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

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
9-13th 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
9-12th December. Poster presentation. Abstract # 244
The role of exogenous TNF alpha in dengue virus replication

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