

**Altered responses of Dengue virus  
infected cells to TNF- $\alpha$  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- $\alpha$ ) plays a significant role in the pathogenesis of DENV infection with elevated levels of TNF- $\alpha$  in the sera of DENV infected patients that parallel the severity of disease and release of TNF- $\alpha$  coincident with the peak of DENV production from infected monocyte-derived-macrophages (MDM) *in vitro*. However, the effect of TNF- $\alpha$  on DENV replication is not fully clarified. In this study we aimed to determine (1) the effect of TNF- $\alpha$  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- $\alpha$  in regulating DENV replication in MDM was investigated. While pre-treatment of MDM with TNF- $\alpha$  had a minor inhibitory effect, addition of TNF- $\alpha$  to MDM with established DENV-infection had no effect on DENV replication as measured by DENV RNA levels or virion production. Blocking endogenous TNF- $\alpha$  using TNF- $\alpha$  antibodies or TNF- $\alpha$  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- $\alpha$ . Additionally, normal cellular TNF- $\alpha$  signalling, measured by quantitation of TNF- $\alpha$ -induced stimulation of transcription from a nuclear factor-kappa B (NF- $\kappa$ B) responsive reporter plasmid or NF- $\kappa$ B protein nuclear translocation, was blocked in DENV-infected MDM. Thus, DENV replication in MDM is not affected by TNF- $\alpha$ , and infected cells do not respond normally to TNF- $\alpha$  stimulation. It is therefore unlikely that the increased production of TNF- $\alpha$  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- $\alpha$  seen in DENV-infection does not influence DENV replication (2) the cellular responses to TNF- $\alpha$  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

## Acknowledgements

I would like to firstly gratefully acknowledge my chief supervisor Dr Jillian Carr for her brilliant supervision. With her vast knowledge and much focussed manner of scientific thinking, she has been an admirable mentor. I would also like to thank my co-supervisors Professor Christopher Burrell and Dr Li Peng for their combined support and critical reading of this thesis.

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## ABBREVIATIONS

2D – 2 dimensional  
°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 diarrhoea 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<sub>2</sub>O – 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<sub>2</sub> and MgSO<sub>4</sub>  
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- $\kappa$ B – 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 – phorbol 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- $\alpha$  - tumor necrosis factor alpha  
 $\mu$ g – microgram  
 $\mu$ M – 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

- 2007      **Satiya Wati, Peng Li, Christopher Burrell, Jillian Carr.**  
Australian Society of Virology meeting, Fraser Island, Queensland  
9-13th December. Poster presentation. Abstract # 244  
**Cellular responses to dengue virus (DENV) infection (*in vitro*) studies**
- 2007      **Satiya Wati, Peng Li, Christopher Burrell, Jillian Carr**  
Australian Society of Microbiology meeting, Adelaide  
9-13<sup>th</sup> July. Poster presentation. Abstract # 14.12  
**Dengue virus (DV) replication in monocyte derived macrophages is not affected by TNF alpha, and DV infection induces altered responsiveness to TNF alpha stimulation. The role of exogenous TNF alpha in dengue virus replication**
- 2004      **Satiya Wati, Jillian Carr, Peng Li, Christopher Burrell**  
Australian Society of Virology meeting, Philip Island, Victoria  
9-12<sup>th</sup> December. Poster presentation. Abstract # 244  
**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 Disposito, 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- $\alpha$  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- $\alpha$  release coincides with the peak of DENV production from infected MDM (Carr *et al.*, 2003) and high levels of TNF- $\alpha$  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