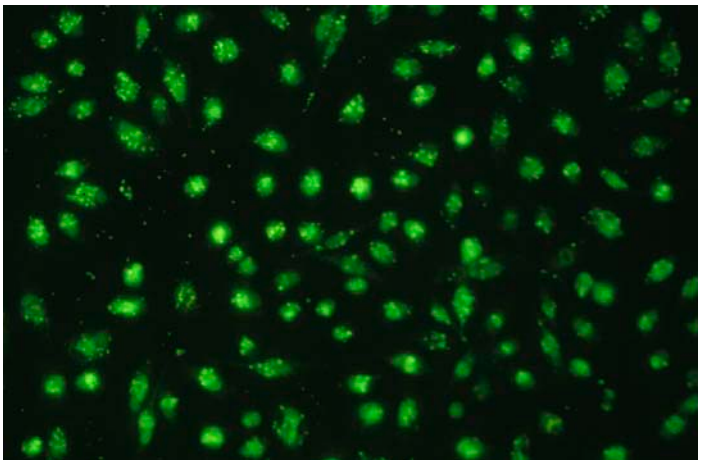
	C	RNA	Ct	Calculated concentration ng/reaction
Standards in pg		0.02	21.52	246.129541
		0.02	21.63	226.886185
		0.002	26.00	18.324981
		0.002	25.02	22.008398
		0.0002	28.53	1.967946
		0.0002	28.00	2.837649
		sample 1(2 pg)	21.47	254.894513
		sample 1(2 pg)	21.48	251.998691
		water control	>35	
		water control	>35	

D

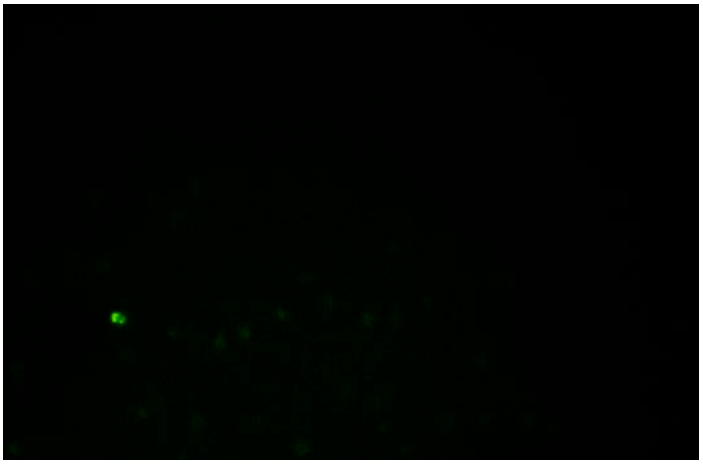


Appendix IV. TNF-α mRNA RT real-time PCR quantitation. Quantitation curves **(A)** and calculated TNF-α mRNA ng/reaction **(C)** based on standard curve **(B)** are shown for RT real-time PCR of RNA extracted from LPS stimulated macrophages (standards) and DENV infected macrophages 32 hrs after infection (sample 1). Melting curves **(D)** show one peak at 81.7^{0}C.

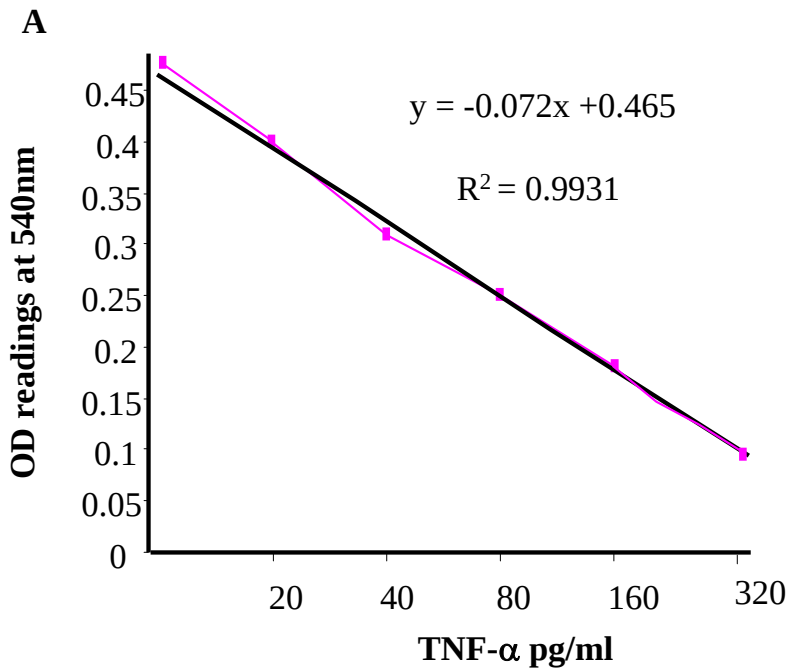
A

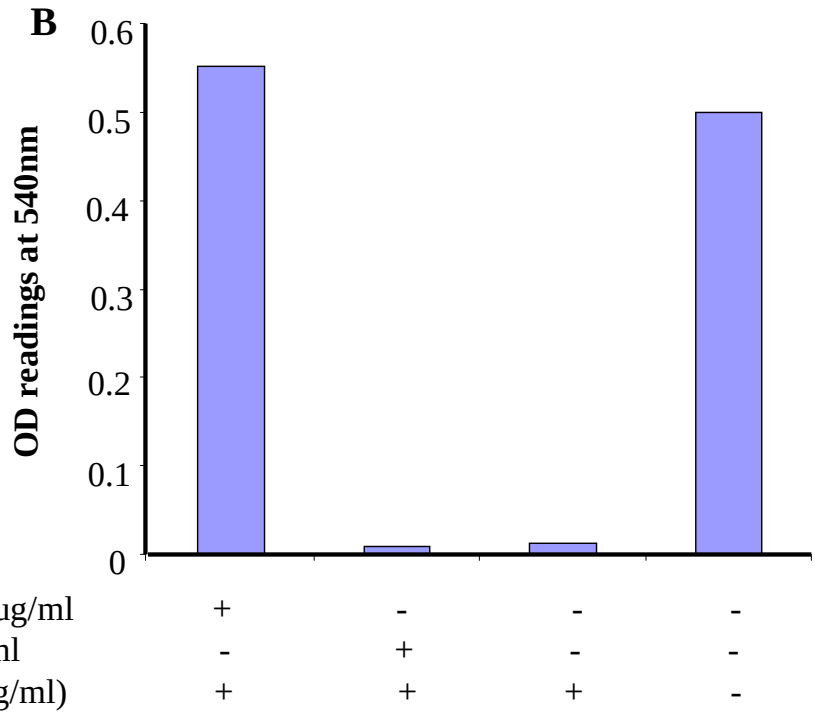


B

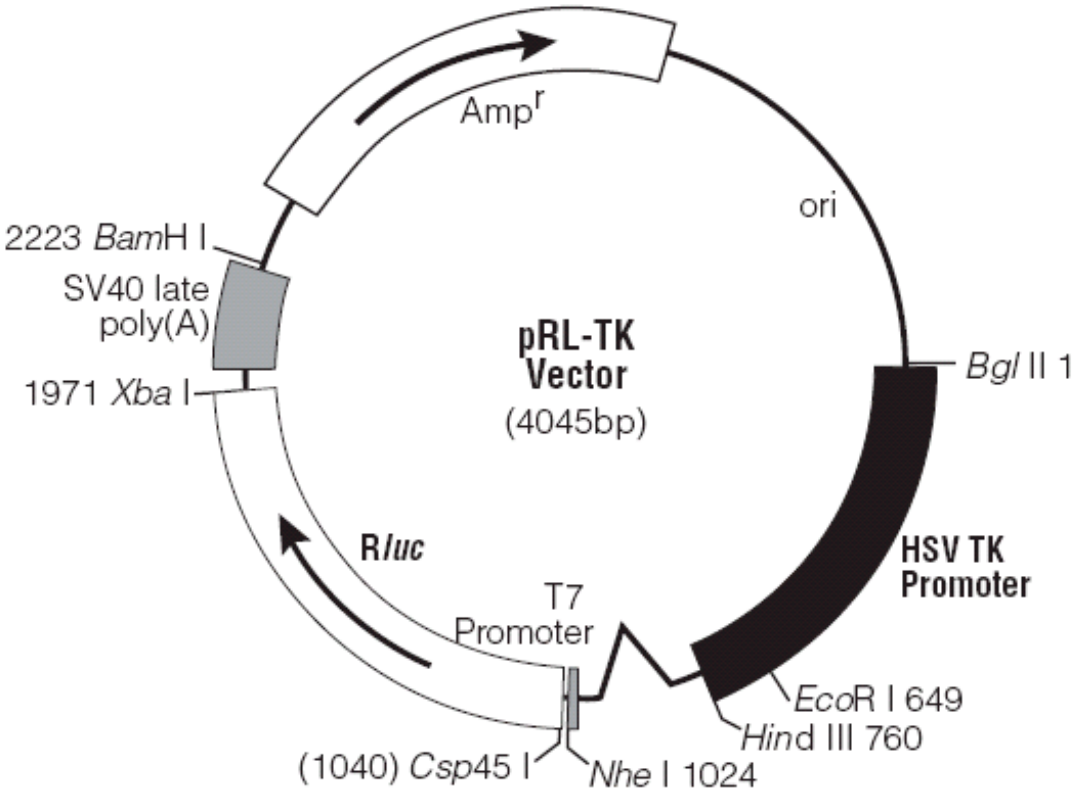


Appendix V. **Greater than 90% transfection efficiency was observed using Block-it™ flourescent labelled oligonucleotides (Invitrogen) in MDM.** MDM were transfected with 20 pmole of fluorescent labelled oligonucleotides and lipofectamine **(A)** > 90 % of cells fluoresced when observed under a fluorescence microscope. **(B)** Negative control – MDM not transfected with fluorescent labelled oligonucleotides.

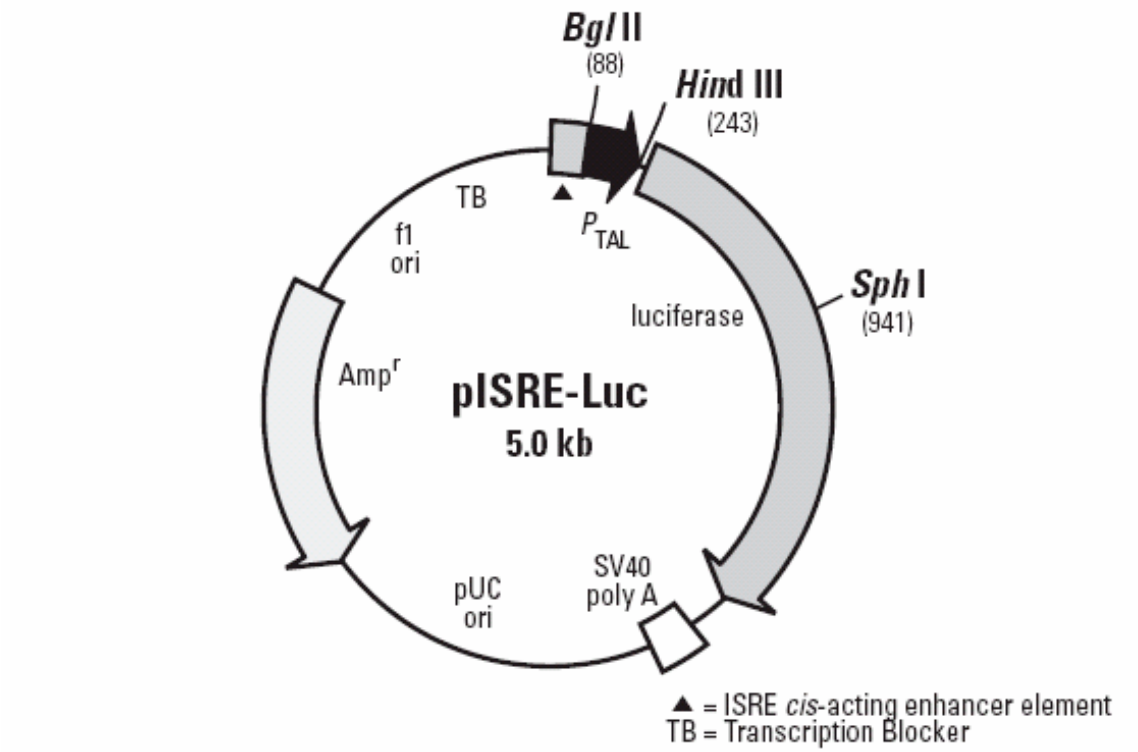




Appendix VI. (A) L929 TNF-α Bioassay. Standard curve of a TNF-α cytotoxic bioassay using L929 fibroblast cell lines. Various concentrations of TNF-α in the presence of actinomycin D were incubated with the L929 cells. After 24 hrs incubation, the viable cells were stained using crystal violet. High OD's indicate minimal effects of TNF-α. **(B) Inhibition of TNF-α activity by 2.5 μg/ml of TNF antibody.**



Appendix VII. pRL-TK map (Promega)



Appendix VIII. pISRE-Luc (Clonetech Laboratories)

Index	Accession	Mass	Score	Description
1.	GRP78_HUMAN	72402	242	78 kDa glucose-regulated protein precursor (GRP 78) (Heat shock 70 kDa protein 5) (Immunoglobulin heavy chain-binding protein) (BiP) (Endoplasmic reticulum lum
2.	PRS10_HUMAN	44430	47	26S protease regulatory subunit S10B (Proteasome 26S subunit ATPase 6) (Proteasome subunit p42) - Homo sapiens (Human)
3.	LRC29_HUMAN	24352	46	Leucine-rich repeat-containing protein 29 (F-box/LRR-repeat protein 9) (F-box and leucine-rich repeat protein 9) (F-box protein FBL9) - Homo sapiens (Human)
4.	MYH9_HUMAN	227515	43	Myosin-9 (Myosin heavy chain, nonmuscle IIa) (Nonmuscle myosin heavy chain IIa) (NMMHC II-a) (NMMHC-IIA) (Cellular myosin heavy chain, type A) (Nonmuscle myosi
5.	ATM_HUMAN	355494	43	Serine-protein kinase ATM (EC 2.7.11.1) (Ataxia telangiectasia mutated) (A-T, mutated) - Homo sapiens (Human)
6.	IF32_HUMAN	36878	40	Eukaryotic translation initiation factor 3 subunit 2 (eIF-3 beta) (eIF3 p36) (eIF3i) (TGF-beta receptor-interacting protein 1) (TRIP-1) - Homo sapiens (Human)
7.	OFD1_HUMAN	117055	40	Oral-facial-digital syndrome 1 protein (Protein 71-7A) - Homo sapiens (Human)
8.	MK04_HUMAN	63040	38	Mitogen-activated protein kinase 4 (EC 2.7.11.24) (Extracellular signal-regulated kinase 4) (ERK-4) (MAP kinase isoform p63) (p63-MAPK) - Homo sapiens (Human)
9.	COMD1_HUMAN	21222	38	COMM domain-containing protein 1 (Protein Murr1) - Homo sapiens (Human)
10.	MRP1_HUMAN	172877	37	Multidrug resistance-associated protein 1 (ATP-binding cassette sub-family C member 1) (Leukotriene C(4) transporter) (LTC4 transporter) - Homo sapiens (Human)
11.	APC_HUMAN	313622	37	Adenomatous polyposis coli protein (Protein APC) - Homo sapiens (Human)
12.	SNPH_HUMAN	58752	36	Syntaphilin - Homo sapiens (Human)
13.	CCD38_HUMAN	65445	36	Coiled-coil domain-containing protein 38 - Homo sapiens (Human)
14.	GBP1_HUMAN	68373	36	Interferon-induced guanylate-binding protein 1 (GTP-binding protein 1) (Guanine nucleotide-binding protein 1) (GBP-1) (HuGBP-1) - Homo sapiens (Human)
15.	DYN3_HUMAN	97021	36	Dynamin-3 (EC 3.6.5.5) (Dynamin, testicular) (T-dynamin) - Homo sapiens (Human)
16.	TGM4_HUMAN	77951	36	Protein-glutamine gamma-glutamyltransferase 4 (EC 2.3.2.13) (Transglutaminase-4) (TGase-4) (Prostate transglutaminase) (TGP) (TG(P)) (Prostate-specific transgl
17.	FRMD7_HUMAN	82417	35	FERM domain-containing protein 7 - Homo sapiens (Human)
18.	NTAL_HUMAN	26819	35	Linker for activation of T-cells family member 2 (Non-T-cell activation linker) (Linker for activation of B-cells) (Membrane-associated adapter molecule) (Will
19.	AURKB_HUMAN	39541	34	Serine/threonine-protein kinase 12 (EC 2.7.11.1) (Aurora-B) (Aurora- and Ipl1-like midbody-associated protein 1) (AIM-1) (Aurora/IPL1-related kinase 2) (Aurora
20.	ZN623_HUMAN	63065	34	Zinc finger protein 623 - Homo sapiens (Human)

Appendix IX. Top 20 data base matches for MS results. Any mouse score >54 indicates a significant match. The highest and only significant data base match is for GRP78.

Results List

Observed	Mr(expt)	Mr(calc)	Delta	Start	End	Miss	Peptide
986.4902	985.4829	985.5080	-0.0251	533	540	0	R.LTPEEIER.M
997.4893	996.4820	996.5101	-0.0280	298	306	0	R.ALSSQHQAR.I
1106.6314	1105.6241	1105.6535	-0.0294	114	122	1	K.FLPFKVVEK.K
1153.5848	1152.5775	1152.6112	-0.0337	297	306	1	K.RALSSQHQAR.I
1191.6193	1190.6120	1190.6295	-0.0175	465	474	0	K.VYEGERPLTK.D
1210.5681	1209.5608	1209.5778	-0.0169	377	386	1	K.EFFNGKEPSR.G
1233.6123	1232.6050	1232.6183	-0.0133	186	197	0	K.DAGTIAGLNVMR.I + Oxidation (M)
1329.5897	1328.5824	1328.6070	-0.0247	327	336	0	K.FEELNMDLFR.S + Oxidation (M)
1397.7602	1396.7529	1396.7813	-0.0284	622	633	0	K.ELEEIVQPIISK.L
1460.7264	1459.7191	1459.7518	-0.0327	354	367	0	K.SDIDEIVLVGGSTR.I
1512.7170	1511.7097	1511.7442	-0.0344	325	336	1	R.AKFEELNMDLFR.S
1528.7172	1527.7099	1527.7391	-0.0292	325	336	1	R.AKFEELNMDLFR.S + Oxidation (M)
1552.7581	1551.7508	1551.7854	-0.0346	139	152	0	K.TFAPEEISAMVLTK.M + Oxidation (M)
1555.7581	1554.7509	1554.7862	-0.0353	47	60	1	K.NGRVEIIANDQGNR.I
1566.7553	1565.7481	1565.7725	-0.0245	61	74	0	R.ITPSYVAFTPEGER.L
1588.8101	1587.8028	1587.8467	-0.0439	353	367	1	K.KSDIDEIVLVGGSTR.I
1604.8269	1603.8196	1603.8569	-0.0373	124	138	0	K.TKPYIQVDIGGGQTK.T
1661.8232	1660.8159	1660.8566	-0.0407	182	197	1	R.QATKDAGTIAGLNVMR.I + Oxidation (M)
1677.7804	1676.7731	1676.8005	-0.0275	82	96	0	K.NQLTSNPENTVFDAK.R
1732.9038	1731.8965	1731.9519	-0.0553	123	138	1	K.KTKPYIQVDIGGGQTK.T
1801.8584	1800.8511	1800.8893	-0.0383	559	573	1	R.IDTRNELESYAYSLK.N
1815.9622	1814.9549	1814.9890	-0.0341	198	214	1	R.IINEPTAAAIAYGLDKR.E
1833.8811	1832.8739	1832.9016	-0.0278	82	97	1	K.NQLTSNPENTVFDAKR.L
1887.9361	1886.9288	1886.9638	-0.0350	165	181	0	K.VTHAVVTVPAYFNDAQR.Q
1933.9850	1932.9777	1933.0057	-0.0280	475	492	0	K.DNHLLGTFDLTGIPPAPR.G
1999.0533	1998.0461	1998.0785	-0.0324	493	510	0	R.GVPQIEVTFEIDVNGILR.V
2016.0365	2015.0293	2015.0588	-0.0295	164	181	1	K.KVTHAVVTVPAYFNDAQR.Q
2164.9550	2163.9477	2163.9847	-0.0370	307	324	0	R.IEIESFYEGEDFSETLTR.A
2175.9499	2174.9427	2174.9855	-0.0428	634	654	1	K.LYGSAGPPPTGEEDTAEKDEL.-
2235.1327	2234.1254	2234.1582	-0.0328	61	81	1	R.ITPSYVAFTPEGERLIGDAAK.N
2259.2136	2258.2063	2258.2191	-0.0128	3	23	1	K.LSLVAAMLLLLSAARAEEEDK.K + Oxidation (M)
3009.4465	3008.4392	3008.5454	-0.1062	448	474	1	K.SQIFSTASDNQPTVTIKVYEGERPLTK.D
3106.5071	3105.4998	3105.6246	-0.1248	465	492	1	K.VYEGERPLTKDNHLLGTFDLTGIPPAPR.G

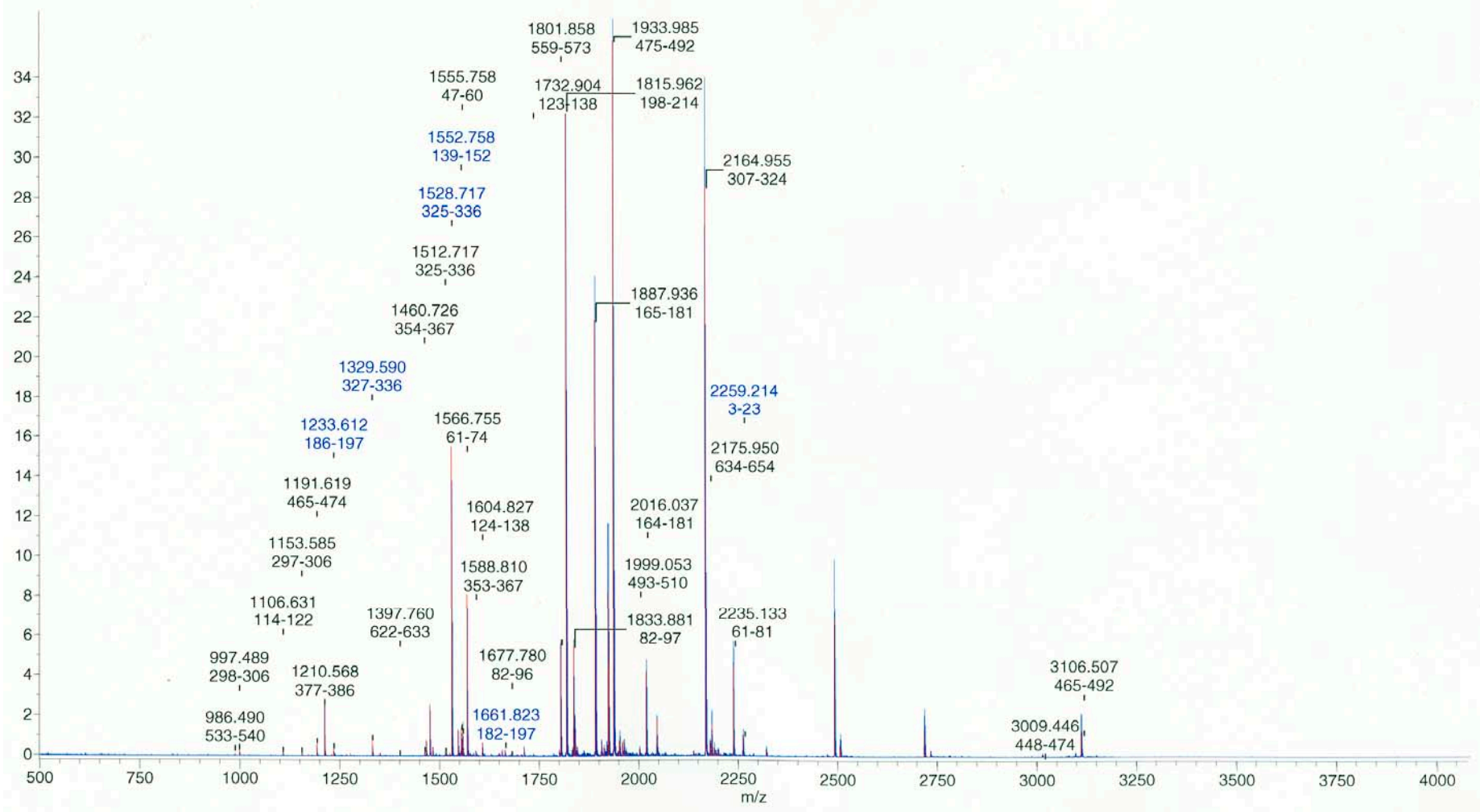
No match to: 1169.5749, 1211.5247, 1227.5729, 1265.6174, 1274.9317, 1348.6375, 1464.7268, 1465.2004, 1473.9729, 1480.7283, 1488.7578, 1515.7850, 1523.7083, 1544.7135, 1550.7164, 1556.7233, 1573.7150, 1582.7512, 1626.8050, 1647.7808, 1653.9381, 1691.8249, 1701.8459, 1709.8243, 1797.9468, 1835.8770, 1837.9267, 1841.9687, 1901.9349, 1903.9366, 1909.9224, 1919.9913, 1921.9868, 1939.9728, 1947.9828, 1949.9819, 1955.9609, 1959.9998, 1965.9815, 2032.0235, 2043.0128, 2134.9448, 2146.9528, 2178.9442, 2180.9557, 2186.9382, 2190.9725, 2196.0345, 2197.9234,

Appendix X. Molecular weight of peptides identified by mass spectrum analysis and the respective sequence.

2211.0565, 2275.1874, 2317.1550, 2417.1878, 2469.2243, 2488.2594, 2502.2338, 2504.2391, 2510.2155, 2514.2721, 2714.2783, 2716.2442, 2716.2486, 2730.2508, 3074.3362, 3091.8693, 3144.3580

Search Parameters

Type of search	: Peptide Mass Fingerprint
Enzyme	: Trypsin
Fixed modifications	: Carbamidomethyl (C)
Variable modifications	: Oxidation (M)
Mass values	: Monoisotopic
Protein Mass	: Unrestricted
Peptide Mass Tolerance	: ± 100 ppm
Peptide Charge State	: 1+
Max Missed Cleavages	: 1
Number of queries	: 99



Appendix XI. Spectrum analysis report. The peaks highlighted in the spectrum represent those that were matched to the identified protein based on the MS data. Sequence name (P11021) GRP78. Number of peaks 99.

REFERENCES

Aaskov, J. (2003) Dengue. Infectious Diseases, ADF Health 2003(4), 66-71.

Aderka, D., Engelmann, H., Maor, Y., Brakebusch, C. and Wallach, D. (1992) Stabilization of the bioactivity of tumor necrosis factor by its soluble receptors. J Exp Med 175(2), 323-9.

Alcon-LePoder, S., Drouet, M.T., Roux, P., Frenkiel, M.P., Arborio, M., Durand-Schneider, A.M., Maurice, M., Le Blanc, I., Gruenberg, J. and Flamand, M. (2005) The secreted form of dengue virus nonstructural protein NS1 is endocytosed by hepatocytes and accumulates in late endosomes: implications for viral infectivity. J Virol 79(17), 11403-11.

Alvarez, D.E., Lodeiro, M.F., Luduena, S.J., Pietrasanta, L.I. and Gamarnik, A.V. (2005) Long-range RNA-RNA interactions circularize the dengue virus genome. J Virol 79(11), 6631-43.

An, J., Kimura-Kuroda, J., Hirabayashi, Y. and Yasui, K. (1999) Development of a novel mouse model for dengue virus infection. Virology 263(1), 70-7.

An, J., Zhou, D.S., Zhang, J.L., Morida, H., Wang, J.L. and Yasui, K. (2004) Dengue-specific CD8+ T cells have both protective and pathogenic roles in dengue virus infection. Immunol Lett 95(2), 167-74.

Anderson, L. and Seilhamer, J. (1997) A comparison of selected mRNA and protein abundances in human liver. Electrophoresis 18(3-4), 533-7.

Anderson, R., King, A.D. and Innis, B.L. (1992) Correlation of E protein binding with cell susceptibility to dengue 4 virus infection. J Gen Virol 73 (Pt 8), 2155-9.

Anderson, R., Wang, S., Osiowy, C. and Issekutz, A.C. (1997a) Activation of endothelial cells via antibody-enhanced dengue virus infection of peripheral blood monocytes. Journal of Virology 71(6), 4226-32.

Anderson, R., Wang, S., Osiowy, C. and Issekutz, A.C. (1997b) Activation of endothelial cells via antibody-enhanced dengue virus infection of peripheral blood monocytes. J Virol 71(6), 4226-32.

Andersson, L.C., Nilsson, K. and Gahmberg, C.G. (1979) K562--a human erythroleukemic cell line. Int J Cancer 23(2), 143-7.

Andrews, B.S., Theofilopoulos, A.N., Peters, C.J., Loskutoff, D.J., Brandt, W.E. and Dixon, F.J. (1978) Replication of dengue and junin viruses in cultured rabbit and human endothelial cells. Infect Immun 20(3), 776-81.

Arias, C.F., Preugschat, F. and Strauss, J.H. (1993) Dengue 2 virus NS2B and NS3 form a stable complex that can cleave NS3 within the helicase domain. Virology 193(2), 888-99.

Atrasheuskaya, A., Petzelbauer, P., Fredeking, T.M. and Ignatyev, G. (2003) Anti-TNF antibody treatment reduces mortality in experimental dengue virus infection. FEMS Immunol Med Microbiol 35(1), 33-42.

Avirutnan, P., Malasit, P., Seliger, B., Bhakdi, S. and Husmann, M. (1998) Dengue virus infection of human endothelial cells leads to chemokine production, complement activation, and apoptosis. Journal of Immunology 161(11), 6338-46.

Avirutnan, P., Punyadee, N., Noisakran, S., Komoltri, C., Thiemmeca, S., Auethavornanan, K., Jairungsri, A., Kanlaya, R., Tangthawornchaikul, N., Puttikhunt, C., Pattanakitsakul, S.N., Yenchitsomanus, P.T., Mongkolsapaya, J., Kasinrerk, W., Sittisombut, N., Husmann, M., Blettner, M., Vasanawathana, S., Bhakdi, S. and Malasit, P. (2006) Vascular leakage in severe dengue virus infections: a potential role for the nonstructural viral protein NS1 and complement. J Infect Dis 193(8), 1078-88.

Balmaseda, A., Hammond, S.N., Perez, L., Tellez, Y., Saborio, S.I., Mercado, J.C., Cuadra, R., Rocha, J., Perez, M.A., Silva, S., Rocha, C. and Harris, E. (2006) Serotype-specific differences in clinical manifestations of dengue. Am J Trop Med Hyg 74(3), 449-56.

Bandyopadhyay, S., Lum, L.C. and Kroeger, A. (2006) Classifying dengue: a review of the difficulties in using the WHO case classification for dengue haemorrhagic fever. Trop Med Int Health 11(8), 1238-55.

Barry, R.C., Alsaker, B.L., Robison-Cox, J.F. and Dratz, E.A. (2003) Quantitative evaluation of sample application methods for semipreparative separations of basic proteins by two-dimensional gel electrophoresis. Electrophoresis 24(19-20), 3390-404.

Bartelma, G. and Padmanabhan, R. (2002) Expression, purification, and characterization of the RNA 5'-triphosphatase activity of dengue virus type 2 nonstructural protein 3. Virology 299(1), 122-32.

Benali-Furet, N.L., Chami, M., Houel, L., De Giorgi, F., Vernejoul, F., Lagorce, D., Buscail, L., Bartenschlager, R., Ichas, F., Rizzuto, R. and Paterlini-Brechot, P. (2005) Hepatitis C virus core triggers apoptosis in liver cells by inducing ER stress and ER calcium depletion. Oncogene 24(31), 4921-33.

Benarroch, D., Selisko, B., Locatelli, G.A., Maga, G., Romette, J.L. and Canard, B. (2004) The RNA helicase, nucleotide 5'-triphosphatase, and RNA 5'-triphosphatase activities of Dengue virus protein NS3 are Mg2+-dependent and require a functional Walker B motif in the helicase catalytic core. Virology 328(2), 208-18.

Bente, D.A., Melkus, M.W., Garcia, J.V. and Rico-Hesse, R. (2005) Dengue fever in humanized NOD/SCID mice. J Virol 79(21), 13797-9.

Bente, D.A. and Rico-Hesse, R. (2006) Models of dengue virus infection. Drug Discov Today Dis Models 3(1), 97-103.

Bethell, D.B., Flobbe, K., Cao, X.T., Day, N.P., Pham, T.P., Buurman, W.A., Cardosa, M.J., White, N.J. and Kwiatkowski, D. (1998) Pathophysiologic and prognostic role of cytokines in dengue hemorrhagic fever. J Infect Dis 177(3), 778-82.

Bjork, J., Hugli, T.E. and Smedegard, G. (1985) Microvascular effects of anaphylatoxins C3a and C5a. J Immunol 134(2), 1115-9.

Boeuf, P., Vigan-Womas, I., Jublot, D., Loizon, S., Barale, J.C., Akanmori, B.D., Mercereau-Puijalon, O. and Behr, C. (2005) CyProQuant-PCR: a real time RT-PCR technique for profiling human cytokines, based on external RNA standards, readily automatable for clinical use. BMC Immunol 6(1), 5.

Bokisch, V.A., Top, F.H., Jr., Russell, P.K., Dixon, F.J. and Muller-Eberhard, H.J. (1973) The potential pathogenic role of complement in dengue hemorrhagic shock syndrome. N Engl J Med 289(19), 996-1000.

Breiman, A., Grandvaux, N., Lin, R., Ottone, C., Akira, S., Yoneyama, M., Fujita, T., Hiscott, J. and Meurs, E.F. (2005) Inhibition of RIG-I-dependent signaling to the interferon pathway during hepatitis C virus expression and restoration of signaling by IKKepsilon. J Virol 79(7), 3969-78.

Brinton, M.A. and Dispoto, J.H. (1988) Sequence and secondary structure analysis of the 5'-terminal region of flavivirus genome RNA. Virology 162(2), 290-9.

Broquet, A.H., Lenoir, C., Gardet, A., Sapin, C., Chwetzoff, S., Jouniaux, A.M., Lopez, S., Trugnan, G., Bachelet, M. and Thomas, G. (2007) Hsp70 negatively controls rotavirus protein bioavailability in caco-2 cells infected by the rotavirus RF strain. J Virol 81(3), 1297-304.

Buchkovich, N.J., Maguire, T.G., Yu, Y., Paton, A.W., Paton, J.C. and Alwine, J.C. (2007) HCMV specifically controls the levels of the endoplasmic reticulum chaperone BiP/GRP78 which is required for virion assembly. J Virol.

Bukowski, J.F., Kurane, I., Lai, C.J., Bray, M., Falgout, B. and Ennis, F.A. (1989) Dengue virus-specific cross-reactive CD8+ human cytotoxic T lymphocytes. Journal of Virology 63(12), 5086-91.

Burke, D.S., Nisalak, A., Johnson, D.E. and Scott, R.M. (1988) A prospective study of dengue infections in Bangkok. Am J Trop Med Hyg 38(1), 172-80.

Cabrera-Hernandez, A., Thepparit, C., Suksanpaisan, L. and Smith, D.R. (2007) Dengue virus entry into liver (HepG2) cells is independent of hsp90 and hsp70. J Med Virol 79(4), 386-92.

Campostrini, N., Areces, L.B., Rappsilber, J., Pietrogrande, M.C., Dondi, F., Pastorino, F., Ponzoni, M. and Righetti, P.G. (2005) Spot overlapping in two-dimensional maps: a serious problem ignored for much too long. Proteomics 5(9), 2385-95.

Cardier, J.E., Marino, E., Romano, E., Taylor, P., Liprandi, F., Bosch, N. and Rothman, A.L. (2005) Proinflammatory factors present in sera from patients with acute dengue infection induce activation and apoptosis of human microvascular endothelial cells: possible role of TNF-alpha in endothelial cell damage in dengue. Cytokine 30(6), 359-65.

Carr, J.M., Hocking, H., Bunting, K., Wright, P.J., Davidson, A., Gamble, J., Burrell, C.J. and Li, P. (2003) Supernatants from dengue virus type-2 infected macrophages induce permeability changes in endothelial cell monolayers. J Med Virol 69(4), 521-8.

Chambers, T.J., Hahn, C.S., Galler, R. and Rice, C.M. (1990) Flavivirus genome organization, expression, and replication. Annu Rev Microbiol 44, 649-88.

Chang, T.H., Liao, C.L. and Lin, Y.L. (2006) Flavivirus induces interferon-beta gene expression through a pathway involving RIG-I-dependent IRF-3 and PI3K-dependent NF-kappaB activation. Microbes Infect 8(1), 157-71.

Chareonsirisuthigul, T., Kalayanarooj, S. and Ubol, S. (2007) Dengue virus (DENV) antibody-dependent enhancement of infection upregulates the production of anti-inflammatory cytokines, but suppresses anti-DENV free radical and pro-inflammatory cytokine production, in THP-1 cells. J Gen Virol 88(Pt 2), 365-75.

Charnsilpa, W., Takhampunya, R., Endy, T.P., Mammen, M.P., Jr., Libraty, D.H. and Ubol, S. (2005) Nitric oxide radical suppresses replication of wild-type dengue 2 viruses in vitro. J Med Virol 77(1), 89-95.

Chaturvedi, U.C., Agarwal, R., Elbishbishi, E.A. and Mustafa, A.S. (2000) Cytokine cascade in dengue hemorrhagic fever: implications for pathogenesis. FEMS Immunol Med Microbiol 28(3), 183-8.

Chaturvedi, U.C., Nagar, R. and Mathur, A. (1983) Effect of dengue virus infection on Fc-receptor functions of mouse macrophages. Journal of General Virology 64(Pt 11), 2399-407.

Chen, H.C., Hofman, F.M., Kung, J.T., Lin, Y.D. and Wu-Hsieh, B.A. (2007) Both virus and tumor necrosis factor alpha are critical for endothelium damage in a mouse model of dengue virus-induced hemorrhage. J Virol 81(11), 5518-26.

Chen, Y., Maguire, T. and Marks, R.M. (1996) Demonstration of binding of dengue virus envelope protein to target cells. Journal of Virology 70(12), 8765-72.

Chen, Y.C. and Wang, S.Y. (2002) Activation of terminally differentiated human monocytes/macrophages by dengue virus: productive infection, hierarchical production of innate cytokines and chemokines, and the synergistic effect of lipopolysaccharide. J Virol 76(19), 9877-87.

Chen, Y.C., Wang, S.Y. and King, C.C. (1999) Bacterial lipopolysaccharide inhibits dengue virus infection of primary human monocytes/macrophages by blockade of virus entry via a CD14-dependent mechanism. Journal of Virology 73(4), 2650-7.

Cheng, Y., King, N.J. and Kesson, A.M. (2004) The role of tumor necrosis factor in modulating responses of murine embryo fibroblasts by flavivirus, West Nile. Virology 329(2), 361-70.

Chisari, F.V. (2000) Rous-Whipple Award Lecture. Viruses, immunity, and cancer: lessons from hepatitis B. Am J Pathol 156(4), 1117-32.

Choi, S.H., Park, K.J., Ahn, B.Y., Jung, G., Lai, M.M. and Hwang, S.B. (2006) Hepatitis C virus nonstructural 5B protein regulates tumor necrosis factor alpha signaling through effects on cellular IkappaB kinase. Mol Cell Biol 26(8), 3048-59.

Choukhi, A., Ung, S., Wychowski, C. and Dubuisson, J. (1998) Involvement of endoplasmic reticulum chaperones in the folding of hepatitis C virus glycoproteins. J Virol 72(5), 3851-8.

Chu, P.W. and Westaway, E.G. (1985) Replication strategy of Kunjin virus: evidence for recycling role of replicative form RNA as template in semiconservative and asymmetric replication. Virology 140(1), 68-79.

Chua, J.J., Ng, M.M. and Chow, V.T. (2004) The non-structural 3 (NS3) protein of dengue virus type 2 interacts with human nuclear receptor binding protein and is associated with alterations in membrane structure. Virus Res 102(2), 151-63.

Churdboonchart, V., Bhamarapravati, N., Peampramprecha, S. and Sirinavin, S. (1991) Antibodies against dengue viral proteins in primary and secondary dengue hemorrhagic fever. Am J Trop Med Hyg 44(5), 481-93.

Cleaves, G.R., Ryan, T.E. and Schlesinger, R.W. (1981) Identification and characterization of type 2 dengue virus replicative intermediate and replicative form RNAs. Virology 111(1), 73-83.

Clyde, K., Kyle, J.L. and Harris, E. (2006) Recent advances in deciphering viral and host determinants of dengue virus replication and pathogenesis. J Virol 80(23), 11418-31.

Cologna, R. and Rico-Hesse, R. (2003) American genotype structures decrease dengue virus output from human monocytes and dendritic cells. J Virol 77(7), 3929-38.

Couvelard, A., Marianneau, P., Bedel, C., Drouet, M.T., Vachon, F., Henin, D. and Deubel, V. (1999) Report of a fatal case of dengue infection with hepatitis: demonstration of dengue antigens in hepatocytes and liver apoptosis. Hum Pathol 30(9), 1106-10.

Cseh, K. and Beutler, B. (1989) Alternative cleavage of the cachectin/tumor necrosis factor propeptide results in a larger, inactive form of secreted protein. J Biol Chem 264(27), 16256-60.

Daughaday, C.C., Brandt, W.E., McCown, J.M. and Russell, P.K. (1981) Evidence for two mechanisms of dengue virus infection of adherent human monocytes: trypsin-sensitive virus receptors and trypsin-resistant immune complex receptors. Infect Immun 32(2), 469-73.

Deen, J.L., Harris, E., Wills, B., Balmaseda, A., Hammond, S.N., Rocha, C., Dung, N.M., Hung, N.T., Hien, T.T. and Farrar, J.J. (2006) The WHO dengue classification and case definitions: time for a reassessment. Lancet 368(9530), 170-3.

Diamond, M.S., Edgil, D., Roberts, T.G., Lu, B. and Harris, E. (2000a) Infection of human cells by dengue virus is modulated by different cell types and viral strains. J Virol 74(17), 7814-23.

Diamond, M.S. and Harris, E. (2001) Interferon inhibits dengue virus infection by preventing translation of viral RNA through a PKR-independent mechanism. Virology 289(2), 297-311.

Diamond, M.S., Roberts, T.G., Edgil, D., Lu, B., Ernst, J. and Harris, E. (2000b) Modulation of Dengue virus infection in human cells by alpha, beta, and gamma interferons. J Virol 74(11), 4957-66.

Earl, P.L., Moss, B. and Doms, R.W. (1991) Folding, interaction with GRP78-BiP, assembly, and transport of the human immunodeficiency virus type 1 envelope protein. J Virol 65(4), 2047-55.

Egloff, M.P., Benarroch, D., Selisko, B., Romette, J.L. and Canard, B. (2002) An RNA cap (nucleoside-2'-O-)-methyltransferase in the flavivirus RNA polymerase NS5: crystal structure and functional characterization. Embo J 21(11), 2757-68.

Ekkapongpisit, M., Wannatung, T., Susantad, T., Triwitayakorn, K. and Smith, D.R. (2007) cDNA-AFLP analysis of differential gene expression in human hepatoma cells (HepG2) upon dengue virus infection. J Med Virol 79(5), 552-61.

Lanford, R.E., Sureau, C., Jacob, J.R., White, R. and Fuerst, T.R. (1994) Demonstration of in vitro infection of chimpanzee hepatocytes with hepatitis C virus using strand-specific RT/PCR. Virology 202(2), 606-14.

Lee, D.H., Tam, S.S., Benyoucef, S., De Groote, D., Deubel, V. and Wattre, P. (1996) Enhanced TNF alpha production by monocytic-like cells exposed to dengue virus antigens. Immunol Lett 53(2-3), 115-20.

Lerat, H., Berby, F., Trabaud, M.A., Vidalin, O., Major, M., Trepo, C. and Inchauspe, G. (1996) Specific detection of hepatitis C virus minus strand RNA in hematopoietic cells. J Clin Invest 97(3), 845-51.

Li, H., Clum, S., You, S., Ebner, K.E. and Padmanabhan, R. (1999) The serine protease and RNA-stimulated nucleoside triphosphatase and RNA helicase functional domains of dengue virus type 2 NS3 converge within a region of 20 amino acids. J Virol 73(4), 3108-16.

Liao, G., Simone, J. and Simon, S.R. (1994) Paracrine downregulation of Fc gamma RIII in human monocyte-derived macrophages induced by phagocytosis of nonopsonized particles. Blood 83(8), 2294-304.

Libraty, D.H., Endy, T.P., Houng, H.S., Green, S., Kalayanarooj, S., Suntayakorn, S., Chansiriwongs, W., Vaughn, D.W., Nisalak, A., Ennis, F.A. and Rothman, A.L. (2002a) Differing influences of virus burden and immune activation on disease severity in secondary dengue-3 virus infections. J Infect Dis 185(9), 1213-21.

Libraty, D.H., Pichyangkul, S., Ajariyakhajorn, C., Endy, T.P. and Ennis, F.A. (2001) Human dendritic cells are activated by dengue virus infection: enhancement by gamma interferon and implications for disease pathogenesis. J Virol 75(8), 3501-8.

Libraty, D.H., Young, P.R., Pickering, D., Endy, T.P., Kalayanarooj, S., Green, S., Vaughn, D.W., Nisalak, A., Ennis, F.A. and Rothman, A.L. (2002b) High circulating levels of the dengue virus nonstructural protein NS1 early in dengue illness correlate with the development of dengue hemorrhagic fever. J Infect Dis 186(8), 1165-8.

Eram, S., Setyabudi, Y., Sadono, T.I., Sutrisno, D.S., Gubler, D.J. and Sulianti Saroso, J. (1979) Epidemic dengue hemorrhagic fever in rural Indonesia. II. Clinical studies. Am J Trop Med Hyg 28(4), 711-6.

Espina, L.M., Valero, N.J., Hernandez, J.M. and Mosquera, J.A. (2003) Increased apoptosis and expression of tumor necrosis factor-alpha caused by infection of cultured human monocytes with dengue virus. Am J Trop Med Hyg 68(1), 48-53.

Falconar, A.K. (1997) The dengue virus nonstructural-1 protein (NS1) generates antibodies to common epitopes on human blood clotting, integrin/adhesin proteins and binds to human endothelial cells: potential implications in haemorrhagic fever pathogenesis. Arch Virol 142(5), 897-916.

Falgout, B., Pethel, M., Zhang, Y.M. and Lai, C.J. (1991) Both nonstructural proteins NS2B and NS3 are required for the proteolytic processing of dengue virus nonstructural proteins. J Virol 65(5), 2467-75.

Feinstein, D.L., Galea, E. and Reis, D.J. (1997) Suppression of glial nitric oxide synthase induction by heat shock: effects on proteolytic degradation of IkappaB-alpha. Nitric Oxide 1(2), 167-76.

Filomatori, C.V., Lodeiro, M.F., Alvarez, D.E., Samsa, M.M., Pietrasanta, L. and Gamarnik, A.V. (2006) A 5' RNA element promotes dengue virus RNA synthesis on a circular genome. Genes Dev 20(16), 2238-49.

Fink, J., Gu, F., Ling, L., Tolfvenstam, T., Olfat, F., Chin, K.C., Aw, P., George, J., Kuznetsov, V.A., Schreiber, M., Vasudevan, S.G. and Hibberd, M.L. (2007) Host gene expression profiling of dengue virus infection in cell lines and patients. PLoS Negl Trop Dis 1(2), e86.

Flamand, M., Megret, F., Mathieu, M., Lepault, J., Rey, F.A. and Deubel, V. (1999) Dengue virus type 1 nonstructural glycoprotein NS1 is secreted from mammalian cells as a soluble hexamer in a glycosylation-dependent fashion. J Virol 73(7), 6104-10.

Foy, E., Li, K., Wang, C., Sumpter, R., Jr., Ikeda, M., Lemon, S.M. and Gale, M., Jr. (2003) Regulation of interferon regulatory factor-3 by the hepatitis C virus serine protease. Science 300(5622), 1145-8.

Frese, M., Barth, K., Kaul, A., Lohmann, V., Schwarzle, V. and Bartenschlager, R. (2003) Hepatitis C virus RNA replication is resistant to tumour necrosis factor-alpha. J Gen Virol 84(Pt 5), 1253-9.

Fuchs, B., Zhang, K., Rock, M.G., Bolander, M.E. and Sarkar, G. (1999) High temperature cDNA synthesis by AMV reverse transcriptase improves the specificity of PCR. Mol Biotechnol 12(3), 237-40.

Gagnon, S.J., Ennis, F.A. and Rothman, A.L. (1999) Bystander target cell lysis and cytokine production by dengue virus-specific human CD4(+) cytotoxic T-lymphocyte clones. J Virol 73(5), 3623-9.

Gagnon, S.J., Mori, M., Kurane, I., Green, S., Vaughn, D.W., Kalayanarooj, S., Suntayakorn, S., Ennis, F.A. and Rothman, A.L. (2002) Cytokine gene expression and protein production in peripheral blood mononuclear cells of children with acute dengue virus infections. J Med Virol 67(1), 41-6.

Gaudin, Y. (1997) Folding of rabies virus glycoprotein: epitope acquisition and interaction with endoplasmic reticulum chaperones. J Virol 71(5), 3742-50.

Gibbons, R.V. and Vaughn, D.W. (2002) Dengue: an escalating problem. Bmj 324(7353), 1563-6.

Gilles, P.N., Fey, G. and Chisari, F.V. (1992) Tumor necrosis factor alpha negatively regulates hepatitis B virus gene expression in transgenic mice. J Virol 66(6), 3955-60.

Gollins, S.W. and Porterfield, J.S. (1985) Flavivirus infection enhancement in macrophages: an electron microscopic study of viral cellular entry. J Gen Virol 66 (Pt 9), 1969-82.

Gollins, S.W. and Porterfield, J.S. (1986) The uncoating and infectivity of the flavivirus West Nile on interaction with cells: effects of pH and ammonium chloride. J Gen Virol 67 (Pt 9), 1941-50.

Green, S., Vaughn, D.W., Kalayanarooj, S., Nimmannitya, S., Suntayakorn, S., Nisalak, A., Lew, R., Innis, B.L., Kurane, I., Rothman, A.L. and Ennis, F.A. (1999a) Early immune activation in acute dengue illness is related to development of plasma leakage and disease severity. J Infect Dis 179(4), 755-62.

Green, S., Vaughn, D.W., Kalayanarooj, S., Nimmannitya, S., Suntayakorn, S., Nisalak, A., Rothman, A.L. and Ennis, F.A. (1999b) Elevated plasma interleukin-10 levels in acute dengue correlate with disease severity. J Med Virol 59(3), 329-34.

Gualano, R.C., Pryor, M.J., Cauchi, M.R., Wright, P.J. and Davidson, A.D. (1998) Identification of a major determinant of mouse neurovirulence of dengue virus type 2 using stably cloned genomic-length cDNA. J Gen Virol 79 (Pt 3), 437-46.

Gubler, D.J. (1998) Dengue and dengue hemorrhagic fever. Clin Microbiol Rev 11(3), 480-96.

Guilhot, S., Guidotti, L.G. and Chisari, F.V. (1993) Interleukin-2 downregulates hepatitis B virus gene expression in transgenic mice by a posttranscriptional mechanism. J Virol 67(12), 7444-9.

Guirakhoo, F., Weltzin, R., Chambers, T.J., Zhang, Z.X., Soike, K., Ratterree, M., Arroyo, J., Georgakopoulos, K., Catalan, J. and Monath, T.P. (2000) Recombinant chimeric yellow fever-dengue type 2 virus is immunogenic and protective in nonhuman primates. J Virol 74(12), 5477-85.

Gulati, L., Chaturvedi, U.C. and Mathur, A. (1982) Depressed macrophage functions in dengue virus-infected mice: role of the cytotoxic factor. Br J Exp Pathol 63(2), 194-202.

Gunji, T., Kato, N., Hijikata, M., Hayashi, K., Saitoh, S. and Shimotohno, K. (1994) Specific detection of positive and negative stranded hepatitis C viral RNA using chemical RNA modification. Arch Virol 134(3-4), 293-302.

Guzhova, I.V., Darieva, Z.A., Melo, A.R. and Margulis, B.A. (1997) Major stress protein Hsp70 interacts with NF-kB regulatory complex in human T-lymphoma cells. Cell Stress Chaperones 2(2), 132-9.

Guzman, M.G. and Kouri, G. (2002) Dengue: an update. Lancet Infect Dis 2(1), 33-42.

Guzman, M.G., Kouri, G., Valdes, L., Bravo, J., Vazquez, S. and Halstead, S.B. (2002) Enhanced severity of secondary dengue-2 infections: death rates in 1981 and 1997 Cuban outbreaks. Rev Panam Salud Publica 11(4), 223-7.

Hales, S., de Wet, N., Maindonald, J. and Woodward, A. (2002) Potential effect of population and climate changes on global distribution of dengue fever: an empirical model. Lancet 360(9336), 830-4.

Hall, W.C., Crowell, T.P., Watts, D.M., Barros, V.L., Kruger, H., Pinheiro, F. and Peters, C.J. (1991) Demonstration of yellow fever and dengue antigens in formalin-fixed paraffin-embedded human liver by immunohistochemical analysis. Am J Trop Med Hyg 45(4), 408-17.

Halstead, S.B. (1989) Antibody, macrophages, dengue virus infection, shock, and hemorrhage: a pathogenetic cascade. Rev Infect Dis 11 Suppl 4, S830-9.

Halstead, S.B., Lan, N.T., Myint, T.T., Shwe, T.N., Nisalak, A., Kalyanarooj, S., Nimmannitya, S., Soegijanto, S., Vaughn, D.W. and Endy, T.P. (2002) Dengue hemorrhagic fever in infants: research opportunities ignored. Emerg Infect Dis 8(12), 1474-9.

Halstead, S.B., O'Rourke, E.J. and Allison, A.C. (1977) Dengue viruses and mononuclear phagocytes. II. Identity of blood and tissue leukocytes supporting in vitro infection. J Exp Med 146(1), 218-29.

Halstead, S.B., Streit, T.G., Lafontant, J.G., Putvatana, R., Russell, K., Sun, W., Kanesa-Thasan, N., Hayes, C.G. and Watts, D.M. (2001) Haiti: absence of dengue hemorrhagic fever despite hyperendemic dengue virus transmission. Am J Trop Med Hyg 65(3), 180-3.

Hase, T., Summers, P.L. and Eckels, K.H. (1989) Flavivirus entry into cultured mosquito cells and human peripheral blood monocytes. Archives of Virology 104(1-2), 129-43.

He, B. (2006) Viruses, endoplasmic reticulum stress, and interferon responses. Cell Death Differ 13(3), 393-403.

He, R.T., Innis, B.L., Nisalak, A., Usawattanakul, W., Wang, S., Kalayanarooj, S. and Anderson, R. (1995) Antibodies that block virus attachment to Vero cells are a major component of the human neutralizing antibody response against dengue virus type 2. J Med Virol 45(4), 451-61.

Heim, M.H., Moradpour, D. and Blum, H.E. (1999) Expression of hepatitis C virus proteins inhibits signal transduction through the Jak-STAT pathway. J Virol 73(10), 8469-75.

Heimbach, J.K., Reznikov, L.L., Calkins, C.M., Robinson, T.N., Dinarello, C.A., Harken, A.H. and Meng, X. (2001) TNF receptor I is required for induction of macrophage heat shock protein 70. Am J Physiol Cell Physiol 281(1), C241-7.

Heinz, F.X. and Allison, S.L. (2003) Flavivirus structure and membrane fusion. Adv Virus Res 59, 63-97.

Henchal, E.A., Henchal, L.S. and Schlesinger, J.J. (1988) Synergistic interactions of anti-NS1 monoclonal antibodies protect passively immunized mice from lethal challenge with dengue 2 virus. J Gen Virol 69 (Pt 8), 2101-7.

Henchal, E.A. and Putnak, J.R. (1990) The dengue viruses. Clin Microbiol Rev 3(4), 376-96.

Herbein, G. and O'Brien, W.A. (2000) Tumor necrosis factor (TNF)-alpha and TNF receptors in viral pathogenesis. Proc Soc Exp Biol Med 223(3), 241-57.

Hilgard, P., Czaja, M.J., Gerken, G. and Stockert, R.J. (2004) Proapoptotic function of protein kinase CK2alpha" is mediated by a JNK signaling cascade. Am J Physiol Gastrointest Liver Physiol 287(1), G192-201.

Hiscott, J., Kwon, H. and Genin, P. (2001) Hostile takeovers: viral appropriation of the NF-kappaB pathway. J Clin Invest 107(2), 143-51.

Ho, L.J., Wang, J.J., Shaio, M.F., Kao, C.L., Chang, D.M., Han, S.W. and Lai, J.H. (2001) Infection of human dendritic cells by dengue virus causes cell maturation and cytokine production. J Immunol 166(3), 1499-506.

Hober, D., Delannoy, A.S., Benyoucef, S., De Groote, D. and Wattre, P. (1996a) High levels of sTNFR p75 and TNF alpha in dengue-infected patients. Microbiol Immunol 40(8), 569-73.

Hober, D., Poli, L., Roblin, B., Gestas, P., Chungue, E., Granic, G., Imbert, P., Pecarere, J.L., Vergez-Pascal, R., Wattre, P. and et al. (1993) Serum levels of tumor necrosis factor-alpha (TNF-alpha), interleukin-6 (IL-6), and interleukin-1 beta (IL-1 beta) in dengue-infected patients. Am J Trop Med Hyg 48(3), 324-31.

Hober, D., Shen, L., Benyoucef, S., De Groote, D., Deubel, V. and Wattre, P. (1996b) Enhanced TNF alpha production by monocytic-like cells exposed to dengue virus antigens. Immunol Lett 53(2-3), 115-20.

Holden, K.L., Stein, D.A., Pierson, T.C., Ahmed, A.A., Clyde, K., Iversen, P.L. and Harris, E. (2006) Inhibition of dengue virus translation and RNA synthesis by a morpholino oligomer targeted to the top of the terminal 3' stem-loop structure. Virology 344(2), 439-52.

Hopken, U.E., Lu, B., Gerard, N.P. and Gerard, C. (1996) The C5a chemoattractant receptor mediates mucosal defence to infection. Nature 383(6595), 86-9.

Huang, K.J., Li, S.Y., Chen, S.C., Liu, H.S., Lin, Y.S., Yeh, T.M., Liu, C.C. and Lei, H.Y. (2000a) Manifestation of thrombocytopenia in dengue-2-virus-infected mice. J Gen Virol 81(Pt 9), 2177-82.

Huang, Y.H., Chang, B.I., Lei, H.Y., Liu, H.S., Liu, C.C., Wu, H.L. and Yeh, T.M. (1997) Antibodies against dengue virus E protein peptide bind to human plasminogen and inhibit plasmin activity. Clinical & Experimental Immunology 110(1), 35-40.

Huang, Y.H., Lei, H.Y., Liu, H.S., Lin, Y.S., Liu, C.C. and Yeh, T.M. (2000b) Dengue virus infects human endothelial cells and induces IL-6 and IL-8 production. Am J Trop Med Hyg 63(1-2), 71-5.

Hung, S.L., Lee, P.L., Chen, H.W., Chen, L.K., Kao, C.L. and King, C.C. (1999) Analysis of the steps involved in Dengue virus entry into host cells. Virology 257(1), 156-67.

Hurtley, S.M., Bole, D.G., Hoover-Litty, H., Helenius, A. and Copeland, C.S. (1989) Interactions of misfolded influenza virus hemagglutinin with binding protein (BiP). J Cell Biol 108(6), 2117-26.

Iordanskiy, S., Zhao, Y., Dubrovsky, L., Iordanskaya, T., Chen, M., Liang, D. and Bukrinsky, M. (2004) Heat shock protein 70 protects cells from cell cycle arrest and apoptosis induced by human immunodeficiency virus type 1 viral protein R. J Virol 78(18), 9697-704.

Jaattela, M. (1993) Overexpression of major heat shock protein hsp70 inhibits tumor necrosis factor-induced activation of phospholipase A2. J Immunol 151(8), 4286-94.

Jaattela, M. and Wissing, D. (1993) Heat-shock proteins protect cells from monocyte cytotoxicity: possible mechanism of self-protection. J Exp Med 177(1), 231-6.

Jarvis, M.A., Borton, J.A., Keech, A.M., Wong, J., Britt, W.J., Magun, B.E. and Nelson, J.A. (2006) Human cytomegalovirus attenuates interleukin-1beta and tumor

necrosis factor alpha proinflammatory signaling by inhibition of NF-kappaB activation. J Virol 80(11), 5588-98.

Jiang, X.S., Tang, L.Y., Dai, J., Zhou, H., Li, S.J., Xia, Q.C., Wu, J.R. and Zeng, R. (2005) Quantitative analysis of severe acute respiratory syndrome (SARS)-associated coronavirus-infected cells using proteomic approaches: implications for cellular responses to virus infection. Mol Cell Proteomics 4(7), 902-13.

Jindadamrongwech, S., Thepparit, C. and Smith, D.R. (2004) Identification of GRP 78 (BiP) as a liver cell expressed receptor element for dengue virus serotype 2. Arch Virol 149(5), 915-27.

Johnson, A.J. and Roehrig, J.T. (1999) New mouse model for dengue virus vaccine testing. J Virol 73(1), 783-6.

Jones, C.T., Ma, L., Burgner, J.W., Groesch, T.D., Post, C.B. and Kuhn, R.J. (2003) Flavivirus capsid is a dimeric alpha-helical protein. J Virol 77(12), 7143-9.

Jones, M., Davidson, A., Hibbert, L., Gruenwald, P., Schlaak, J., Ball, S., Foster, G.R. and Jacobs, M. (2005) Dengue virus inhibits alpha interferon signaling by reducing STAT2 expression. J Virol 79(9), 5414-20.

Jordan, R., Wang, L., Graczyk, T.M., Block, T.M. and Romano, P.R. (2002) Replication of a cytopathic strain of bovine viral diarrhea virus activates PERK and induces endoplasmic reticulum stress-mediated apoptosis of MDBK cells. J Virol 76(19), 9588-99.

Juffrie, M., Meer, G.M., Hack, C.E., Haasnoot, K., Sutaryo, Veerman, A.J. and Thijs, L.G. (2001) Inflammatory mediators in dengue virus infection in children: interleukin-6 and its relation to C-reactive protein and secretory phospholipase A2. Am J Trop Med Hyg 65(1), 70-5.

Kangwanpong, D., Bhamarapravati, N. and Lucia, H.L. (1995) Diagnosing dengue virus infection in archived autopsy tissues by means of the in situ PCR method: a case report. Clin Diagn Virol 3(2), 165-72.

Kapoor, M., Zhang, L., Ramachandra, M., Kusukawa, J., Ebner, K.E. and Padmanabhan, R. (1995) Association between NS3 and NS5 proteins of dengue virus type 2 in the putative RNA replicase is linked to differential phosphorylation of NS5. J Biol Chem 270(32), 19100-6.

Katze, M.G., He, Y. and Gale, M., Jr. (2002) Viruses and interferon: a fight for supremacy. Nat Rev Immunol 2(9), 675-87.

Kaufman, B.M., Summers, P.L., Dubois, D.R., Cohen, W.H., Gentry, M.K., Timchak, R.L., Burke, D.S. and Eckels, K.H. (1989) Monoclonal antibodies for dengue virus prM glycoprotein protect mice against lethal dengue infection. Am J Trop Med Hyg 41(5), 576-80.

Kaufman, B.M., Summers, P.L., Dubois, D.R. and Eckels, K.H. (1987) Monoclonal antibodies against dengue 2 virus E-glycoprotein protect mice against lethal dengue infection. Am J Trop Med Hyg 36(2), 427-34.

Kaufman, R.J., Scheuner, D., Schroder, M., Shen, X., Lee, K., Liu, C.Y. and Arnold, S.M. (2002) The unfolded protein response in nutrient sensing and differentiation. Nat Rev Mol Cell Biol 3(6), 411-21.

Khromykh, A.A., Sedlak, P.L. and Westaway, E.G. (2000) cis- and trans-acting elements in flavivirus RNA replication. J Virol 74(7), 3253-63.

Killen, H. and O'Sullivan, M.A. (1993) Detection of dengue virus by in situ hybridization. J Virol Methods 41(2), 135-46.

King, A.D., Nisalak, A., Kalayanrooj, S., Myint, K.S., Pattanapanyasat, K., Nimmannitya, S. and Innis, B.L. (1999) B cells are the principal circulating mononuclear cells infected by dengue virus. Southeast Asian J Trop Med Public Health 30(4), 718-28.

King, C.A., Anderson, R. and Marshall, J.S. (2002) Dengue virus selectively induces human mast cell chemokine production. J Virol 76(16), 8408-19.

Kittigul, L., Temprom, W., Sujirarat, D. and Kittigul, C. (2000) Determination of tumor necrosis factor-alpha levels in dengue virus infected patients by sensitive biotin-streptavidin enzyme-linked immunosorbent assay. J Virol Methods 90(1), 51-7.

Klein, E., Ben-Bassat, H., Neumann, H., Ralph, P., Zeuthen, J., Polliack, A. and Vanky, F. (1976) Properties of the K562 cell line, derived from a patient with chronic myeloid leukemia. Int J Cancer 18(4), 421-31.

Kliks, S. (1990) Antibody-enhanced infection of monocytes as the pathogenetic mechanism for severe dengue illness. AIDS Res Hum Retroviruses 6(8), 993-8.

Kliks, S.C., Nimmanitya, S., Nisalak, A. and Burke, D.S. (1988) Evidence that maternal dengue antibodies are important in the development of dengue hemorrhagic fever in infants. Am J Trop Med Hyg 38(2), 411-9.

Kliks, S.C., Nisalak, A., Brandt, W.E., Wahl, L. and Burke, D.S. (1989) Antibody-dependent enhancement of dengue virus growth in human monocytes as a risk factor for dengue hemorrhagic fever. American Journal of Tropical Medicine & Hygiene 40(4), 444-51.

Kochel, T.J., Raviprakash, K., Hayes, C.G., Watts, D.M., Russell, K.L., Gozalo, A.S., Phillips, I.A., Ewing, D.F., Murphy, G.S. and Porter, K.R. (2000) A dengue virus serotype-1 DNA vaccine induces virus neutralizing antibodies and provides protection from viral challenge in Aotus monkeys. Vaccine 18(27), 3166-73.

Konishi, E., Yamaoka, M., Kurane, I. and Mason, P.W. (2000) A DNA vaccine expressing dengue type 2 virus premembrane and envelope genes induces neutralizing antibody and memory B cells in mice. Vaccine 18(11-12), 1133-9.

Kontny, U., Kurane, I. and Ennis, F.A. (1988) Gamma interferon augments Fc gamma receptor-mediated dengue virus infection of human monocytic cells. J Virol 62(11), 3928-33.

Kuhn, R.J., Zhang, W., Rossmann, M.G., Pletnev, S.V., Corver, J., Lenches, E., Jones, C.T., Mukhopadhyay, S., Chipman, P.R., Strauss, E.G., Baker, T.S. and Strauss,

J.H. (2002) Structure of dengue virus: implications for flavivirus organization, maturation, and fusion. Cell 108(5), 717-25.

Kurane, I., Innis, B.L., Nimmannitya, S., Nisalak, A., Meager, A., Janus, J. and Ennis, F.A. (1991) Activation of T lymphocytes in dengue virus infections. High levels of soluble interleukin 2 receptor, soluble CD4, soluble CD8, interleukin 2, and interferon-gamma in sera of children with dengue. Journal of Clinical Investigation 88(5), 1473-80.

Kurane, I., Innis, B.L., Nisalak, A., Hoke, C., Nimmannitya, S., Meager, A. and Ennis, F.A. (1989) Human T cell responses to dengue virus antigens. Proliferative responses and interferon gamma production. J Clin Invest 83(2), 506-13.

Kurane, I., Janus, J. and Ennis, F.A. (1992) Dengue virus infection of human skin fibroblasts in vitro production of IFN-beta, IL-6 and GM-CSF. Archives of Virology 124(1-2), 21-30.

Kurane, I., Kontny, U., Janus, J. and Ennis, F.A. (1990) Dengue-2 virus infection of human mononuclear cell lines and establishment of persistent infections. Arch Virol 110(1-2), 91-101.

Kurane, I., Rothman, A.L., Livingston, P.G., Green, S., Gagnon, S.J., Janus, J., Innis, B.L., Nimmannitya, S., Nisalak, A. and Ennis, F.A. (1994) Immunopathologic mechanisms of dengue hemorrhagic fever and dengue shock syndrome. Arch Virol Suppl 9, 59-64.

Kuruvilla, J.G., Troyer, R.M., Devi, S. and Akkina, R. (2007) Dengue virus infection and immune response in humanized RAG2(-/-)gamma(c)(-/-) (RAG-hu) mice. Virology 369(1), 143-52.

Lane, B.R., Markovitz, D.M., Woodford, N.L., Rochford, R., Strieter, R.M. and Coffey, M.J. (1999) TNF-alpha inhibits HIV-1 replication in peripheral blood monocytes and alveolar macrophages by inducing the production of RANTES and decreasing C-C chemokine receptor 5 (CCR5) expression. J Immunol 163(7), 3653-61.

Lidbury, B.A. and Mahalingam, S. (2000) Specific ablation of antiviral gene expression in macrophages by antibody-dependent enhancement of Ross River virus infection. J Virol 74(18), 8376-81.

Liew, K.J. and Chow, V.T. (2006) Microarray and real-time RT-PCR analyses of a novel set of differentially expressed human genes in ECV304 endothelial-like cells infected with dengue virus type 2. J Virol Methods 131(1), 47-57.

Lin, C.F., Lei, H.Y., Shiau, A.L., Liu, C.C., Liu, H.S., Yeh, T.M., Chen, S.H. and Lin, Y.S. (2003) Antibodies from dengue patient sera cross-react with endothelial cells and induce damage. J Med Virol 69(1), 82-90.

Lin, Y.L., Lei, H.Y., Lin, Y.S., Yeh, T.M., Chen, S.H. and Liu, H.S. (2002a) Heparin inhibits dengue-2 virus infection of five human liver cell lines. Antiviral Res 56(1), 93-6.

Lin, Y.L., Liao, C.L., Chen, L.K., Yeh, C.T., Liu, C.I., Ma, S.H., Huang, Y.Y., Huang, Y.L., Kao, C.L. and King, C.C. (1998) Study of Dengue virus infection in SCID mice engrafted with human K562 cells. Journal of Virology 72(12), 9729-37.

Lin, Y.L., Liu, C.C., Lei, H.Y., Yeh, T.M., Lin, Y.S., Chen, R.M. and Liu, H.S. (2000) Infection of five human liver cell lines by dengue-2 virus. J Med Virol 60(4), 425-31.

Lin, Y.W., Wang, K.J., Lei, H.Y., Lin, Y.S., Yeh, T.M., Liu, H.S., Liu, C.C. and Chen, S.H. (2002b) Virus replication and cytokine production in dengue virus-infected human B lymphocytes. J Virol 76(23), 12242-9.

Lindenbach, B.D. and Rice, C.M. (2001) Flaviviridae: The viruses and their replication, Vol. 2.

Littaua, R., Kurane, I. and Ennis, F.A. (1990) Human IgG Fc receptor II mediates antibody-dependent enhancement of dengue virus infection. J Immunol 144(8), 3183-6.

Liu, H.S., Lin, Y.L. and Chen, C.C. (1997) Comparison of various methods of detection of different forms of dengue virus type 2 RNA in cultured cells. Acta Virol 41(6), 317-24.

Lobigs, M. (1993) Flavivirus premembrane protein cleavage and spike heterodimer secretion require the function of the viral proteinase NS3. Proc Natl Acad Sci U S A 90(13), 6218-22.

Lozzio, C.B. and Lozzio, B.B. (1975) Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome. Blood 45(3), 321-34.

Luo, S., Mao, C., Lee, B. and Lee, A.S. (2006) GRP78/BiP is required for cell proliferation and protecting the inner cell mass from apoptosis during early mouse embryonic development. Mol Cell Biol 26(15), 5688-97.

Ma, L., Jones, C.T., Groesch, T.D., Kuhn, R.J. and Post, C.B. (2004) Solution structure of dengue virus capsid protein reveals another fold. Proc Natl Acad Sci U S A 101(10), 3414-9.

Mackenzie, J.M., Jones, M.K. and Young, P.R. (1996) Immunolocalization of the dengue virus nonstructural glycoprotein NS1 suggests a role in viral RNA replication. Virology 220(1), 232-40.

Mackenzie, J.S., Gubler, D.J. and Petersen, L.R. (2004) Emerging flaviviruses: the spread and resurgence of Japanese encephalitis, West Nile and dengue viruses. Nat Med 10(12 Suppl), S98-109.

Mahalingam, S. and Lidbury, B.A. (2002) Suppression of lipopolysaccharide-induced antiviral transcription factor (STAT-1 and NF-kappa B) complexes by antibody-dependent enhancement of macrophage infection by Ross River virus. Proc Natl Acad Sci U S A 99(21), 13819-24.

Mairuhu, A.T., Wagenaar, J., Brandjes, D.P. and Van Gorp, E.C. (2004) Dengue: an arthropod-borne disease of global importance. Eur J Clin Microbiol Infect Dis 23(6), 425-33.

Mandl, C.W., Heinz, F.X., Stockl, E. and Kunz, C. (1989) Genome sequence of tick-borne encephalitis virus (Western subtype) and comparative analysis of nonstructural proteins with other flaviviruses. Virology 173(1), 291-301.

Mangada, M.M., Endy, T.P., Nisalak, A., Chunsuttiwat, S., Vaughn, D.W., Libraty, D.H., Green, S., Ennis, F.A. and Rothman, A.L. (2002) Dengue-specific T cell responses in peripheral blood mononuclear cells obtained prior to secondary dengue virus infections in Thai schoolchildren. J Infect Dis 185(12), 1697-703.

Mangada, M.M. and Rothman, A.L. (2005) Altered cytokine responses of dengue-specific CD4+ T cells to heterologous serotypes. J Immunol 175(4), 2676-83.

Mangada, M.N. and Igarashi, A. (1998) Molecular and in vitro analysis of eight dengue type 2 viruses isolated from patients exhibiting different disease severities. Virology 244(2), 458-66.

Marianneau, P., Cardona, A., Edelman, L., Deubel, V. and Despres, P. (1997) Dengue virus replication in human hepatoma cells activates NF-kappaB which in turn induces apoptotic cell death. Journal of Virology 71(4), 3244-9.

Marianneau, P., Megret, F., Olivier, R., Morens, D.M. and Deubel, V. (1996) Dengue 1 virus binding to human hepatoma HepG2 and simian Vero cell surfaces differs. J Gen Virol 77 (Pt 10), 2547-54.

Marianneau, P., Steffan, A.M., Royer, C., Drouet, M.T., Jaeck, D., Kirn, A. and Deubel, V. (1999) Infection of primary cultures of human Kupffer cells by Dengue virus: no viral progeny synthesis, but cytokine production is evident. Journal of Virology 73(6), 5201-6.

Marianneau, P., Steffan, A.M., Royer, C., Drouet, M.T., Kirn, A. and Deubel, V. (1998) Differing infection patterns of dengue and yellow fever viruses in a human hepatoma cell line. J Infect Dis 178(5), 1270-8.

Marovich, M., Grouard-Vogel, G., Louder, M., Eller, M., Sun, W., Wu, S.J., Putvatana, R., Murphy, G., Tassaneetrithep, B., Burgess, T., Birx, D., Hayes, C., Schlesinger-

Frankel, S. and Mascola, J. (2001) Human dendritic cells as targets of dengue virus infection. J Investig Dermatol Symp Proc 6(3), 219-24.

Martinez, E., Guzman, M.G., Valdes, M., Soler, M. and Kouri, G. (1993) [Dengue fever and hemorrhagic dengue in infants with a primary infection]. Rev Cubana Med Trop 45(2), 97-101.

Marusawa, H., Hijikata, M., Chiba, T. and Shimotohno, K. (1999) Hepatitis C virus core protein inhibits Fas- and tumor necrosis factor alpha-mediated apoptosis via NF-kappaB activation. J Virol 73(6), 4713-20.

Mathew, A., Kurane, I., Green, S., Stephens, H.A., Vaughn, D.W., Kalayanarooj, S., Suntayakorn, S., Chandanayingyong, D., Ennis, F.A. and Rothman, A.L. (1998) Predominance of HLA-restricted cytotoxic T-lymphocyte responses to serotype-cross-reactive epitopes on nonstructural proteins following natural secondary dengue virus infection. J Virol 72(5), 3999-4004.

Matsumura, T., Shiraki, K., Sashikata, T. and Hotta, S. (1977) Morphogenesis of dengue-1 virus in cultures of a human leukemic leukocyte line (J-111). Microbiol Immunol 21(6), 329-34.

Matsumura, T., Stollar, V. and Schlesinger, R.W. (1971) Studies on the nature of dengue viruses. V. Structure and development of dengue virus in Vero cells. Virology 46(2), 344-55.

Mayer, M.P. (2005) Recruitment of Hsp70 chaperones: a crucial part of viral survival strategies. Rev Physiol Biochem Pharmacol 153, 1-46.

McBride, J.H. (1999) Dengue fever. An Australian perspective. Aust Fam Physician 28(4), 319-23.

Medin, C.L., Fitzgerald, K.A. and Rothman, A.L. (2005) Dengue virus nonstructural protein NS5 induces interleukin-8 transcription and secretion. J Virol 79(17), 11053-61.

Melen, K., Fagerlund, R., Nyqvist, M., Keskinen, P. and Julkunen, I. (2004) Expression of hepatitis C virus core protein inhibits interferon-induced nuclear import of STATs. J Med Virol 73(4), 536-47.

Mellor, J., Haydon, G., Blair, C., Livingstone, W. and Simmonds, P. (1998) Low level or absent in vivo replication of hepatitis C virus and hepatitis G virus/GB virus C in peripheral blood mononuclear cells. J Gen Virol 79 (Pt 4), 705-14.

Mentor, N.A. and Kurane, I. (1997) Dengue virus infection of human T lymphocytes. Acta Virol 41(3), 175-6.

Messer, W.B., Gubler, D.J., Harris, E., Sivananthan, K. and de Silva, A.M. (2003) Emergence and global spread of a dengue serotype 3, subtype III virus. Emerg Infect Dis 9(7), 800-9.

Minagawa, H., Hashimoto, K. and Yanagi, Y. (2004) Absence of tumour necrosis factor facilitates primary and recurrent herpes simplex virus-1 infections. J Gen Virol 85(Pt 2), 343-7.

Moreno-Altamirano, M.M., Sanchez-Garcia, F.J. and Munoz, M.L. (2002) Non Fc receptor-mediated infection of human macrophages by dengue virus serotype 2. J Gen Virol 83(Pt 5), 1123-30.

Morens, D.M., Sather, G.E., Gubler, D.J., Rammohan, M. and Woodall, J.P. (1987) Dengue shock syndrome in an American traveler with primary dengue 3 infection. Am J Trop Med Hyg 36(2), 424-6.

Mosquera, J.A., Hernandez, J.P., Valero, N., Espina, L.M. and Anez, G.J. (2005) Ultrastructural studies on dengue virus type 2 infection of cultured human monocytes. Virol J 2, 26.

Munoz-Jordan, J.L., Laurent-Rolle, M., Ashour, J., Martinez-Sobrido, L., Ashok, M., Lipkin, W.I. and Garcia-Sastre, A. (2005) Inhibition of alpha/beta interferon signaling by the NS4B protein of flaviviruses. J Virol 79(13), 8004-13.

Munoz-Jordan, J.L., Sanchez-Burgos, G.G., Laurent-Rolle, M. and Garcia-Sastre, A. (2003) Inhibition of interferon signaling by dengue virus. Proc Natl Acad Sci U S A 100(24), 14333-8.

Mustafa, A.S., Elbishbishi, E.A., Agarwal, R. and Chaturvedi, U.C. (2001) Elevated levels of interleukin-13 and IL-18 in patients with dengue hemorrhagic fever. FEMS Immunol Med Microbiol 30(3), 229-33.

Neves-Souza, P.C., Azeredo, E.L., Zagne, S.M., Valls-de-Souza, R., Reis, S.R., Cerqueira, D.I., Nogueira, R.M. and Kubelka, C.F. (2005) Inducible nitric oxide synthase (iNOS) expression in monocytes during acute Dengue Fever in patients and during in vitro infection. BMC Infect Dis 5, 64.

Nguyen, T.H., Lei, H.Y., Nguyen, T.L., Lin, Y.S., Huang, K.J., Le, B.L., Lin, C.F., Yeh, T.M., Do, Q.H., Vu, T.Q., Chen, L.C., Huang, J.H., Lam, T.M., Liu, C.C. and Halstead, S.B. (2004) Dengue hemorrhagic fever in infants: a study of clinical and cytokine profiles. J Infect Dis 189(2), 221-32.

Nimmannitya, S. (1987) Clinical spectrum and management of dengue haemorrhagic fever. Southeast Asian J Trop Med Public Health 18(3), 392-7.

Nomaguchi, M., Ackermann, M., Yon, C., You, S. and Padmanabhan, R. (2003) De novo synthesis of negative-strand RNA by Dengue virus RNA-dependent RNA polymerase in vitro: nucleotide, primer, and template parameters. J Virol 77(16), 8831-42.

Nordeen, S.K. (1988) Luciferase reporter gene vectors for analysis of promoters and enhancers. Biotechniques 6(5), 454-8.

O'Sullivan, M.A. and Killen, H.M. (1994) The differentiation state of monocytic cells affects their susceptibility to infection and the effects of infection by dengue virus. J Gen Virol 75 (Pt 9), 2387-92.

Paes, M.V., Pinhao, A.T., Barreto, D.F., Costa, S.M., Oliveira, M.P., Nogueira, A.C., Takiya, C.M., Farias-Filho, J.C., Schatzmayr, H.G., Alves, A.M. and Barth, O.M.

(2005) Liver injury and viremia in mice infected with dengue-2 virus. Virology 338(2), 236-46.

Palmer, D.R., Sun, P., Celluzzi, C., Bisbing, J., Pang, S., Sun, W., Marovich, M.A. and Burgess, T. (2005) Differential effects of dengue virus on infected and bystander dendritic cells. J Virol 79(4), 2432-9.

Park, K.J., Choi, S.H., Lee, S.Y., Hwang, S.B. and Lai, M.M. (2002) Nonstructural 5A protein of hepatitis C virus modulates tumor necrosis factor alpha-stimulated nuclear factor kappa B activation. J Biol Chem 277(15), 13122-8.

Pavic, I., Polic, B., Crnkovic, I., Lucin, P., Jonjic, S. and Koszinowski, U.H. (1993) Participation of endogenous tumour necrosis factor alpha in host resistance to cytomegalovirus infection. J Gen Virol 74 (Pt 10), 2215-23.

Pestova, T.V., Shatsky, I.N., Fletcher, S.P., Jackson, R.J. and Hellen, C.U. (1998) A prokaryotic-like mode of cytoplasmic eukaryotic ribosome binding to the initiation codon during internal translation initiation of hepatitis C and classical swine fever virus RNAs. Genes Dev 12(1), 67-83.

Peyrefitte, C.N., Pastorino, B., Bessaud, M., Tolou, H.J. and Couissinier-Paris, P. (2003) Evidence for in vitro falsely-primed cDNAs that prevent specific detection of virus negative strand RNAs in dengue-infected cells: improvement by tagged RT-PCR. J Virol Methods 113(1), 19-28.

Phillips, B., Abravaya, K. and Morimoto, R.I. (1991) Analysis of the specificity and mechanism of transcriptional activation of the human hsp70 gene during infection by DNA viruses. J Virol 65(11), 5680-92.

Phuong, C.X., Nhan, N.T., Kneen, R., Thuy, P.T., van Thien, C., Nga, N.T., Thuy, T.T., Solomon, T., Stepniewska, K. and Wills, B. (2004) Clinical diagnosis and assessment of severity of confirmed dengue infections in Vietnamese children: is the world health organization classification system helpful? Am J Trop Med Hyg 70(2), 172-9.

Polyak, S.J., Khabar, K.S., Paschal, D.M., Ezelle, H.J., Duverlie, G., Barber, G.N., Levy, D.E., Mukaida, N. and Gretch, D.R. (2001) Hepatitis C virus nonstructural 5A protein induces interleukin-8, leading to partial inhibition of the interferon-induced antiviral response. J Virol 75(13), 6095-106.

Pryor, M.J., Carr, J.M., Hocking, H., Davidson, A.D., Li, P. and Wright, P.J. (2001) Replication of dengue virus type 2 in human monocyte-derived macrophages: comparisons of isolates and recombinant viruses with substitutions at amino acid 390 in the envelope glycoprotein. American Journal of Tropical Medicine & Hygiene 65(5), 427-34.

Pryor, M.J., Rawlinson, S.M., Butcher, R.E., Barton, C.L., Waterhouse, T.A., Vasudevan, S.G., Bardin, P.G., Wright, P.J., Jans, D.A. and Davidson, A.D. (2007) Nuclear Localization of Dengue Virus Nonstructural Protein 5 Through Its Importin alpha/beta-Recognized Nuclear Localization Sequences is Integral to Viral Infection. Traffic 8(7), 795-807.

Purdy, D.E. and Chang, G.J. (2005) Secretion of noninfectious dengue virus-like particles and identification of amino acids in the stem region involved in intracellular retention of envelope protein. Virology 333(2), 239-50.

Raghupathy, R., Chaturvedi, U.C., Al-Sayer, H., Elbishbishi, E.A., Agarwal, R., Nagar, R., Kapoor, S., Misra, A., Mathur, A., Nusrat, H., Azizieh, F., Khan, M.A. and Mustafa, A.S. (1998) Elevated levels of IL-8 in dengue hemorrhagic fever. J Med Virol 56(3), 280-5.

Randolph, V.B. and Stollar, V. (1990) Low pH-induced cell fusion in flavivirus-infected Aedes albopictus cell cultures. J Gen Virol 71 (Pt 8), 1845-50.

Raviprakash, K., Porter, K.R., Kochel, T.J., Ewing, D., Simmons, M., Phillips, I., Murphy, G.S., Weiss, W.R. and Hayes, C.G. (2000) Dengue virus type 1 DNA vaccine induces protective immune responses in rhesus macaques. J Gen Virol 81(Pt 7), 1659-67.

Reyes-Del Valle, J., Chavez-Salinas, S., Medina, F. and Del Angel, R.M. (2005) Heat shock protein 90 and heat shock protein 70 are components of dengue virus receptor complex in human cells. J Virol 79(8), 4557-67.

Rico-Hesse, R., Harrison, L.M., Salas, R.A., Tovar, D., Nisalak, A., Ramos, C., Boshell, J., de Mesa, M.T., Nogueira, R.M. and da Rosa, A.T. (1997) Origins of dengue type 2 viruses associated with increased pathogenicity in the Americas. Virology 230(2), 244-51.

Rigau-Perez, J.G., Clark, G.G., Gubler, D.J., Reiter, P., Sanders, E.J. and Vorndam, A.V. (1998) Dengue and dengue haemorrhagic fever. Lancet 352(9132), 971-7.

Rosen, L., Drouet, M.T. and Deubel, V. (1999) Detection of dengue virus RNA by reverse transcription-polymerase chain reaction in the liver and lymphoid organs but not in the brain in fatal human infection. Am J Trop Med Hyg 61(5), 720-4.

Rosen, L., Khin, M.M. and U, T. (1989) Recovery of virus from the liver of children with fatal dengue: reflections on the pathogenesis of the disease and its possible analogy with that of yellow fever. Res Virol 140(4), 351-60.

Rossol-Voth, R., Rossol, S., Schutt, K.H., Corridori, S., de Cian, W. and Falke, D. (1991) In vivo protective effect of tumour necrosis factor alpha against experimental infection with herpes simplex virus type 1. J Gen Virol 72 (Pt 1), 143-7.

Rothman, A.L. (2004) Dengue: defining protective versus pathologic immunity. J Clin Invest 113(7), 946-51.

Rothman, A.L. and Ennis, F.A. (1999) Immunopathogenesis of Dengue hemorrhagic fever. Virology 257(1), 1-6.

Rothwell, S.W., Putnak, R. and La Russa, V.F. (1996) Dengue-2 virus infection of human bone marrow: characterization of dengue-2 antigen-positive stromal cells. American Journal of Tropical Medicine & Hygiene 54(5), 503-10.

Saito, K., Meyer, K., Warner, R., Basu, A., Ray, R.B. and Ray, R. (2006) Hepatitis C virus core protein inhibits tumor necrosis factor alpha-mediated apoptosis by a protective effect involving cellular FLICE inhibitory protein. J Virol 80(9), 4372-9.

Schmitt, E., Gehrmann, M., Brunet, M., Multhoff, G. and Garrido, C. (2007) Intracellular and extracellular functions of heat shock proteins: repercussions in cancer therapy. J Leukoc Biol 81(1), 15-27.

Scott, R.M., Nimmannitya, S., Bancroft, W.H. and Mansuwan, P. (1976) Shock syndrome in primary dengue infections. Am J Trop Med Hyg 25(6), 866-74.

Scott, R.M., Nisalak, A., Cheamudon, U., Seridhoranakul, S. and Nimmannitya, S. (1980) Isolation of dengue viruses from peripheral blood leukocytes of patients with hemorrhagic fever. J Infect Dis 141(1), 1-6.

Se-Thoe, S.Y., Ng, M.M. and Ling, A.E. (1999) Retrospective study of Western blot profiles in immune sera of natural dengue virus infections. J Med Virol 57(3), 322-30.

Shresta, S., Kyle, J.L., Snider, H.M., Basavapatna, M., Beatty, P.R. and Harris, E. (2004) Interferon-dependent immunity is essential for resistance to primary dengue virus infection in mice, whereas T- and B-cell-dependent immunity are less critical. J Virol 78(6), 2701-10.

Shresta, S., Sharar, K.L., Prigozhin, D.M., Beatty, P.R. and Harris, E. (2006) Murine model for dengue virus-induced lethal disease with increased vascular permeability. J Virol 80(20), 10208-17.

Silverstein, S.C., Steinman, R.M. and Cohn, Z.A. (1977) Endocytosis. Annu Rev Biochem 46, 669-722.

Simmons, C.P., Popper, S., Dolocek, C., Chau, T.N., Griffiths, M., Dung, N.T., Long, T.H., Hoang, D.M., Chau, N.V., Thao le, T.T., Hien, T.T., Relman, D.A. and Farrar, J. (2007) Patterns of host genome-wide gene transcript abundance in the

peripheral blood of patients with acute dengue hemorrhagic fever. J Infect Dis 195(8), 1097-107.

Sittisombut, N., Sistayanarain, A., Cardosa, M.J., Salminen, M., Damrongdachakul, S., Kalayanarooj, S., Rojanasuphot, S., Supawadee, J. and Maneekarn, N. (1997) Possible occurrence of a genetic bottleneck in dengue serotype 2 viruses between the 1980 and 1987 epidemic seasons in Bangkok, Thailand. Am J Trop Med Hyg 57(1), 100-8.

Souza, L.J., Alves, J.G., Nogueira, R.M., Gicovate Neto, C., Bastos, D.A., Siqueira, E.W., Souto Filho, J.T., Cezario Tde, A., Soares, C.E. and Carneiro Rda, C. (2004) Aminotransferase changes and acute hepatitis in patients with dengue fever: analysis of 1,585 cases. Braz J Infect Dis 8(2), 156-63.

Stadler, K., Allison, S.L., Schalich, J. and Heinz, F.X. (1997) Proteolytic activation of tick-borne encephalitis virus by furin. J Virol 71(11), 8475-81.

Stocks, C.E. and Lobigs, M. (1995) Posttranslational signal peptidase cleavage at the flavivirus C-prM junction in vitro. J Virol 69(12), 8123-6.

Stocks, C.E. and Lobigs, M. (1998) Signal peptidase cleavage at the flavivirus C-prM junction: dependence on the viral NS2B-3 protease for efficient processing requires determinants in C, the signal peptide, and prM. J Virol 72(3), 2141-9.

Stout, R.D. and Suttles, J. (2004) Functional plasticity of macrophages: reversible adaptation to changing microenvironments. J Leukoc Biol 76(3), 509-13.

Su, H.L., Liao, C.L. and Lin, Y.L. (2002) Japanese encephalitis virus infection initiates endoplasmic reticulum stress and an unfolded protein response. J Virol 76(9), 4162-71.

Suharti, C., van Gorp, E.C., Dolmans, W.M., Setiati, T.E., Hack, C.E., Djokomoeljanto, R. and van der Meer, J.W. (2003) Cytokine patterns during dengue shock syndrome. Eur Cytokine Netw 14(3), 172-7.

Sundstrom, C. and Nilsson, K. (1976) Establishment and characterization of a human histiocytic lymphoma cell line (U-937). Int J Cancer 17(5), 565-77.

Tan, B.H., Fu, J., Sugrue, R.J., Yap, E.H., Chan, Y.C. and Tan, Y.H. (1996) Recombinant dengue type 1 virus NS5 protein expressed in Escherichia coli exhibits RNA-dependent RNA polymerase activity. Virology 216(2), 317-25.

Tassaneetrithep, B., Burgess, T.H., Granelli-Piperno, A., Trumpfheller, C., Finke, J., Sun, W., Eller, M.A., Pattanapanyasat, K., Sarasombath, S., Birx, D.L., Steinman, R.M., Schlesinger, S. and Marovich, M.A. (2003) DC-SIGN (CD209) mediates dengue virus infection of human dendritic cells. J Exp Med 197(7), 823-9.

Taylor, R. (2003) Dengue pathogenesis:Characterisation of dengue virus type-2, replication in human monocyte derived macrophages. Honours thesis, University of Adelaide.

Theofilopoulos, A.N., Brandt, W.E., Russell, P.K. and Dixon, F.T. (1976) Replication of dengue-2 virus in cultured human lymphoblastoid cells and subpopulations of human peripheral leukocytes. J Immunol 117(3), 953-61.

Tolou, H. (1994) Reverse-transcription polymerase chain reaction for selective detection of RNA of single polarity: the role of reverse-transcription incubation temperature. Anal Biochem 220(1), 216-7.

Valdes, K., Alvarez, M., Pupo, M., Vazquez, S., Rodriguez, R. and Guzman, M.G. (2000) Human Dengue antibodies against structural and nonstructural proteins. Clin Diagn Lab Immunol 7(5), 856-7.

van den Broek, M.F., Muller, U., Huang, S., Aguet, M. and Zinkernagel, R.M. (1995) Antiviral defense in mice lacking both alpha/beta and gamma interferon receptors. J Virol 69(8), 4792-6.

Vaughn, D.W., Green, S., Kalayanarooj, S., Innis, B.L., Nimmannitya, S., Suntayakorn, S., Endy, T.P., Raengsakulrach, B., Rothman, A.L., Ennis, F.A. and Nisalak, A. (2000)

Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. J Infect Dis 181(1), 2-9.

Wang, T., Town, T., Alexopoulou, L., Anderson, J. F., Fikrig, E. and Flavell, R. A. (2004) Toll-like receptor 3 mediates West Nile virus entry into the brain causing lethal encephalitis. Nat Med 10(12), 1366-1373

Wang, W.K., Lin, S.R., Lee, C.M., King, C.C. and Chang, S.C. (2002a) Dengue type 3 virus in plasma is a population of closely related genomes: quasispecies. J Virol 76(9), 4662-5.

Wang, W.K., Sung, T.L., Lee, C.N., Lin, T.Y. and King, C.C. (2002b) Sequence diversity of the capsid gene and the nonstructural gene NS2B of dengue-3 virus in vivo. Virology 303(1), 181-91.

Wang, W.K., Sung, T.L., Tsai, Y.C., Kao, C.L., Chang, S.M. and King, C.C. (2002c) Detection of dengue virus replication in peripheral blood mononuclear cells from dengue virus type 2-infected patients by a reverse transcription-real-time PCR assay. J Clin Microbiol 40(12), 4472-8.

Warke, R.V., Martin, K.J., Giaya, K., Shaw, S.K., Rothman, A.L. and Bosch, I. (2008) TRAIL is a novel antiviral protein against dengue virus. J Virol 82(1), 555-64.

Warke, R.V., Xhaja, K., Martin, K.J., Fournier, M.F., Shaw, S.K., Brizuela, N., De Bosch, N., Lapointe, D., Ennis, F.A., Rothman, A.L. and Bosch, I. (2003) Dengue virus induces novel changes in gene expression of human umbilical vein endothelial cells. J Virol 77(21), 11822-32.

White, N.J. (1999) Variation in virulence of dengue virus. Lancet 354(9188), 1401-2.

Whitehead, S.S., Blaney, J.E., Durbin, A.P. and Murphy, B.R. (2007) Prospects for a dengue virus vaccine. Nat Rev Microbiol 5(7), 518-28.

WHO. (1997) Dengue haemorrhagic fever: Diagnosis, treatment, prevention and control. 2nd edition. Geneva : World Health Organization. 1997. 2nd edition. WHO. Geneva.

Wichmann, O., Hongsiriwon, S., Bowonwatanuwong, C., Chotivanich, K., Sukthana, Y. and Pukrittayakamee, S. (2004) Risk factors and clinical features associated with severe dengue infection in adults and children during the 2001 epidemic in Chonburi, Thailand. Trop Med Int Health 9(9), 1022-9.

Winkler, G., Maxwell, S.E., Ruemmler, C. and Stollar, V. (1989) Newly synthesized dengue-2 virus nonstructural protein NS1 is a soluble protein but becomes partially hydrophobic and membrane-associated after dimerization. Virology 171(1), 302-5.

Winkler, G., Randolph, V.B., Cleaves, G.R., Ryan, T.E. and Stollar, V. (1988) Evidence that the mature form of the flavivirus nonstructural protein NS1 is a dimer. Virology 162(1), 187-96.

Wissing, D., Mouritzen, H., Egeblad, M., Poirier, G.G. and Jaattela, M. (1997) Involvement of caspase-dependent activation of cytosolic phospholipase A2 in tumor necrosis factor-induced apoptosis. Proc Natl Acad Sci U S A 94(10), 5073-7.

Wittke, V., Robb, T.E., Thu, H.M., Nisalak, A., Nimmannitya, S., Kalayanrooj, S., Vaughn, D.W., Endy, T.P., Holmes, E.C. and Aaskov, J.G. (2002) Extinction and rapid emergence of strains of dengue 3 virus during an interepidemic period. Virology 301(1), 148-56.

Wu, S.F., Liao, C.L., Lin, Y.L., Yeh, C.T., Chen, L.K., Huang, Y.F., Chou, H.Y., Huang, J.L., Shaio, M.F. and Sytwu, H.K. (2003) Evaluation of protective efficacy and immune mechanisms of using a non-structural protein NS1 in DNA vaccine against dengue 2 virus in mice. Vaccine 21(25-26), 3919-29.

Wu, S.J., Grouard-Vogel, G., Sun, W., Mascola, J.R., Brachtel, E., Putvatana, R., Louder, M.K., Filgueira, L., Marovich, M.A., Wong, H.K., Blauvelt, A., Murphy, G.S., Robb, M.L., Innes, B.L., Birx, D.L., Hayes, C.G. and Frankel, S.S. (2000) Human skin Langerhans cells are targets of dengue virus infection. Nat Med 6(7), 816-20.

Xu, A., Bellamy, A.R. and Taylor, J.A. (1998) BiP (GRP78) and endoplasmin (GRP94) are induced following rotavirus infection and bind transiently to an endoplasmic reticulum-localized virion component. J Virol 72(12), 9865-72.

Xu, W., Liu, L., Charles, I.G. and Moncada, S. (2004) Nitric oxide induces coupling of mitochondrial signalling with the endoplasmic reticulum stress response. Nat Cell Biol 6(11), 1129-34.

Yang, K.D., Lee, C.S. and Shaio, M.F. (1995) A higher production of platelet activating factor in ex vivo heterologously secondary dengue-2 virus infections. Acta Microbiol Immunol Hung 42(4), 403-7.

Yon, C., Teramoto, T., Mueller, N., Phelan, J., Ganesh, V.K., Murthy, K.H. and Padmanabhan, R. (2005) Modulation of the nucleoside triphosphatase/RNA helicase and 5'-RNA triphosphatase activities of Dengue virus type 2 nonstructural protein 3 (NS3) by interaction with NS5, the RNA-dependent RNA polymerase. J Biol Chem 280(29), 27412-9.

You, S., Falgout, B., Markoff, L. and Padmanabhan, R. (2001) In vitro RNA synthesis from exogenous dengue viral RNA templates requires long range interactions between 5'- and 3'-terminal regions that influence RNA structure. J Biol Chem 276(19), 15581-91.

You, S. and Padmanabhan, R. (1999) A novel in vitro replication system for Dengue virus. Initiation of RNA synthesis at the 3'-end of exogenous viral RNA templates requires 5'- and 3'-terminal complementary sequence motifs of the viral RNA. J Biol Chem 274(47), 33714-22.

Yu, I.M., Zhang, W., Holdaway, H.A., Li, L., Kostyuchenko, V.A., Chipman, P.R., Kuhn, R.J., Rossmann, M.G. and Chen, J. (2008) Structure of the immature dengue virus at low pH primes proteolytic maturation. Science 319(5871), 1834-7.

Zhang, Y., Corver, J., Chipman, P.R., Zhang, W., Pletnev, S.V., Sedlak, D., Baker, T.S., Strauss, J.H., Kuhn, R.J. and Rossmann, M.G. (2003) Structures of immature flavivirus particles. Embo J 22(11), 2604-13.

Zilm, P.S., Bagley, C.J., Rogers, A.H., Milne, I.R. and Gully, N.J. (2007) The proteomic profile of Fusobacterium nucleatum is regulated by growth pH. Microbiology 153(Pt 1), 148-59.

Zivna, I., Green, S., Vaughn, D.W., Kalayanarooj, S., Stephens, H.A., Chandanayingyong, D., Nisalak, A., Ennis, F.A. and Rothman, A.L. (2002) T cell responses to an HLA-B*07-restricted epitope on the dengue NS3 protein correlate with disease severity. J Immunol 168(11), 5959-65.