THE IMPACT OF INNATE IMMUNE CELLS ON IMMUNOPATHOLOGY IN DENGUE

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D.PHIL

October 2014

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ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. Kerstin Luhn for her unending support throughout my D.Phil, I could not have come this far without it! I am especially grateful for her compassion in helping me through the hardest times over the last few years.

I would also like to thank my co-supervisor Prof. Benedikt Kessler for the collaboration on mass spectrometry analysis and for all his help and advice, especially in the lead up to submission.

I am very grateful to Prof. Vincenzo Cerundolo for his support during the final stages of my D.Phil, especially after my funding ran out! I really appreciate that he always had time to help me out, even with such a busy schedule.

I am also thankful to the Rehwinkel lab for all their advice and guidance throughout the final stages of my research, it was very much appreciated and I will miss working on the bench next door.

I also want to mention all my family and friends who were so patient over the years when I never had time to spare for meeting up or had to cancel at the last minute because of my ever changing schedule. I couldn't have completed this D.Phil without their constant support and understanding.

Finally, I would like to dedicate all my work over the last few years to my Mother who I only wish was here to see me finish. I know she would be very proud and I just hope I do her justice....

ABSTRACT

Dengue virus (DENV) is an arthropod-borne virus and has become a worldwide problem with steadily rising annual infection rates. Patients present with a range of symptoms from mild fever to, in some cases, life-threatening hemorrhagic fever and shock syndrome. The most severe cases require emergency hospital care and currently, there is no effective drug treatment or vaccine for dengue. As severe symptoms appear post-peak viremia, immuno-pathology is thought to be the cause and a potential trigger of this is differential activation of the immune response upon recognition of DENV. This could be due to a combination of factors including varying receptors, signalling pathways and immune regulation mechanisms. In order to understand DENV infection better, it is imperative to study the mechanisms of activation and control of immune responses triggered by the virus.

Very early events in viral infection (after 10 min stimulation) were studied aiming to identify proteins involved in differential activation of immune responses. Phosphorylated proteins were isolated from cells post-stimulation and analysed by mass spectrometry. More than 200 proteins were differentially regulated by phosphorylation in response to DENV stimulation as compared to Mock, Influenza A virus and LPS stimulation.

The effect of two specific proteins, namely Calpain-2 and Importin-5, identified to be differentially phosphorylated was investigated further. Calpain-2 was seen to be vital in the efficient production of progeny virions and the transcription of Mx1, an anti-viral interferon stimulated gene. Importin-5 is known to transport DENV NS5 into the nucleus during infection and was seen to co-precipitate with many host proteins.

In summary, it is imperative that novel treatments and vaccines are developed for dengue as it is one of the world's most prevalent arthropod-borne viruses. It was discovered here that many proteins undergo phosphorylation/de-phosphorylation in response to DENV stimulation to a differing degree than other stimuli. Calpain-2 plays a vital role DENV infection, potentially influencing the potency of immune response. Importin-5 associates with various host proteins during DENV infection, potentially altering their function or the function of Importin-5 itself. Research into targeted inhibition of Calpain-2 function or Importin-5 interaction with DENV NS5 could lead to a successful anti-viral treatment for DENV infection.

ABBREVIATIONS

µA	Microamp
µg	Microgramme
µM	Micromolar
Ab	Antibody
ADE	Antibody dependent enhancement
ATP	Adenosine triphosphate
AIF	Apoptosis-inducing factor
APC	Antigen presenting cell
BSA	Bovine serum albumin
C	Capsid protein
CD	Cluster of differentiation
cDNA	Complimentary DNA
CLEC5A	C-type lectin domain family 5 member A
CXCL	Chemokine (C-X-C motif) ligand
DAB	3,3'-diaminobenzidine
DC	Dendritic cell
DC-SIGN	DC-specific intercellular adhesion molecule-3-grabbing non-integrin
DENV	Dengue virus
DF	Dengue fever
DHF	Dengue hemorrhagic fever
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
DSS	Dengue shock syndrome
DTT	Dithiothreitol
E	Envelope protein
EDTA	Ethylene diamine tetra acetic acid
EGFR	Epidermal growth factor receptor
ELISA	Enzyme linked immunosorbent assay
ER	Endoplasmic reticulum
ESI	Electrospray ionisation
FACS	Fluorescent activated cell sorter
FCS	Foetal calf serum
FFU	Focus forming unit
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte-macrophage colony stimulating factor
GDP	Guanosine diphosphate
GTP	Guanosine triphosphate
HI	Heat inactivated
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
HSP	Heat shock protein
IAV	Influenza A virus
ICAM	Intercellular adhesion molecule
IEF	Iso-electric focussing
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin

IP	Immuno-precipitation
IRF	Interferon response factor
ISG	Interferon stimulated gene
IPG	Immobilised pH gradient
IPS	Interferon-β promoter stimulator
JE	Japanese encephalitis
kDa	Kilodalton
LC	Liquid chromatography
LPS	Lipopolysaccharide
M	Membrane protein
MACS	Magnetic cell sorting buffer
MDA	Melanoma differentiation-associated protein 5
MDDC	Monocyte derived dendritic cell
MEM	Minimal essential medium
mg	Milligramme
MIP	Macrophage inflammatory protein
ml	Millilitres
mM	Millimolar
MOI	Multiplicity of infection
mRNA	messenger RNA
MS	Mass spectrometry
NDV	Newcastle disease virus
NLS	Nuclear localisation signal
NS	Non-structural protein
ORF	Open reading frame
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PE	Phycoerythrin
PerCP-Cy5.5	Peridinin chlorophyll protein-cyanine5
PSM	Peptide sequence match
PVDF	Polyvinylidene difluoride
qPCR	Quantitative PCR
RIG-1	Retinoic acid-inducible gene 1
RNA	Ribonucleic acid
SARS-CoV	Severe acute respiratory syndrome-coronavirus
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
siRNA	Small interfering RNA
STAT	Signal transducer and activator of transcription
TBST	Tris-buffered saline-tween20
TGN	Trans-golgi network
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TPCK	Tosyl phenylalanyl chloromethyl ketone
tRNA	Transfer RNA
WCL	Whole cell lysate
WHO	World health organisation

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CHAPTER ONE - INTRODUCTION

Dengue - Overview

Dengue virus (DENV) is emerging as one of the fastest spreading arboviruses, most prevalent in tropical and subtropical areas as seen in Fig. 1, causing approximately 390 million dengue cases per year of which nearly 100 million present with symptomatic disease (Bhatt et al., 2013).

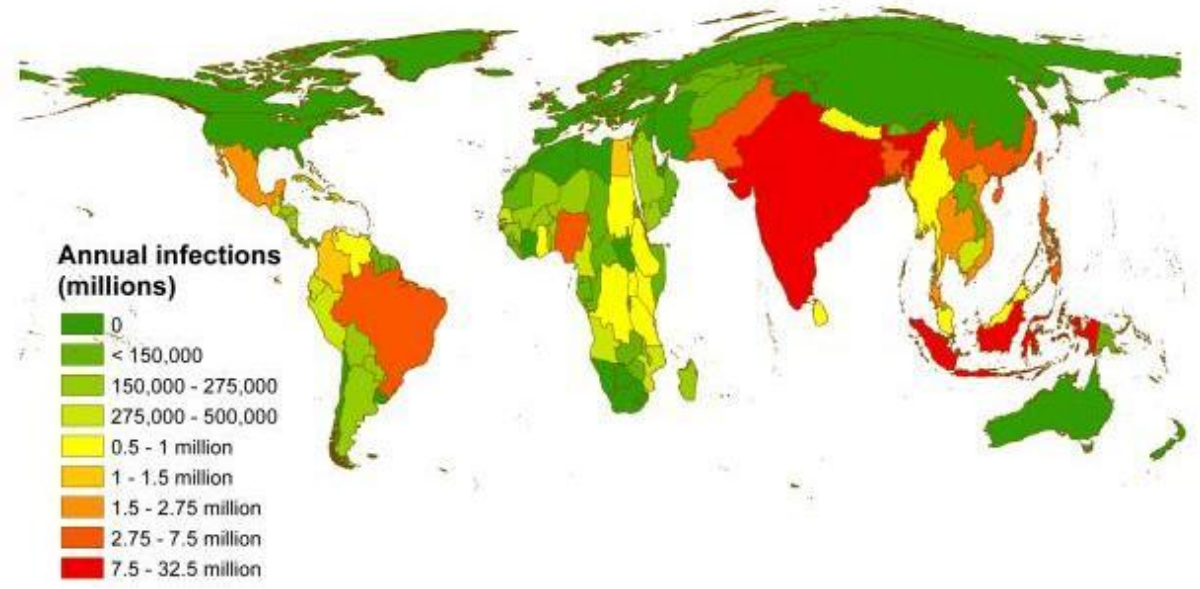


Fig. 1: ***Distribution of annual reported cases of dengue***
Dengue is most prevalent in sub-tropical and tropical areas with the vast majority of reported cases appearing in these areas (source: WHO)

These figures far exceed the WHO estimate of dengue burden which falls at approximately 100 million cases annually with around 500,000 requiring hospital treatment (source: Dengue Guidelines for diagnosis, treatment, prevention and control, New Edition, WHO, 2009).

Symptomatic dengue ranges from mild dengue fever (much like Influenza symptoms) to the potentially life threatening dengue hemorrhagic fever or shock syndrome. In the most severe cases, patients experience plasma leakage and cytokine storm leading to hypovolemic shock which can only be treated by fluid replacement therapy. At the present time there are no antiviral drugs or other treatment options available for dengue, neither is there a vaccine to reduce the risk of infection.

The virus is transmitted primarily by *Aedes* aegypti mosquitoes (with *Aedes* albopictus spreading virus to a lesser extent) where it replicates within the mid-gut and subsequently migrates to the salivary glands from where it is passed into humans during the process of an infectious bite. The virus is delivered subcutaneously and therefore the primary target cells are thought to be immature dendritic cells in the skin, likely followed by monocytes/macrophages later in infection (Kyle et al., 2007; Schmid and Harris, 2014).

Dengue viruses can be one of four serotypes (DENV 1-4) and these share 60-70% genetic homology. Infection with one type of DENV affords life-long immunity to that specific serotype however, not to subsequent infection with a differing serotype. As hemorrhagic fever emerges post the point of peak viremia and oftentimes secondary heterologous infection correlates with the most severe cases of DENV infection, this suggests immuno-pathology has a role in causing the more serious symptoms of disease. The most severe form of DENV infection is commonly attributed to the phenomenon named ‘antibody dependent enhancement’ (Halstead et al., 1970) whereby antibodies generated in response to the initial serotype of virus are able to opsonise virus particles of other serotypes but do not meet the threshold of neutralisation. This leaves two possibilities, either the opsonised virions gain an additional route of entry into target cells (innate immune cells such as dendritic cells and macrophages) via Fc receptors and

phagocytosis leading to an increased viral load and subsequently a heightened immune response, or another possibility is that this alternative method of entry could in itself trigger a variation in immune response which may alter the subsequent outcome of infection (Halstead et al., 2010; Ubol and Halstead, 2010). Possible variations include differential activation of pathways in response to opsonised virions (rather than virus particles alone) (Modhiran et al., 2010), varying methods of physical entry resulting in differential localisation of virus particles within the cell or the interaction of antibody coated virions with proteins in the cell which do not recognise virion alone.

Clinical features of dengue disease

Dengue can present with a large range of symptoms from asymptomatic disease to the most severe cases of shock and hemorrhagic fever. The virus has an incubation period of around 4-10 days (Althouse et al., 2014) and milder symptoms usually last for 3-7 days, with severe symptoms appearing between 3-7 days after onset of initial symptoms.

The mildest form of symptomatic dengue is known as dengue fever which is characterised by high temperature and other symptoms which vary between patients, including muscle and joint pain, nausea and vomiting, rash, severe headache, pain behind the eyes and swollen glands.

Severe dengue is characterised by a subsequent decrease in temperature and can be identified by a range of symptoms including persistent vomiting, severe abdominal pain, bleeding gums, blood in vomit, rapid breathing, fatigue and restlessness. These symptoms are caused by plasma leakage, severe bleeding, fluid accumulation and organ impairment and can be fatal if left untreated (source: WHO Dengue Control Program).

Management

There are currently no preventative vaccines for dengue and no effective treatments for this disease. Many dengue cases are self-limiting illnesses which do not require specialised treatment however, cases which progress to hemorrhagic fever or shock syndrome need specific hospitalised care. It is imperative that severe dengue is diagnosed as quickly as possible in order to enhance the chance of survival.

Patients presenting with mild symptoms and no warning signs of severe disease can manage their condition at home with increased fluid intake until symptoms are reduced. In the case of more severe symptoms which suggest the onset of severe disease, patients are referred to hospital where they are monitored and encouraged to take fluids orally or if this is not possible intravenously until the period of risk is over. Patients who progress to severe disease require emergency treatment in hospital which consists of continual maintenance of fluid levels until the patient recovers from severe symptoms. There are no other treatment options and this approach is effective in most cases.

It is thought that approximately 2.5% of patients admitted to hospital with severe DENV infection will die of the disease.

Prevention and control

In order to combat DENV spread, various measures have been taken to reduce the ability of mosquitoes to breed/spread disease. Methods such as using mosquito nets, insect repellent and reducing the amount of stagnant water lying around have been encouraged in endemic areas. Also, methods to control the mosquito population biologically have been employed in certain areas such as release of *Wolbachia* bacteria

which symbiotically infect mosquitoes and inhibit DENV replication, thereby resulting in a lower level of disease transmission.

The biggest effort in managing DENV is centred around controlling the mosquito population and limiting vector-host interaction. This is done by frequent emptying of water containers reducing the amount of stagnant water available for breeding sites, installation of water distribution systems removing the need for outdoor water containers and use of mosquito screens on doors and windows alongside using mosquito nets whilst sleeping.

These methods are capable only of reducing the rate of spread and infection therefore it is imperative that treatments and vaccines are formulated to aid the elimination of this disease. The range of serotypes and the fact that immunity is not cross-effective between them is a major obstacle to vaccine development as any effective vaccine must provide neutralising efficacy for all four serotypes simultaneously. Also, the lack of an animal model which adequately correlates to infection in humans and the lack of understanding relating to identification of what constitutes effective protection *in-vivo* are proving to be difficult obstacles to overcome.

The most promising candidate so far has been a tetravalent chimeric virus with a yellow fever backbone containing DENV prM and E proteins which reached phase IIb clinical trials and results have recently been reported on the phase III trial. The vaccine is known as CYD TDV and has been developed by Sanofi Pasteur (Wilder-Smith, 2014). Phase III trials include testing in multiple countries on over 10,000 children using an immunisation schedule of one dose in months 0, 6 and 12.

The vaccine showed relatively high levels of efficacy against DENV 3 and 4 however DENV 1 and 2 were not so well neutralised. Conferring adequate protection

against serotype 2 was most challenging and remains to be accomplished with this vaccine.

It was positive to note that the vaccine did not elicit severe responses from participants and does not seem to prime for ADE upon subsequent exposure to DENV, though longer term analysis needs to be carried out to ensure priming does not occur later in life.

It seems from these results that the vaccine is much more effective in individuals who have encountered DENV in the past and therefore have primed immune systems which are boosted in response to this vaccine. The efficacy for DENV naive individuals is significantly lower and therefore this vaccine would be more suited to areas where DENV is endemic rather than being available to those wishing to travel to at risk areas. However, it shows a very high level of protection against severe disease (approx 80%) even if it is not particularly protective in regard to primary DENV 2 infection and therefore would still be of immense benefit in reducing the number of cases of severe dengue and therefore the number of deaths occurring from infection.

Dengue virion

The DENV virion is made up of a single-stranded RNA genome, surrounded by capsid protein and encased in a membrane containing the structural proteins M (membrane) and E (envelope) in association with host derived lipids. The virions are either immature, partly mature or fully mature (see Fig. 2), with a high percentage of immature virions thought to be released in DENV infection (van der Schaar et al., 2007). The status of maturity is determined by the configuration of the envelope and membrane proteins (Junjhon et al., 2010).

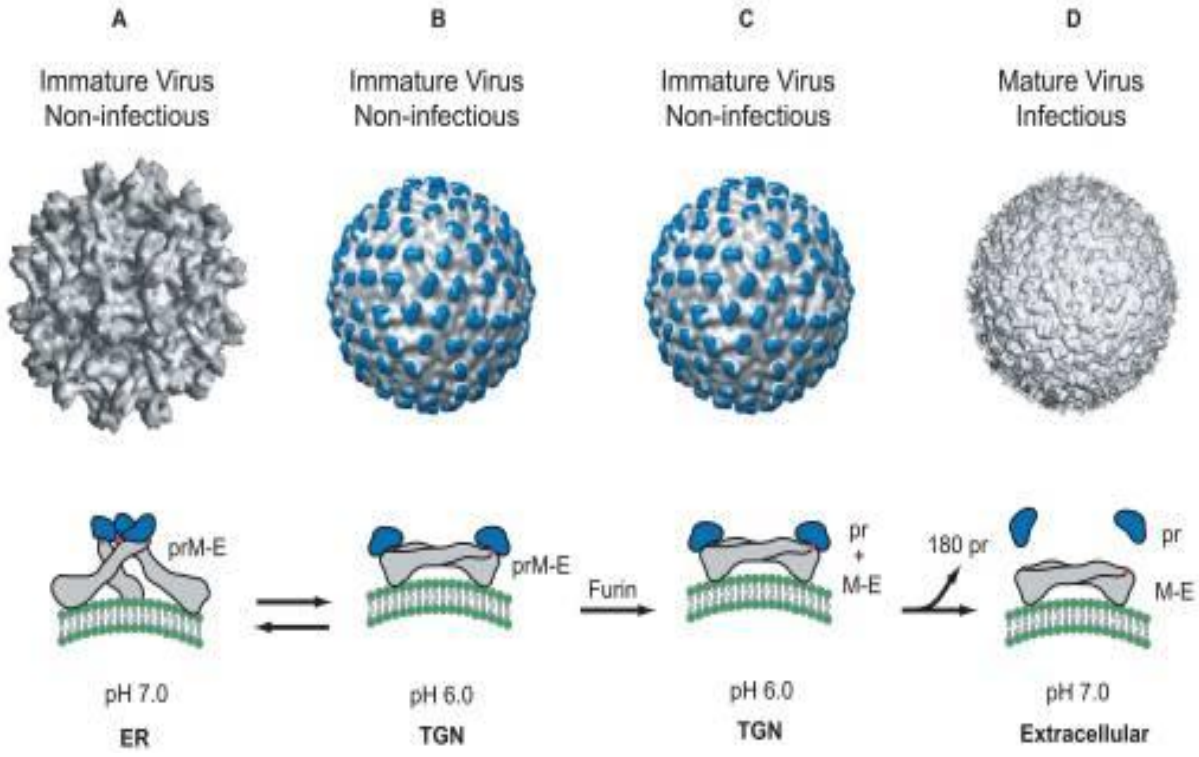


Fig. 2: ***Maturation of DENV particles***
DENV virions are initially produced in an immature state and throughout maturation prM is cleaved by host protein Furin, resulting in removal of the pr fragment leaving mature M protein and E protein only. This cleavage allows the surface proteins to alter from a spiked to a smooth arrangement and renders the virion infectious (Perera and Kuhn, 2008)

The DENV genome is encased within these virions and is a single-stranded, positive-sense RNA genome. It is approximately 11 kb in size and is transcribed as a polyprotein encoding 10 viral proteins (see Fig. 3). These are split into 3 structural (C (capsid), M (membrane) and E (envelope)) and 7 non-structural (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) proteins.

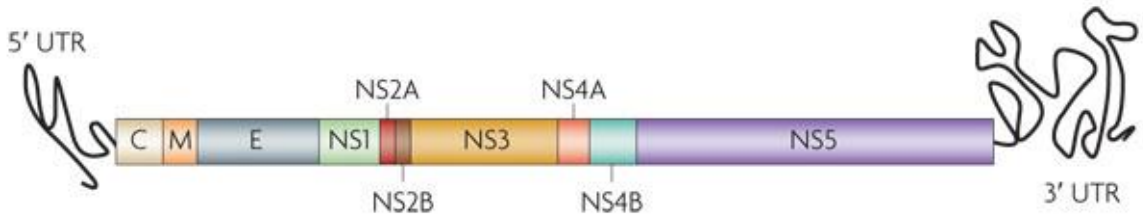


Fig. 3: ***DENV genome***
The DENV genome comprises a single ORF (open reading frame) and encodes 10 proteins, 3 structural and 7 non-structural **(Guzman et al., 2010)**

Viral infection

DENV is recognised at the cell surface by a variety of receptors including DC-SIGN (presented highly on immature dendritic cells, which are the initial target cells of DENV), mannose receptor and CLEC5A. Viral entry is thought to be via clathrin-mediated endocytosis (Acosta et al., 2008) whereby upon recognition at the cell surface, clathrin molecules gather around the virion forming clathrin coated pits in the membrane. The membrane subsequently indents into the cell before budding off into the cytoplasm as an endosome containing the virion. These early endosomes have a pH of approximately 6-6.5 and travel through the cytoplasm, with a gradually lowering pH throughout maturation of the endosome by loss of Rab-5 (which blocks acidification) and gain of Rab-7. Once the endosomal pH reaches a threshold level of acidity (low enough pH) the viral membrane fuses with the endosomal membrane, releasing the RNA genome into the cytoplasm, where viral replication takes place (Acosta et al., 2012; Salonen et al., 2005; Smit et al., 2011). Replication proceeds via a negative sense single stranded RNA intermediate which goes on to serve as a template for more positive sense RNA genomes. The genome is both transcribed into templates for more genomes and translated into a polypeptide simultaneously, leading to efficient viral replication utilising both host and viral proteins.

Replication takes place in the cytoplasm and viral assembly occurs in ER associated virus induced membrane compartments (Welsch et al., 2009). These compartments are thought to contain viral proteins and viral RNA and they could serve to concentrate components of progeny virions, allowing more efficient replication, whilst protecting virion production from host cell immune defences (Chatel-Chaix and Bartenschlager, 2014). Membrane proteins are produced as a precursor and at a late stage of progeny release prM is cleaved by a host protein called Furin (Zheng et al., 2014). This process leads to release of progeny in a fully infectious mature form rather than immature non-infectious particles being released whereby cleavage has not been completed fully.

Dengue virus receptors

There are a number of cell surface molecules that have been shown to play a part in recognition, adsorption, initiation of signalling cascades and entry of DENV particles (see Fig. 4) with each individual receptor showing different profiles on various cell types and varying degrees of recognition between DENV serotypes. These receptors are described to have a role in infection in varying capacities ranging from purely recognition, to concentration of virus particles on the cell surface, to instigating the cellular uptake of virions. This variation suggests that DENV uses a complex combination of receptors during infection leading to various outcomes depending on the environment of infection e.g. the cell type targeted and serotype of virus inoculated.

Receptor	Properties	Cell/Tissue expression	Serotype
Heparan sulfate	Sulfated glycosaminoglycan	Vero cells, BHK-21 cells SW-13 cells	DENV1–4
nLc_4Cer	Glycosphingolipid	Vero cells, BHK-21 cells, K562 cells	DENV1–4
DC-SIGN/L-SIGN	Dendritic cell-specific lectin, CD209	Dendritic cells, Macrophage	DENV-1–4
Mannose receptor	Protein with lectin activity	Macrophage	DENV-1–4
HSP70/HSP90	Expression on plasma membrane Heat-shock proteins	HepG2 cells, SK-SY-5Y cells, Macrophage	DENV-2
GRP78	Expression on plasma membrane Chaperon	HepG2 cells	DENV-2
Laminin receptor	High-affinity laminin receptor MW: 37/67 kDa	PS Clone D cells, HepG2 cells	DENV-1–3
CD14-associated protein	Protein associated with LPS receptor	Monocyte, Macrophage	DENV-2
Unknown glycoproteins	CHO-dpd binding MW: 44/74 kDa	Vero cells	DENV-4

Fig. 4: ***Overview of receptors involved in DENV infection***
There are a number of cell surface receptors which have been identified to play a role in DENV infection in varying capacities, a selection of these are shown here (Hidari and Suzuki, 2011)

These receptors may play a vital role in the outcome of infection as they not only have an effect on the efficiency of infection but can also trigger numerous cell signalling pathways within the cell upon recognition of viral epitopes. These include immune response pathways which ultimately influence the quality and quantity of the immune response mounted by infected cells.

It is also interesting to note that glycosylation is an important post-translational modification in DENV infection whereby viral proteins are glycosylated in the ER/Golgi and this has an effect on subsequent receptor recognition and viral function (Sun and Kochel, 2013) . These effects can lead to differences in DENV pathogenicity in different cell types, for example glycosylation of E protein is required for DENV recognition and association with DC-SIGN (Mondotte et al., 2007).

The most notable cell surface receptors involved in DENV infection and those which could play a role in the triggering of severe immuno-pathology are described below.

Lectins:

DC-SIGN

DC-SIGN is the primary DENV receptor on dendritic cells and is expressed primarily on immature DC. DC-SIGN is very highly expressed on immature DC but during the process of maturation, DC-SIGN expression on the cell surface is substantially down-regulated.

DC SIGN recognises N-glycosylation on the E-protein of DENV. E protein is blocked by prM on immature virions hence the need for Furin cleavage to occur in order for DENV to be infectious (Zybert et al., 2008).

Although immature DENV particles are considered non-infectious it has been shown that infectivity can be 'rescued' (albeit at a low level) by interaction with DC-SIGN (Richter et al., 2014). Infection of immature DC with immature DENV may play a role in increasing viremia levels and subsequent increase in immune responses.

This could play a role in the high levels of immuno-pathology as if particles that are normally not able to establish infection can begin to infect, this will lead to a higher viral load and subsequently higher levels of immune response. This could then result in a more severe immune response and subsequently trigger hemorrhagic fever and shock syndrome given DC-SIGN is expressed on target cells (Tassaneetrithep et al., 2003).

On this note, it is also important to consider that immature virions are recognised by antibodies at the same rate as mature particles and therefore, when opsonised upon

secondary infection they may be taken up by phagocytes such as DC and rendered infectious (da Silva Voorham et al., 2012), thereby contributing to viral load and infection levels whereas during a primary infection with no pre-existing, cross-reactive antibodies they would be non-infectious. This process is shown to require the presence of host cell Furin within endosomes used by virus for entry to the cell (Rodenhuis-Zybert et al., 2010). Whilst Furin is predominantly found in the TGN (where immature virions are matured before exit), some is also seen shuttling between early endosomes and the cell surface. If Furin is captured within endosomes used by DENV for entry, the cleavage event required for maturation can occur here rendering immature progeny infectious in secondary DENV infection.

CLEC5A

CLEC5A is a receptor found on the surface of macrophages which has been shown to have a role in signalling pathways upon recognition of DENV (Kingeter and Lin, 2012) but that does not have a role in viral infection levels (i.e. it is not thought to be an entry receptor). The existence of such receptors gives strength to the hypothesis that there may be receptors or a combination of receptors which contribute to severe disease by eliciting a different immune response compared to that triggered by viral recognition and entry via expected DENV receptors such as DC-SIGN. CLEC5A has effects on cytokines released in response to recognition of DENV at the cell surface and this in turn has an effect on the immuno-pathology induced by the virus (Chen et al., 2008). It could be that a certain combination of specific receptors/co-receptors and certain DENV serotypes, results in severe DENV infection due to differential profiles of cytokine secretion by target cells.

Mannose receptor

Mannose receptor has also been described as a receptor for DENV and recognises glycosylated residues on the viral E protein (Miller et al., 2008). This receptor seems to have a role in viral recognition and replication but does not seem to elicit immune signalling events. Nonetheless, differential interaction of DENV with this receptor could increase or decrease viral titre, potentially leading to increased infectivity and subsequently increased immune responses.

As seen above there are an array of receptors described to recognise DENV at the cell surface. This may be important in the study of early signalling events as various receptors might initiate varying signalling pathways and therefore different receptors or combinations of receptors used in each individual case of DENV, or used in DENV but not in other viral infections, could trigger differing outcomes of infection. This study may highlight proteins which are phosphorylated differentially due to recognition of DENV by a specific set of receptors which is not utilised in recognition of other viruses such as Influenza A virus (IAV).

There are also a number of co-receptors for DENV including carbohydrate containing molecules like glycosphingolipids and glycosaminoglycans (Lee et al., 2006), these can theoretically contribute to the aforementioned hypothesis of differential immune activation along with their function of increasing the efficiency of viral infection.

Hepran Sulphate

This glycosaminoglycan is used by many viruses such as Herpes Simplex virus (Shuklaet al., 1999), HIV (Patelet al.,1993), Vaccinia virus (Linet al.,2000), Papillomavirus (Joyceet al.,1999), RSV (Feldmanet al.,2000; Hallaketal.,2000), and Echovirus (Goodfellowet al.,2001). DENV has also been shown to associate with hepran sulphate (Chen et al., 1997), again showing the importance of glycosylation of viral proteins in DENV, as the virtual elimination of binding activity between DENV and hepran sulphate was demonstrated using competing binding molecules to block infection. As this receptor is thought to function by concentrating viral particles at the cell surface, this interaction is likely to be a key player in viral entry/pathogenesis and subsequent infectivity (Chen et al., 1997).

It is also interesting to note that DENV has been proposed to activate immune response pathways in the absence of recognition by specific receptors for RNA/viral membrane proteins. It is thought that the mechanism of membrane fusion in some way triggers innate immune responses by stimulating interferon genes via STING protein (Holm et al., 2012). Novel mechanisms of immune response activation like this could well play a part in increased immuno-pathology seen in dengue patients compared to those infected with other viruses. This study could highlight phosphorylated proteins involved in such pathways, potentially leading to discovery of differential immune activation pathways.

Immune response

Once the virus has been recognised at the cell surface, signalling cascades have been triggered by receptors and viral particles are anchored to the surface in preparation for entry into target cells, the first line of defence is innate immunity. This is especially interesting with respect to DENV as the primary target cells are dendritic cells, the very drivers of the innate immune response (Schmid et al., 2014).

DENV infects a range of cell types including monocytes (Durbin et al., 2008), dendritic cells and macrophages (Kou et al., 2008). It is these cells which transport viral antigen to the lymph nodes to be presented and which shape the subsequent adaptive immune response (Mellman, 2013). Meanwhile, they also mount the initial innate response which involves instigating an 'antiviral state' within cells. This is achieved by the release of chemokines and cytokines such as CXCL10 and TNF-alpha (Dejnirattisai et al., 2008). These molecules serve many functions including attraction of more immune cells to the area of infection and triggering activation of transcription factors encoding proteins which help to fight virus such as interferon stimulated genes. As it is the target cells of DENV infection that instigate immune responses, there are many opportunities for the virus to evolve mechanisms to enhance infectivity which may or may not shape the immune response in its favour. Differential regulation of immune responses to DENV infection may well contribute to severe immuno-pathology and therefore, studies into the basic science of DENV infection are imperative in the development of vaccines and treatments to eliminate disease.

Immune evasion

As mentioned previously, one of the first host defence mechanisms employed in response to viral detection is mounting of the innate immune response. This leads to triggering of the adaptive response and results in priming of the immune system to react readily in the case of secondary infection (Iwasaki and Medzhitov, 2010).

The ability of host cells to control infection is challenged by virally induced methods of subverting the immune response. A vital part of innate immunity is the interferon response. This is mediated by both Type 1 and 2 interferons and results in the aforementioned 'antiviral state' in cells. Type 1 interferons, namely IFN α/β, are produced and secreted by dendritic cells and their function is to inhibit viral pathogenesis. They can act within cells to up/down regulate many transcription factors leading to the production of proteins with virus inhibiting functions. They also act extracellularly by attracting more innate immune cells to the site of infection, allowing a stronger immune response to be mounted and quicker viral clearance (Samuel, 2001).

Interferon response has been shown to be vitally important in DENV infection and this has been illustrated by way of *in-vivo* mouse studies illustrating that mice are not susceptible to DENV unless interferon receptors on cell surfaces are knocked out (or STAT2 is knocked out) (Ashour et al., 2010; Shresta et al., 2005). Many viruses have evolved the ability to subvert the interferon response as it is a key player in the course of infection (Pagni and Fernandez-Sesma, 2012). DENV too has evolved the ability to inhibit this host cell defence mechanism. It has been shown that DENV NS4B plays a role in inhibition of interferon stimulated gene expression (Muñoz-Jordan et al., 2003). Also, DENV NS2B3 protein plays an active role in the inhibition of the Type 1 interferon response by reducing the phosphorylation of IRF3 (Morrison et al., 2012). This results in

a lack of IFNα/β production by the cell. As reported by Rodriguez-Madoz et al., the antagonistic effect is demonstrated as dendritic cells infected with Newcastle Disease Virus (NDM) are able to produce IFNα/β as normal, however if the cells are infected with DENV prior to NDV the IFNα/β are not produced to the same level (Rodriguez-Madoz et al., 2010a). This illustrates that DENV infection is responsible for this immune evasion mechanism, perhaps using a soluble mediator. It has been shown that in contrast to dendritic cells infected only with NDV, those infected with DENV and those infected with DENV followed by NDV had a reduced level of IRF3 phosphorylation. The inhibition of interferon responses occurs within 2 hr of infection and this suggests that an early event in viral infection is responsible for this effect. It was also reported that the DENV NS2B3 protein is responsible for inhibition with only the protease portion of NS2B3 required for reduction of Type 1 interferon production.

The lower levels of interferon secretion resulting from this inhibition reduces the potency of the interferon response on both infected and bystander cells. This in turn reduces the activation of the Jak/STAT pathway which is activated in response to Type 1 interferon binding to Type 1 interferon receptor (IFNAR) on the cell surface.

Jak/STAT pathway

The Jak/STAT pathway is triggered when Type 1 interferon is detected on the cell surface by IFNAR and this docking leads to activation of Janus Kinase 1 (JAK1) and Tyrosine Kinase 2 (TYK2) (see Fig. 5 below).

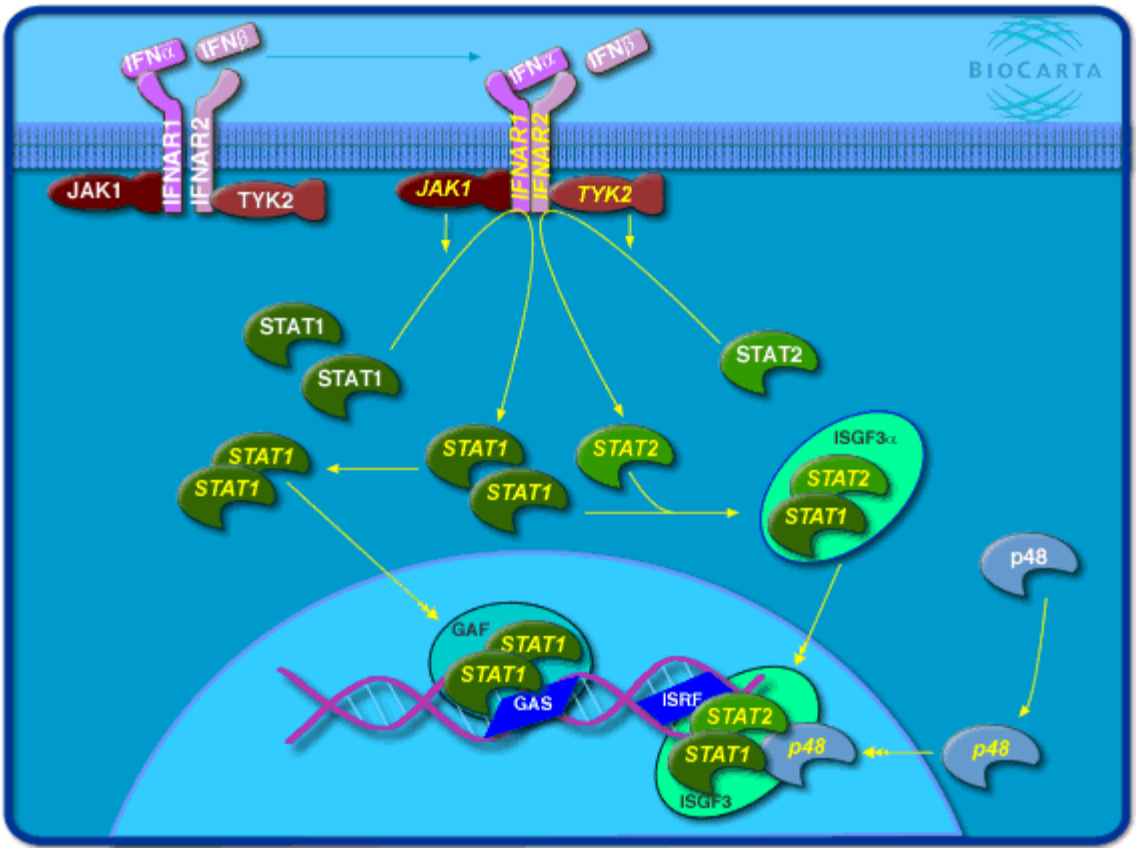


Fig. 5: ***Illustration of proteins involved in the Jak/STAT pathway***
Type 1 interferons are recognised at the cell surface by interferon receptors and subsequently a phosphorylation cascade results in modification of transcription of many genes encoding proteins involved in the immune response. Any alteration in the regulation of this pathway could potentially alter the immune response to viral infection (source: Biocarta pathways)

Both of these kinases facilitate the phosphorylation of STAT1 and STAT2 which in turn associate with IRF9 and this complex is known as interferon-stimulated gene factor 3 (ISGF3). The ISGF3 complex is then able to interact with interferon stimulated response elements (ISRE) corresponding to interferon stimulated genes, these IRSEs facilitate up-regulation of transcription of interferon stimulated genes (Morrison and García-Sastre,

2014). Activation of the Jak/STAT pathway leads to up-regulation of transcription of numerous interferon stimulated genes (Darnell et al., 1994) such as Mx1 (Haller and Kochs, 2002).

DENV NS2B3 not only inhibits signalling via Type 1 interferons but also has an effect on synthesis of Type 1 interferon, this occurs via a protein called STING which resides within the ER and is involved in RIG-I and IPS-1 pathways. Cleavage of STING leads to reduced ability of the cell to control early events in virus production as IPS-1 is involved in Type 1 interferon production and the control of early events in DENV pathogenesis (Aguirre et al., 2012). Interestingly, mouse STING protein is not affected by DENV which may contribute to the lack of susceptibility of mice to DENV infection.

The immune evasion mechanisms described above have been attributed to DENV NS2B3, however non-structural proteins NS2A, NS4A, NS4B and NS5 have also been implicated in antagonising the interferon response pathway. As more than one viral protein is involved it is likely to be an important mechanism for viral pathogenesis.

For example, DENV NS5 protein has been shown to cause degradation of STAT2 in humans with no effect on STAT2 equivalents in mice. Also, DENV infected cells show lower levels of STAT2 protein within the cell (Ashour et al., 2009; Jones et al., 2005; Morrison et al., 2012).

The reduction in production and secretion of IFN α/β and the effect of DENV non-structural proteins on molecules acting downstream of interferon recognition has effects on subsequent immune activation mechanisms as T-cell priming relies on cytokine secretion by infected innate immune cells (Rodriguez-Madoz et al., 2010b). This means that the ability of DENV to subvert innate immune responses carries over into the

adaptive response which as a result is weakened, providing a more DENV permissive environment and altered immune activation.

These differences between murine and human hosts show that the targeting of innate immune responses by DENV in humans potentially holds the key to productive infection and perhaps in turn, de-regulated immune responses.

Interferon stimulated genes

The up-regulation of gene transcription by interferon stimulated processes has a vital role in clearance of viral infection. In order to successfully clear infection, host cells must initiate an antiviral state which reduces infectivity. This involves the up-regulation of transcription factors which induce increased transcription of antiviral genes. These lead to the production of proteins which aid clearance of virus. For example, Mx1 is a potent antiviral which plays a key role in clearance of many viruses including those belonging to the Orthomyxovirus and Bunyavirus families (Haller et al., 1998; Verhelst et al., 2013; Xiao et al., 2013).

Mx1 has also been seen to be up-regulated in response to DENV and this is thought to contribute to establishment of an antiviral state within cells (Sariol et al., 2007).

Mx1 was discovered in the context of Influenza virus whereby it was seen to be down regulated in response to infection, along with the down-regulation of interferon responses (Haye et al., 2009). This could be a method utilised by the virus to evade the immune response and increase levels of infectivity. Virus evolved mechanisms to down-regulate production of antiviral proteins like Mx1 show that these pathways must play a key role in the outcome of infections and are therefore interesting targets for further study

into the immune response of host cells to viral infection (Hoenen et al., 2014; Menachery et al., 2014). These studies could shed light onto how infections are controlled and the resulting immuno-pathology caused.

Viral evasion methods

Another mechanism utilised by DENV, and indeed many other viruses, is to promote the production of ER-derived membrane structures within which many viral non-structural proteins, double stranded RNA and newly synthesised genomes are situated after production (Miller and Krijnse-Locker, 2008). This allows the viral protein within the cell to avoid contact with host cytoplasmic pattern recognition receptors such as RIG-I and MDA-5 and therefore inhibits the induction of cellular response to the presence of foreign substances. These membranes can also function to concentrate the viral proteins in one place allowing efficient progeny assembly and subsequently increased viremia (Morrison et al., 2012).

Also, apoptosis has been identified to occur in response to DENV (Arias et al., 2014; Liao et al., 2010; Martins et al., 2012) usually via the extrinsic pathway which is triggered by extra-cellular ligands of surface receptors which induce apoptotic pathways (but it can also occur via the intrinsic pathway which is triggered by changes in the intra-cellular environment such as calcium levels and ER stress responses (Klomporn et al., 2011)). Apoptosis can be beneficial to both host and virus by killing infected cells to reduce spread (beneficial to host) or, if this is unsuccessful it could in theory lead to viral escape from apoptotic bodies thereby aiding dissemination of progeny virions (beneficial to virus). However, the latter mechanism is a hypothesis and is yet to be reported in the literature.

Proteomics

This thesis uses a novel method in DENV research, comprising of a combined phospho-protein enrichment and mass spectrometry approach using previously described methods of in-gel trypsin digestion (Emaduddin et al., 2008) followed my LC-MS/MS (Batycka et al., 2006). This involved isolation of only phosphorylated proteins from samples and analysis of these by mass spectrometry, revealing the relative level of phosphorylation of each protein in response to varying stimuli. This method allows focussed detection of proteins post-translationally modified by phosphorylation and eliminates background from all other proteins within cells, leading to clearer outcomes whereby differences are not masked by the abundance of general proteins within the cell.

The results obtained from this experimental model will represent an accurate view of proteins present within each sample. This is partly due to peptide sequence matching, allowing the number of peptides associated with the identified protein to be shown (Steen and Mann, 2004). This information provides a level of confidence which can be used to gauge the accuracy of protein 'hits' returned by the analysis.

It can be seen from the literature that methods of mass spectrometry are improving vastly, allowing more and more detailed analysis to take place, for example the ability to detect phosphorylated proteins (Salih, 2005). The use of these phospho-proteomics techniques has been successful in the investigation of various other viral infection processes such as HIV-1 (Wojcechowskyj et al., 2013) and Influenza (Dapat et al., 2014) and therefore, the principle of detecting phosphorylation of proteins during viral infection presents a promising avenue of research.

Label-free phospho-proteomic analysis has also been utilised successfully to investigate other viral infections e.g. PRRSV (Luo et al., 2014) and this shows that this

method of phospho-protein analysis can lead to interesting findings regarding protein regulation by phosphorylation.

Summary

In summary, DENV is the causative agent of dengue which is a rapidly emerging arboviral infection presenting with a wide range of symptoms from mild fever to shock syndrome. There is currently no vaccine or treatment for dengue and with 2.5 billion people estimated to be living at risk of infection it is imperative that researchers develop preventative measures and effective treatments for this disease. The basic science pertaining DENV has not been as well studied as many other viral infections and as yet the exact mechanisms occurring upon infection remain unclear. The innate immune system is likely to play a vital role in the outcome of disease both due to its effect on initial viral inhibition and through its capacity to induce the adaptive immune response. This study aims to highlight the proteins within cells which are modified in response to DENV recognition and could therefore have a unique function in response to DENV as compared to other viruses such as IAV which do not result in such severe immuno-pathology. The phospho-proteomics approach adopted in this study is similar to other studies of protein regulation by viruses and has therefore been seen to produce insightful results pertaining to post-translational modification of proteins in response to viral stimulation of cells. The hypothesis is that DENV encounter triggers a different quality of immune response in specific circumstances and ultimately this leads to the possibility of severe symptoms developing. As no concrete evidence exists as to the trigger of severe disease, it is pertinent to further analyse infection at the cellular level to gain a better understanding of the effects of viral recognition which could in turn shed light on the trigger of immune de-regulation which leads to severe disease.

CHAPTER TWO - MATERIALS AND METHODS

Cell culture

Media: All culture medium was supplemented with 100U/ml Penicillin, 0.1mg/ml Streptomycin (PAA) and 2mM L-Glutamine (PAA).

Media was also supplemented with varying concentrations of FCS.

When referring to media utilised: RPMI-1640 (Sigma) is denoted 'R' and alpha-MEM (Sigma) is denoted 'M', the number following refers to the percentage of heat inactivated FCS (PAA) added.

Incubator: All cells were cultured in 5% CO_2 incubators at 37 ^{0}C with the exception of C6/36 cells, grown at 28^{0}C.

VERO

The Vero cell line is a dengue virus permissive adherent cell line derived from African Green Monkey kidney cells. Cells were cultured in M5 and split 1:5 every 3 days.

U937

U937 is a human non-adherent leukemic monocyte lymphoma cell line displaying a macrophage like phenotype (Sundström and Nilsson, 1976). Cells were cultured in R10 (upstanding culture flask) and split 1:5 every three days.

THP-1

THP-1 cells are a human monocyte-like line derived from an acute monocytic leukemia patient. Cells were cultured in R10 and split 1:5 every 3 days. Cells were grown in upstanding tissue culture flasks.

C6/36

C6/36 are derived from *Aedes albopictus* mosquitoes and are used widely in the propagation of flaviviruses such as dengue virus. Cells were cultured in R10 in large tissue culture grade plastic flasks (Corning) and were split 1:5 every 3 days.

Monocyte derived dendritic cells (MDDC)

Cells were generated from Leukocyte Cones (National Blood Service, Bristol). Blood was diluted 1:3 with RPMI-1640 and separated by density gradient centrifugation using Lymphoprep (Axis Shield). Lymphoprep was underlayed into tubes and these were centrifuged for 20 min at 2000rpm. PBMC were isolated from the gradient and washed with 10ml RPMI-1640 three times. PBMC were subsequently re-suspended in 3ml cold MACS buffer (PBS, 2% FCS, 2mM EDTA) and 250µl CD14+ microbeads (Miltenyi Biotech) were added. Cells were incubated at 4^0C for 20 min, 30ml MACS buffer was added to wash and the pellet was re-suspended in 3ml MACS buffer. CD14+ monocytes were isolated using Miltenyi Biotech magnetic columns, washed with RPMI-1640 and plated in 2x large tissue culture plates per buffy coat in R10 with 40ng/ml each of IL-4 and GM-CSF (Peprotech).

Phenotyping of cell lines and primary cells by flow cytometry

For detection of CD80/83/86/206/209 and HLA-DR, cells were incubated with FITC, PE or PerCP-Cy5.5 conjugated detection antibodies for 20 min at 4^0C. For detection of CD32, cells were incubated with primary anti-CD32 followed by an AlexaFluor-488 conjugated secondary antibody. Cells were washed with PBS/0.1% BSA, fixed with 1% paraformaldehyde (PFA) overnight and acquired on a Cyan flow cytometer. Data was analysed using FlowJo software.

Viral stock production

Dengue virus

Dengue virus strain 16681 (DENV-2) was used in all experiments. Virus was propagated in C6/36 mosquito cells (*Aedes albopictus*) by culturing cells to approximately 80% confluency, removing media and infecting in a 4ml volume (MOI – 0.1, R2.5 diluent) for 2 hr at 28^0C (flasks were tilted every 20 min). Subsequently, 25ml R15 culture media was added and infected cells were incubated for up to 11 days, culture supernatant was harvested, centrifuged at 1500rpm for 5 min, aliquoted and stored at -80^0C until use. Viral stock titres were measured by DENV FFU assay (see page 42).

Mock supernatant was obtained from a separate flask of C6/36 cells grown alongside the infected cells but with no virus added (cell medium was used instead). The culture supernatant was harvested in the same way and at the same time as for infected cells and this was used for Mock infections.

A time-course experiment investigating optimal time-point for viral harvest was conducted, taking aliquots from day 1 to day 15. Harvest on Day 11 resulted in the

highest viral titre and therefore whenever new stocks were generated, all virus was harvested on day 11 post-infection (results not shown).

Influenza A virus

Human Influenza A virus (IAV) (H3N2 Udorn 307/72 human isolate, kindly donated by John McCauley, NIMR, London) was used in all experiments. The virus was propagated in MDCK–SIAT cells in media containing TPCK-Trypsin (Sigma). MDCK-SIAT cells express high levels of sialic acid receptors on their surface (the primary receptor for Influenza viruses) allowing higher levels of virus recognition and subsequent infection, resulting in increased viral titres compared to conventional MDCK cells. Virus containing supernatant was plaqued to determine the concentration of infectious virus particles and stored at -80^{0}C. Plaque assays were carried out by the McCauley lab within the NIMR.

Phospho-proteome analysis

Viral stimulation of cells

MOI used was 1 unless otherwise stated.

Cells were harvested and washed in 5ml RPMI-1640 at 4^{0}C before stimulation with either Mock (supernatant from non-infected C6/36 cells), DENV, IAV or LPS (10ng/ml). For 10 min stimulation: cells were incubated in a 37^{0}C water bath for 2 min and then transferred to a 37^{0}C incubator for 8 min, for longer stimulation: cells were incubated at 37^{0}C in an incubator for the relevant time. All subsequent steps were carried out on ice. Cells were washed in 5ml HEPES buffered saline (HBS) - 500ml distilled water, 4.4g NaCl, 10ml 1M HEPES pH7.4, 500µl phosphatase inhibitor cocktail 3 (Sigma) - twice and subsequently lysed in lysis buffer prepared as per manufacturer

instructions (PhosphoProtein Purification Kit, Qiagen). During the 40 min lysis, cells were vortexed every 10 min. Lysate was then centrifuged at 13000rpm for 30 min at 4^0C to remove cellular debris and supernatant was collected as whole-cell lysate.

Western blot

Whole cell lysate was diluted 1:4 in 4X SDS running buffer (Invitrogen) and boiled at 95^0C for 5 min in a heat block prior to being cooled on ice. Cooled samples were loaded (up to 30µl) into individual lanes of a pre-cast 4-12% SDS-PAGE 10-well gel (Invitrogen) alongside a lane containing 7µl of full range molecular weight marker (GE Healthcare). Gels were submerged in MOPS running buffer (Invitrogen) and were run at 60V for 30 min followed by 120V until completion.

Proteins were transferred onto nitrocellulose PVDF membranes (GE Healthcare) according to manufacturer instructions (Invitrogen transfer module), transfers were run for 1 hr at 30V.

Proteins were visualised on membranes by one of two methods:

1) Blots visualised on photographic film

Membranes were blocked with 3% BSA at 4^0C overnight or in Milk Powder/TBST for actin controls. On the next day the membranes were washed three times in TBST, incubated for 1 hr at RT with primary antibody (diluted to 1:1000 with Milk Powder/TBST), washed again three times and incubated for 1 hr at RT with a 1:1000 dilution of species-specific secondary antibody conjugated to HRP (Sigma). Membranes were then washed three times and soaked in ECL reaction reagent (Amersham) before being visualised on photographic paper (Amersham) developed by Xograph.

2) Blots detected with fluorescent detection system

Membranes were blocked using Odyssey blocking buffer (LiCor) by rotating overnight at 4^0C. The following day, membranes were washed three times in TBST (960ml MiliQ water, 30ml NaCl, 10ml Tris-HCl pH 7.4 and 500µl Tween20) for 10 min on a shaker. Primary antibodies were diluted 1:1000 in 2.5ml LiCor Odyssey blocking buffer and membranes were rotated in this solution overnight at 4^0C. Membranes were again washed in TBST (shaking) before incubation with 1:1000 dilution of secondary antibody (goat anti-rabbit/mouse, LiCor) for 1 hr at room temperature protected from light. Subsequent to secondary antibody incubation, membranes were washed three times in TBST and detected using the LiCor Odyssey detection system (Odyssey CLx for infrared imaging) as per manufacturer instructions.

Primary antibodies used:

Anti-phosphotyrosine-HRP (Zymed), anti-β actin (Sigma), various rabbit derived primary antibodies for validating western blots (Cell Signalling).

Phospho-protein enrichment of whole cell lysate

Purification was carried out as per manufacturer instruction (PhosphoProtein Purification Kit, Qiagen), briefly:

Whole-cell lysate protein concentration was ascertained by Bradford assay (Bio-Rad). Samples were diluted to 0.1mg/ml and passed through a TiO2 (titanium dioxide)/resin-containing column which traps phosphorylated proteins. Columns were washed to remove non-phosphorylated proteins from the resin and phospho-proteins were eluted with provided buffer (see Fig. 6).

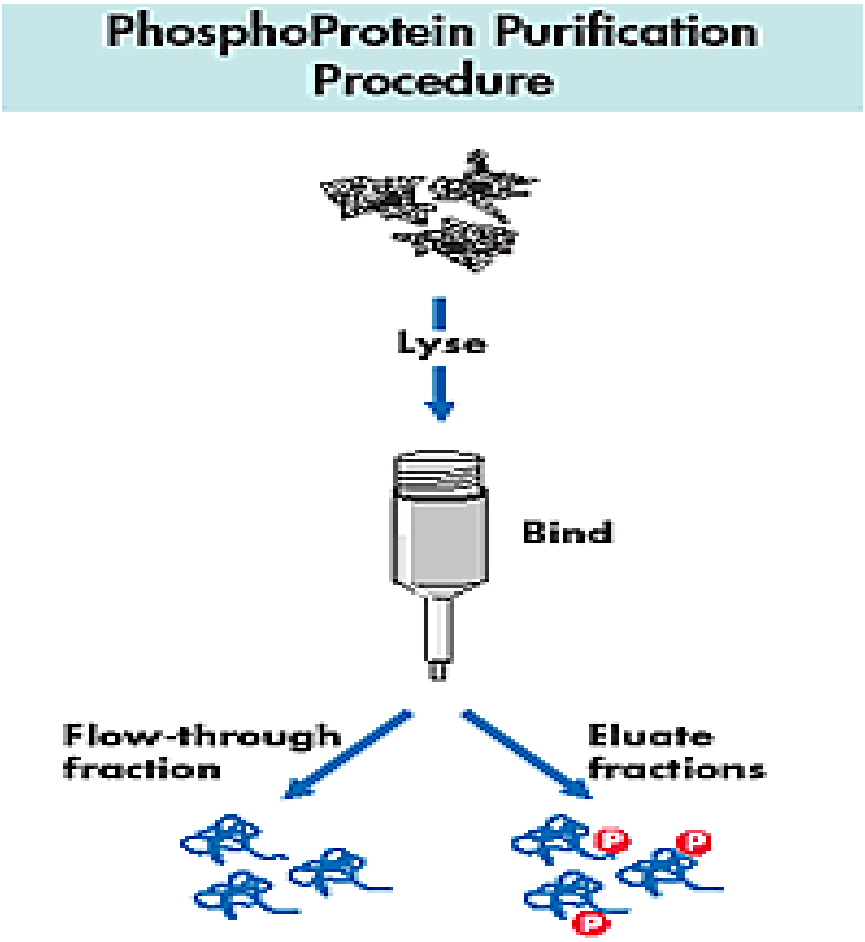


Fig. 6: ***Illustration of phospho-protein isolation procedure***
WCL is passed through a resin containing column to which phosphorylated proteins bind, the column is then washed to remove any non-phosphorylated protein and finally, elution buffer is used to collect the phosphorylated proteins trapped in the resin.

Iso-electric focussing

This process was carried out as per manufacturer instructions (3100 OFF-GEL High Resolution kit, Agilent), briefly:

Eluates from the phospho-purification columns were de-salted using ZEBA spin de-salting columns and concentrated to 500µl volumes using 7K ultra-centrifugal concentrator tubes (Pierce). All samples were adjusted to identical concentrations.

Protein OFFGEL Stock Solution was prepared to 1.25x concentration. This was used to formulate IPG Strip Rehydration Solution and to dilute protein samples to

appropriate concentration. The IPG strip (pH 3-10), frames and electrodes were assembled as described and 40µl IPG rehydration solution was added to each chamber. Electrode pads were placed at each end of the strip and the IPG gel was left to swell for 15 min. Subsequently, 150µl of pre-prepared OFFGEL protein sample was added to each chamber, a cover seal was fitted and mineral oil was used as cover fluid at each end of the strip (added in two stages). Electrodes were fitted to each end of the gel frame and phospho-proteins were separated into 12 fractions by iso-electric focussing at a maximum current of 50µA and maximum power of 200 milliwatts.

Samples were collected from each of the 12 chambers and desalted again using ZEBA columns (Pierce) prior to subsequent analysis to ensure the removal of urea, thiourea and glycerol.

Silver stain of SDS-PAGE gel

For phospho-proteome analysis:

Buffers:

Fix: 50ml MeOH, 5ml HAc, 90ml H_2O

Wash: 50ml MeOH, 50ml H_2O

Sensitiser: 0.02g $Na_2S_2O_3$, 100ml H_2O

Silver Nitrate: 0.1g $AgNO_3$, 100ml H_2O

Developer: 50µl Formalin (35% Formaldehyde), 2.5g Na_2CO_3, 125ml H_2O

5% HAc: 5ml HAc, 95ml H_2O

1% HAc: 1ml HAc, 99ml H_2O

SDS-PAGE gel electrophoresis was carried out on the protein samples as described previously and gels were then immediately fixed overnight in 100ml fixing solution. Gels were washed in 50% methanol for 10 min and subsequently washed again for 2 hr in 100ml ultrapure water. Gels were sensitized in 0.02% Sodium Thiosulphate for 60 seconds and washed twice in 100ml ultrapure water for 60 seconds. Gels were then incubated in cold 0.1% Silver Nitrate for 20 min at 4^0C before being washed for 60 seconds in 100ml ultrapure water. At this point the gel was transferred to a fresh chamber where it was again washed for 60 seconds in 100ml ultrapure water before being developed in 0.04% Formalin and 2% Sodium Carbonate. When the developing solution turned yellow it was discarded and a fresh 100ml was added to the chamber. Once the desired band intensity was reached the development was terminated with 100ml 5% Acetic Acid. The termination solution was refreshed twice and gels were finally stored in 1% Acetic Acid.

Mass Spectrometry

Mass spectrometry analysis was carried out in collaboration with Benedikt Kessler's Group, WTCHG, Oxford.

Individual gel slices representing enriched phosphoproteins were excised and subjected to in-gel trypsin digestion as previously described (Emaduddin et al., 2008). Digested protein material was kept at -20°C until analysis. Sample analysis was performed by LC-MS/MS using an 3000 Ultimate (LC-Packings, Dionex, Amsterdam, NL) HPLC system coupled on-line to a 3D high-capacity ion trap (HCTplus, Bruker Daltonics, Bremen, Germany) mass spectrometer via a pneumatically assisted nano-electrospray source as described previously by (Batycka et al., 2006). MS/MS spectra (peak lists) were searched against the SwissProt database using Mascot version 2.4

(Matrixscience, London, UK) and the following parameters: peptide tolerance 2.5 Da, ^{13}C=0, fragment tolerance 0.8 Da, missed cleavages: 3, instrument type: ESI-TRAP. Individual MS/MS spectra for peptides with a Mascot Mowse score lower than 20 were inspected manually and included in the protein discovery list only if a series of at least 4 continuous y or b ions were observed. Protein ID is also based on the assignment of at least two peptides. In cases where proteins were identified based on one peptide sequence and to verify phosphorylation sites, the corresponding MS/MS spectra were inspected and verified manually.

Identified proteins were initially analysed using Ingenuity Pathway Analysis software in order to identify the common pathways affected by proteins found to be differentially phosphorylated.

Silencing of Calpain-2 using siRNA pool

siRNA was re-suspended as per manufacturer instructions (Dharmacon, Thermo Scientific), briefly:

1X siRNA re-suspension buffer was prepared by diluting Dharmacon 5X siRNA buffer one in five in sterile RNAse free water. Tubes containing lyophilised siRNA were pulse centrifuged and 50µl of 1X siRNA buffer was added to obtain a 100µM stock solution. This was pipetted 5 times and the tube placed on an orbital shaker for 30 min at room temperature. Tubes were pulse centrifuged again and stock was aliquoted for future use and stored at -20^0C.

The required volume of cell suspension was centrifuged at 1500rpm for 5 min. Supernatant was discarded and the pellet re-suspended in Accell delivery media (Dharmacon, Thermo Fisher), this was supplemented with 2% HI FCS and subsequently

split into two 15ml Falcon tubes of equal volume. siRNA targeting Calpain-2 (Dharmacon, Thermo Fisher), scrambled siRNA control or no siRNA (Accell media) was added to the corresponding pellet at a concentration of 1µl/1million cells. The tube was gently inverted to mix. Cells in delivery medium with or without siRNA were then split into wells of a 6-well plate (3million cells/well) and incubated at 37^0C for 72 hr.

To check the level of silencing, whole cell lysate was obtained from dedicated wells and western blots were carried out with Calpain-2 specific antibody used for detection. Band intensity was quantified using the LiCor imaging system.

Focus forming unit assay to measure viral titre

Overlay: 25ml Avicel (Fluke)

22ml 2xMEM (Nitrogen)

1ml $NaCO_2$ (Invitrogen/Gibco)

1ml HI FCS

0.5ml Pen/Strep (100U/ml Penicillin and 0.1mg/ml Streptomycin (PAA))

0.5ml L-Glutamine (2mM)

Fixative: 3.7% Formaldehyde in PBS

Permeabilisation Solution:

1% Triton-X100 in PBS

1^o Antibody: 4G2 hybridoma supernatant (undiluted)

2^o Antibody: Goat or Rabbit anti-mouse Ig conjugated with HRP dilute to 1:1000 with diluent (PBS containing 2% FCS and 0.05% Tween20)

Substrate: 3,3'-diaminobenzidine (DAB) (0.6mg/ml) 5 ml+8% NiCl-PBS 200 µl+30%H_2O_2 5 µl

Vero cells were seeded in 100µl M5 at a confluency of 20,000 cells per well in flat-bottom 96-well plates. Cells were grown overnight at 37^0C with 5% CO_2.

The following day samples were serially diluted 1:10 from 10^{-1} to 10^{-6} in M2.5. Media was removed from wells and 50µl of diluted sample was added to each, after which plates were incubated for 2 hr at 37^0C (5% CO_2). 100µl of overlay medium was then added to each well and plates were again incubated at 37^0C (5% CO_2) for 4/5 days. Overlay medium sets into a lattice formation, thereby immobilising the virus at the cell surface. This occurs as powder particles swell, resulting in a dispersion of cellulose microcrystals which create a stable lattice structure (source: FMC BioPolymer). This immobilisation traps virus particles at their location, inhibiting movement throughout the inoculation/avicel solution. This ensures that each virus particle that successfully infected a cell is capable of generating one focus only.

Staining was then carried out to detect the number of focus forming units. In this procedure, cells were washed three times with 100µl PBS and once with fixing solution. 100µl fixing solution was added to each well and plates were incubated at room temperature for 20 min. Fixing solution was removed and discarded prior to addition of 100µl of permeabilisation solution for 10 min at room temperature. Wells were washed three times with 100µl PBS and 100µl of primary (1^o) anti-DENV envelope antibody solution was added to each well. Plates were incubated at 4^0C overnight. The next day plates were washed with 100µl PBS three times and 50µl HRP-conjugated secondary (2^0) antibody solution was added for 1hr during which plates were incubated at 37^0C. Subsequently, plates were again washed three times with 100µl PBS and 50µl of DAB substrate solution was added to each well for 10-15 min or until dark spots begin to appear. Once spots reached the desired intensity the reaction was stopped by washing plates with 100µl/well distilled water.

Staining is carried out after washing away Avicel/inoculum overlay therefore only viral particles within permeabilised infected cells are detected. In principle, each focus represents an initial infectious virus particle therefore the number of foci represents the number of infectious viral particles within the original sample added (regardless of the variable number of progeny produced by infected cells). It is then possible to calculate the number of infectious virus particles contained per millilitre of sample and this is known as the viral titre.

Measurement of mRNA levels of interferon stimulated genes

Silencing was carried out as described above (in 6-well plates). At 72 hr, supernatant was removed and centrifuged, the cell pellet was re-suspended in R2.5 containing dengue virus at an MOI of 1. The cell/virus suspension was replaced into the well and plates were incubated for 2 hr at 37^0C. Each well was filled to 3ml total volume with R2.5 and plates were incubated at 37^0C for 72 hr. Cells were taken into a 15ml Falcon tube and pelleted for 5 min at 1500rpm. Cells were snap frozen at -80^0C. Subsequently, RNA was extracted using Qiagen RNeasy kit (Mini Protocol, Spin method).

5 million cells were taken per condition and centrifuged at 1500rpm for 5 min after 72 hr infection. Supernatant was carefully removed and the pellet loosened before the addition of 600µl Qiagen buffer RLT. Solutions were vortexed and transferred into a QIAshredder (Qiagen) spin column placed into a 2ml collection tube. The column was centrifuged for 2 min at 13000rpm to homogenise cells. 600µl of 70% RNase-free ethanol (ETOH) was added to the homogenised lysate in the collection tube (column was discarded) and the solution was mixed well by pipette. One RNeasy spin column per condition was placed in a 2ml collection tube and 700µl of homogenised lysate was

added to the corresponding column. Columns were centrifuged for 15seconds at 8000g, flow-through was discarded, columns were placed back into the collection tube and this step was repeated until all lysate had passed through the corresponding column. DNase digestion was then performed by adding 350µl Qiagen buffer RW1 to each spin column and centrifuging for 15seconds at 8000g (flow-through was discarded). Subsequently, 10µl of DNase I stock solution was added to 70µl Qiagen buffer RDD and the tube was inverted gently to mix. Tubes were pulse centrifuged and 80µl of the solution was added directly to each spin column membrane. Tubes were incubated at room temperature for 15 min. 350µl Qiagen buffer RW1 was added to each column and the columns centrifuged for 15seconds at 8000g. Flow-through was discarded prior to adding 500µl buffer RPE to the column. Columns were centrifuged for 15seconds at 8000g and flow-through was discarded. A further 500µl aliquot of buffer RPE was added to the column and tubes were centrifuged for 2 min at 8000g. Columns were placed in a fresh collection tube and centrifuged at 13000rpm for 1 min. The spin columns were then transferred into 1.5ml collection tubes and 30µl RNase-free water was added to each membrane. Columns were centrifuged for 1 min at 8000g to elute RNA. The eluate was taken from the collection tube and reapplied to the membrane, the spin was performed again for 1 min at 8000g to obtain the final eluate.

This eluate was used to measure RNA concentration by Nanodrop.

RNA was diluted in RNase free water to an appropriate concentration, allowing 0.5µg to be used for reverse transcriptase reaction. 0.5 or 1µg of diluted RNA was added to 1µl of random hexamers (100ng/µl) and RNase free water was added to a final reaction volume of 12.5µl. Tubes were incubated at 70^0C for 10 min then cooled on ice.

To produce cDNA a master mix consisting of 4µl 1st strand buffer (5x) (Invitrogen), 2µl 0.1M DTT, 1µl dNTPs (10mM) and 0.5µl of superscript II reverse transcriptase was made. 7.5µl was added to each 12.5µl reverse transcription reaction and one tube of master mix without reverse transcriptase was prepared for control purposes. Reactions were incubated at 42^0C for 60 min, followed by 70^0C for 15 min and held at 4^0C. Samples were diluted to 10ng/µl with RNase free water and stored at -20^0C. qPCR was carried out on samples to determine the level of Mx1 RNA expression: a master mix consisting of 5µl 2X master mix (Promega), 1.5µl RNase-free water and 0.5µl ISG probe was made and 7µl of this master mix was added to each well of a 96-well PCR plate along with 3µl cDNA obtained by reverse transcriptase reaction. Non-template controls were included whereby RNase-free water was added in place of cDNA. Plates were centrifuged to gather the solution at the bottom of the well and then covered with a qPCR compatible seal before analysis.

Immuno-precipitation

Buffers:

Tris-lysis buffer:	50mM Tris-HCl (pH 8.0), 200mM NaCl, 33% Glycerol, 0.5% Igepal CA-630, 1mM DTT, 1mM EDTA, 1X protease inhibitor tablet (Roche Complete Protease Inhibitor)
Binding buffer:	133mM NaCl, 1X protease inhibitor tablet
Wash buffer:	20mM Tris-HCl (pH8.0), 150mM NaCl, 0.1% Igepal CA-630, 1mM EDTA, 1X protease inhibitor tablet.

THP-1 cells were cultured in R10 and split on Day 0 into 4 large tissue culture dishes per condition (total of 100 million cells/condition, 25 ml per dish). These were incubated overnight at 37^0C with 5% CO_2.

On Day 1, cell suspension was centrifuged at 1500rpm for 5 min to pellet and supernatant was discarded. Cells were washed with 5ml PBS and subsequently infected at an MOI of 5 or Mock infected in a volume of 25ml R2.5 for 30 min at 37^0C. Cells were centrifuged again and re-suspended in 5ml of ice cold PBS followed by another centrifugation at 400g for 5 min at 4^0C. Supernatant was discarded and cells were snap frozen at -80^0C.

On Day 2, cells were lysed in 4ml Tris-lysis buffer for 1 hr, rotating at 4^0C. Lysate was centrifuged at 17000g for 5 min at 4^0C. 3.95ml of the resulting soluble fraction was diluted in 7.2ml of binding buffer. 25µl (5µg) of primary antibody was added to this solution and samples were rotated for 2 hr at 4^0C.

Pre-washed Protein G beads were prepared by washing 120µl of slurry per condition and performing this three more times with 1ml wash buffer for 10 min each time. Samples were centrifuged at 400g for 3 min to pellet beads.

Antibody-lysate mix was added to the pre-washed beads after aforementioned 2 hr incubation. This solution was then incubated (rotating) at 4^0C for a further 2 hr.

The solution was then centrifuged at 4^0C for 3 min at 400g to pellet beads, supernatant was discarded and 1ml wash buffer was added for 10 min of rotation at 4^0C. This was repeated for a total of 4 washes and finally, 180µl of 1X SDS loading buffer (diluted in ultrapure water) (Invitrogen) was added to the beads and subsequently samples were boiled at 99^0C for 5 min before being cooled on ice. The solution was centrifuged at 17000g for 5 min and the supernatant was taken as the final eluate.

Silver stain of SDS-PAGE gel

For Immuno-precipitation

Gels were silver stained using the basic protocol from SilverQuest kit (Invitrogen) for IP validation gels:

Buffers:

Fixative: 40ml ETOH, 10ml HAc, 50ml H_2O

Sensitising Solution: 30ml ETOH, 10ml Sensitiser, 60ml H_2O

Staining Solution: 1ml Stainer, 99ml H_2O

Developing Solution: 10ml Developer, 1 drop Developer Enhancer, 90ml H_2O

SDS-PAGE gel electrophoresis was performed as previously described and gels were rinsed in ultrapure water prior to fixation in 100ml of fixative solution (40% ETOH, 10% acetic acid and 50% ultrapure water) overnight. Fixing solution was discarded and gels washed in 100ml 30% ETOH for 10 min. Washing solution was discarded and 100ml sensitising solution was added, gels were incubated for 10 min. The wash step was then repeated (100ml 30% ETOH for 10 min) and the gels were subsequently washed in 100ml ultrapure water for 10 min. 100ml staining solution was added for 15 min followed by 100ml ultrapure water for 60seconds. After removing ultrapure water the gels were incubated in 100ml developing solution until bands appeared. At the desired intensity, 10ml stopper solution was added to developing solution and the gel was rotated for 10 min (stopping of development is indicated by change in colour of solution from pink to clear). Stopping solution was discarded and the gel was washed in ultrapure water for 10 min. Images were captured digitally.

CHAPTER THREE - PHOSPHO-PROTEOME ANALYSIS OF DENDRITIC CELLS 10 MINUTES POST-STIMULATION WITH DENGUE VIRUS

Introduction

As described previously, dengue is a growing public health concern and a deeper understanding of viral pathogenesis needs to be gained in order to improve prevention methods and to make progress in developing effective treatments. To understand the cause of severe immuno-pathology the molecular mechanisms involved in viral infections need to be analysed in order to pinpoint pathways or protein functions which could lead to severe disease. In this study, the hypothesis that DENV elicits a severe immune response due to irregular signalling pathway activation was investigated using various approaches designed to dissect the immune response. By analysing the proteins modified upon stimulation, those involved in pathogenesis can be identified and their role in infection established. Initially, differences in the phospho-proteome were crudely assessed to ascertain that differentially phosphorylated protein profiles are in fact observed after stimulation with DENV in comparison to Mock supernatant. Once this was established I took the approach of analysing the phospho-proteome using mass-spectrometry to identify potential proteins involved with the immune response, giving an unbiased look into all cellular pathways involving phosphorylation. In addition to this, I also took a biased approach, whereby specific immune signalling proteins were targeted to ascertain the level of phosphorylation/protein present to see if these well known players in the immune response may be causing severe reactions in dengue patients. From these approaches, the phospho-proteome analysis was most informative and therefore

further work focused on the analysis of that data set. Identified proteins were validated to ensure the mass spectrometry result was confirmed with another method of analysis, in this case western blot. This was done to give the best chance of selecting proteins that were truly affected upon stimulation and not falsely identified by mass spectrometry. From those identified I ascertained which were most likely to play a significant part in the outcome of infection. Ultimately, identified candidates were chosen for further study into their role in cellular responses to infection and this knowledge may lead to a possible method of inhibiting the effect of such proteins by interfering at various points along functional pathways in which they are involved.

During this study, multiple cell types were used including U937 and primary dendritic cells derived from monocytes isolated from buffy coats. In order to ascertain the suitability of these cells for stimulation and infection with DENV and to ensure the primary cells isolated showed the expected surface marker profile of dendritic cells, each cell type was stained with a panel of fluorescent antibodies against various cell surface markers which included receptors involved in DENV infection (see Fig. 7). These included CD80, CD83, CD86 and HLA-DR (all maturation markers for dendritic cells ensuring immature DC are utilised as these are the primary target cells upon natural infection), CD32 (Fc receptor expressed on DC), CD209 (DC-SIGN - a known receptor for DENV) and CD206 (mannose receptor – another known receptor for DENV).

Surface marker expression of U937 and MDDC analysed by flow cytometry

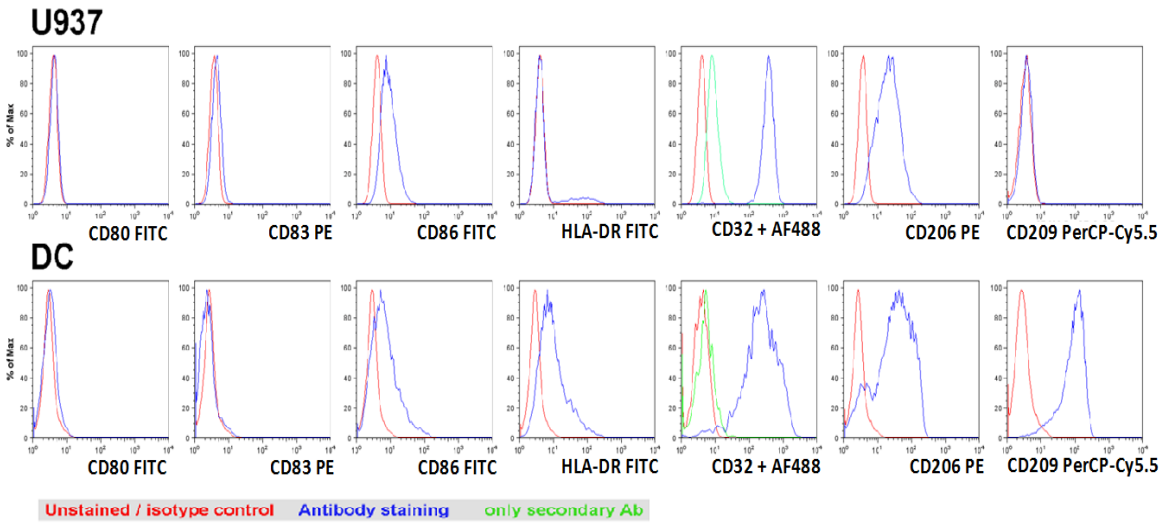


Fig. 7: ***Identification of cell surface markers important in DENV infection***
Cells were stained with fluorochrome conjugated antibodies to markers including: co-stimulatory molecules CD80, 83 and 86, HLA-DR, CD32 (Fcγ Receptor II), DENV receptors CD209 (DC-SIGN) and CD206 (Mannose receptor). Stained cells were washed, fixed with paraformaldehyde and levels of fluorescence were ascertained by flow cytometry. All cells showed an expected profile of surface markers. (FITC, PE, AF488 and PerCP-Cy5.5 indicate the fluorophore-labelled secondary antibody used)

As seen in Fig. 7, the chosen cell lines all displayed an immature phenotype, as desired, given that the DENV encounters immature dendritic cells upon host entry and infects these more readily than mature cells.

All lines also expressed mannose receptor on their surface and as previously mentioned this is a known receptor for DENV. Therefore, this indicates the cells used were all capable of being responsive to DENV stimulation/infection, even if reproductive infection in those cells is still controversial.

It was also observed that immature dendritic cells expressed DC-SIGN, making them highly permissive to infection (Lozach et al., 2005) and as expected the U937 cells did not express this receptor on their surface. Mature dendritic cells down-regulate

expression of DC-SIGN on their surface, rendering them less permissive to infection comparable to immature dendritic cells. This process of surface DC-SIGN reduction occurs as immature dendritic cells are generated in the presence of IL-4, which can induce DC-SIGN expression, however upon activation DC produce molecules such as interferon which are seen to inhibit induction of DC-SIGN expression (Relloso et al., 2002).

All cell lines have been reported to express at least one DENV receptor and are therefore appropriate for use in this study. The aim was to analyse the effects of cell stimulation after DENV recognition by receptors (within 10 min), therefore the presence of one or more receptors is sufficient for investigation of the effect within the cell. I hypothesise that an event occurring very early after recognition of viral particles by receptors on the cell surface is either unique to DENV stimulation or is regulated differently in response to DENV stimulation compared to Mock and IAV stimulation. For this reason, the 10 min time point was chosen in order to capture the phosphorylation status of proteins in the very first stages of immune response and before replication has occurred.

Dendritic cells were chosen for this study as they are the primary target cells for DENV *in vivo* (Wu et al., 2000). As the aim was to understand the basic nature of infection with DENV, primary target cells provide the best method of doing so *in vitro*. Also, dendritic cells are, in part, the instigators of innate immune responses and as it is these responses that will be investigated further, studying the action of cells which initiate them may shed further light on the processes which occur upon infection and leading up to immuno-pathology. In the absence of a suitable DC cell line I used U937 cells for some experiments. U937 cells are human myeloid cells often used as a model system for

monocytes. As monocytes are also targeted by DENV *in vivo* and they share an overlapping DENV receptor profile with DC, they were chosen.

As the outcome of DENV infection could potentially be decided by very early events after stimulation, such as triggering of de-regulated pathways and various post translational modifications of proteins within the cell, the initial targets of infection (before dissemination throughout the body) will theoretically present the most accurate portrayal of early *in vivo* events. If the pathways which are affected in the very first instance can be deciphered, components of these pathways which play a pivotal role in the outcome of the infection could be discovered. Differential signalling pathway activation or inhibition in primary target cells could potentially drive a very different immune response given it is these cells that bridge the gap between innate and adaptive immunity, therefore they may ultimately decide the outcome of infection.

Study design

The aim of this study was to ascertain the involvement of innate immune cells, with a focus on dendritic cells, in the outcome of DENV infection. The hypothesis was that proteins identified as differentially regulated within cells upon recognition of DENV, compared to cells stimulated with Mock or IAV, may play a role in leading to the immuno-pathology observed in severe dengue patients but not in influenza patients.

Many of the initial processes taking place within cells upon viral stimulation utilise phosphorylation as a means of transmitting ‘messages’ which are carried by proteins along a dedicated pathway leading to an effector response. This study utilises this fact in order to analyse the processes occurring within cells by capturing the phospho-proteome of cells 10 min post-stimulation. Most virions will have associated

with clathrin on the cell surface by 2 min after infection (van der Schaar et al., 2008), at about 5 min the virions are detected in late endosomes and fusion occurs at about 12 min post-entry. In light of this time-scale, 10 min post-stimulation was chosen as a suitable time-point as it should allow initial signalling events to occur and be captured before viral genome release and replication takes place.

At such an early time-point post stimulation where viral replication is unlikely to have occurred there will be little effect on the abundance of specific proteins within the cell as de-novo synthesis of anti-viral proteins for example, will not yet have taken place. For this reason, only post-translational modification such as phosphorylation, alkylation, glycosylation, ubiquitination, lipidation or sumoylation etc. will be detectable within the samples taken, meaning analysis of the full cellular proteome would not be required as this remains experimentally challenging.

Phosphorylation was chosen as a read out as the immune response to stimulation is ultimately the process which will be analysed. The immune response is regulated by numerous pathways which instigate varying effector functions and for a significant majority of these, phosphorylation is the method by which signals are transmitted from the point of viral recognition to the effector protein activated/deactivated in response. These proteins function in many ways, one of which being the ability to enter the nucleus and alter the level of transcription of various immune related proteins in response to recognition of virus, this change in transcription is what drives the innate immune response and is ultimately responsible for disease severity. Therefore, if a protein is identified to be differentially phosphorylated in response to DENV as compared to Mock and IAV, it may ultimately play a role in instigating the severe immuno-pathology seen in many dengue patients.

In order to investigate the study hypothesis, a novel phospho-proteomics method was designed to identify only phosphorylated protein within stimulated cell lysates. The experimental procedure is depicted in Fig. 8 below.

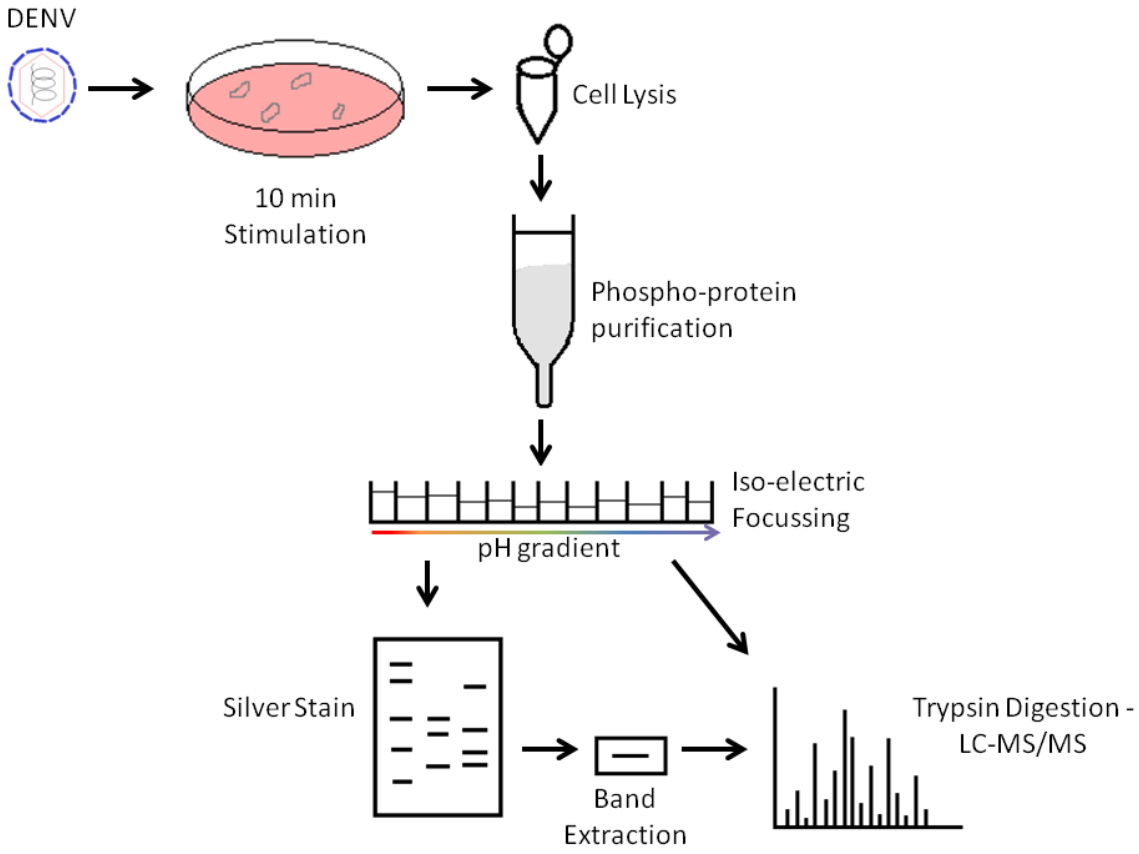


Fig. 8: ***Workflow of experimental procedure***
Dendritic cells were incubated for 10 min with various stimuli, cells were lysed and phosphorylated proteins were isolated, eluates from phospho-purification columns underwent iso-electric focussing and aliquots from each chamber were subject to silver staining, for mass spectrometry analysis a) bands showing the greatest difference between DENV and other conditions were excised and digested and b) aliquots from each chamber with the most striking overall difference in band profiles between DENV and other conditions were also digested, both sample types were subject to LC-MS/MS.

Initial investigation into capacity of cells to differentially phosphorylate proteins in response to Mock, DENV, IAV and LPS

Initially, in order to ascertain the potential of this method to produce reliable results, a crude assessment of differences in phosphorylation was carried out by stimulating U937 cells with Mock, DENV, IAV and LPS before isolating phosphorylated proteins and carrying out a western blot to detect proteins with phosphorylated tyrosine residues (see Fig. 9). This was done in order to investigate the level of differential phosphorylation occurring in cells stimulated with DENV compared to those stimulated with other conditions.

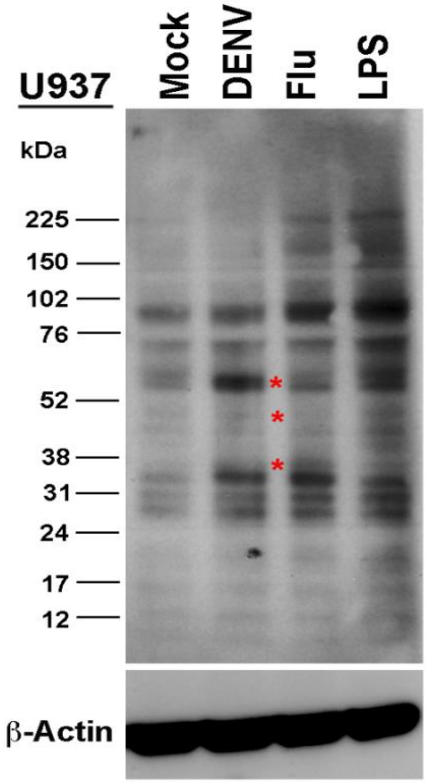


Fig. 9: ***Comparison of phospho-proteome of U937 following stimulation with Mock, DENV, IAV or LPS***
U937 cells were stimulated with Mock, DENV, IAV or LPS for 10 min, lysed and subsequently diluted to 0.1mg/ml before being passed through phospho-protein enrichment columns. Eluates containing phosphorylated proteins only were then subject to western blotting as shown, with anti-phospho-tyrosine antibody conjugated to HRP used for detection. LPS stimulation was included as a positive control for cell stimulation and β-actin as a control ensuring equal protein loading into wells. The asterisks indicate the most prominent differences in protein levels.

This initial phase of the study shows that a number of proteins are differentially phosphorylated in response to DENV stimulation when compared with Mock and IAV stimulation. As differences in the phospho-proteome were detectable even without simplifying the complexity of proteins present in the sample or utilising protein specific antibodies to avoid cross-over between bands, the extent of differential phosphorylation between each condition is likely to be very high.

With this initial success in mind, the next stage of investigation was to reduce sample complexity in order to allow more detailed analysis and identification of specific proteins affected by phosphorylation.

Sample complexity was reduced using iso-electric focussing (IEF) (see Fig. 10) which primarily serves to allow more sensitive detection of proteins by mass spectrometry and to increase the chances of low abundance proteins being identified rather than being lost behind a mask of high abundance proteins. The method also serves to improve the illustration of the level of phosphorylation via silver stain, allowing differential band profiles to be identified by eye and individually analysed by mass spectrometry.

As seen in Fig 10, this process involves splitting each sample into 12 wells of equal volume over a gel strip comprising a pH gradient (pH 3-10). As current passes through the gel the proteins migrate through wells toward the point on the gel strip corresponding to their iso-electric point. They are then held there resulting in 12 chambers, each containing only the phosphorylated proteins with specific iso-electric points, corresponding to the pH of the gel strip at the base. Each protein will be concentrated in one chamber so when the samples are run on a gel the band is more

concentrated and there will be fewer bands on each gel so it reduces masking of one band by another.

This simplification allows the differences to be highlighted more clearly as there will be less merging of bands representing proteins of similar size and therefore less uncertainty as to whether bands are bigger in one condition or if there are two bands merged together. Gels containing simpler solutions of proteins can then be silver stained to illustrate all proteins present. This removes the need for protein specific antibodies or phosphorylation specific antibodies as all proteins present are phosphorylated (isolated by phospho-columns) and the bands are distributed sparsely enough to identify differences without pinpointing a specific protein in order to see only one band per gel.

Iso-electric focussing

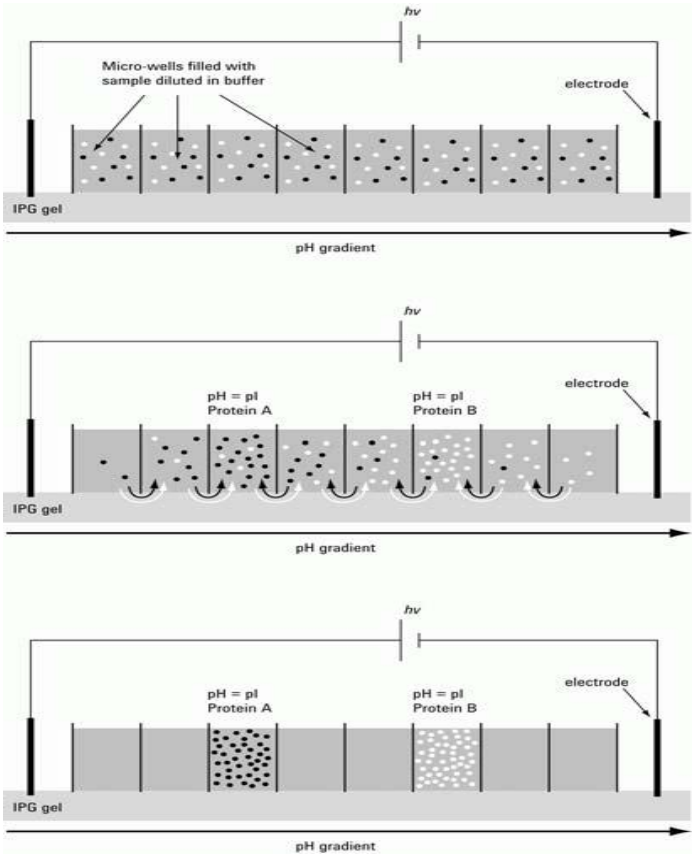


Fig. 10: ***Agilent iso-electric focussing technique***
Schematic diagram of iso-electric focussing whereby samples are diluted in stock buffer and added in equal volume to 12 chambers across a pH graded gel. Proteins are separated by the application of a current, resulting in movement of proteins to their iso-electric point over the gel. Samples from each chamber are harvested for further analysis of proteins with an iso-electric point corresponding to the pH within the chamber (source: Agilent Technologies)

Fractions isolated from iso-electric focussing chambers subject to SDS-PAGE and silver stain to highlight differential phospho-proteome

In order to optimise the technique chosen, initial experiments were carried out using U937 cells and including Mock and DENV stimulation only (see Fig. 11 below). A higher abundance of protein can be generated within samples using U937 cells as larger cell numbers can be generated compared to primary dendritic cells. The use of cell lines rather than primary cells also eliminates donor to donor variation which can play a role in differential immune responses.

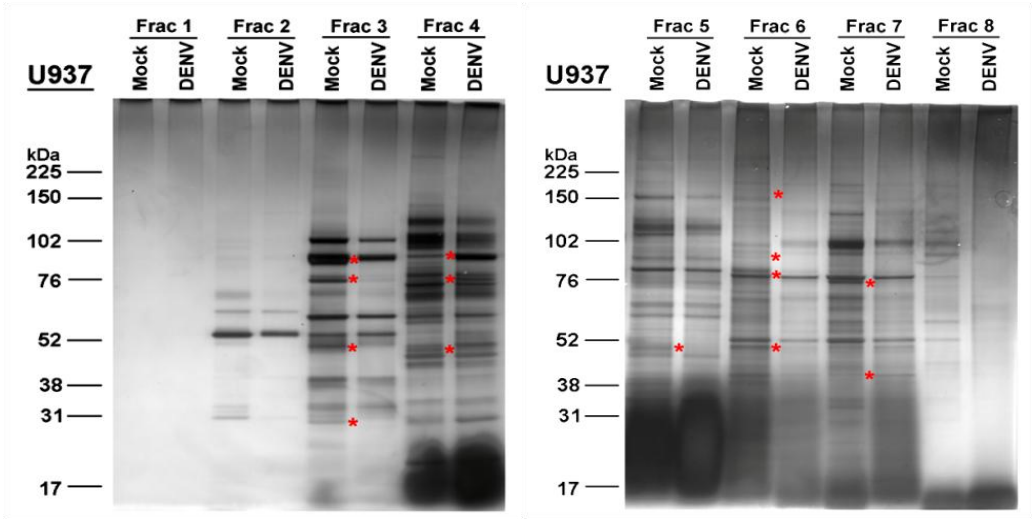


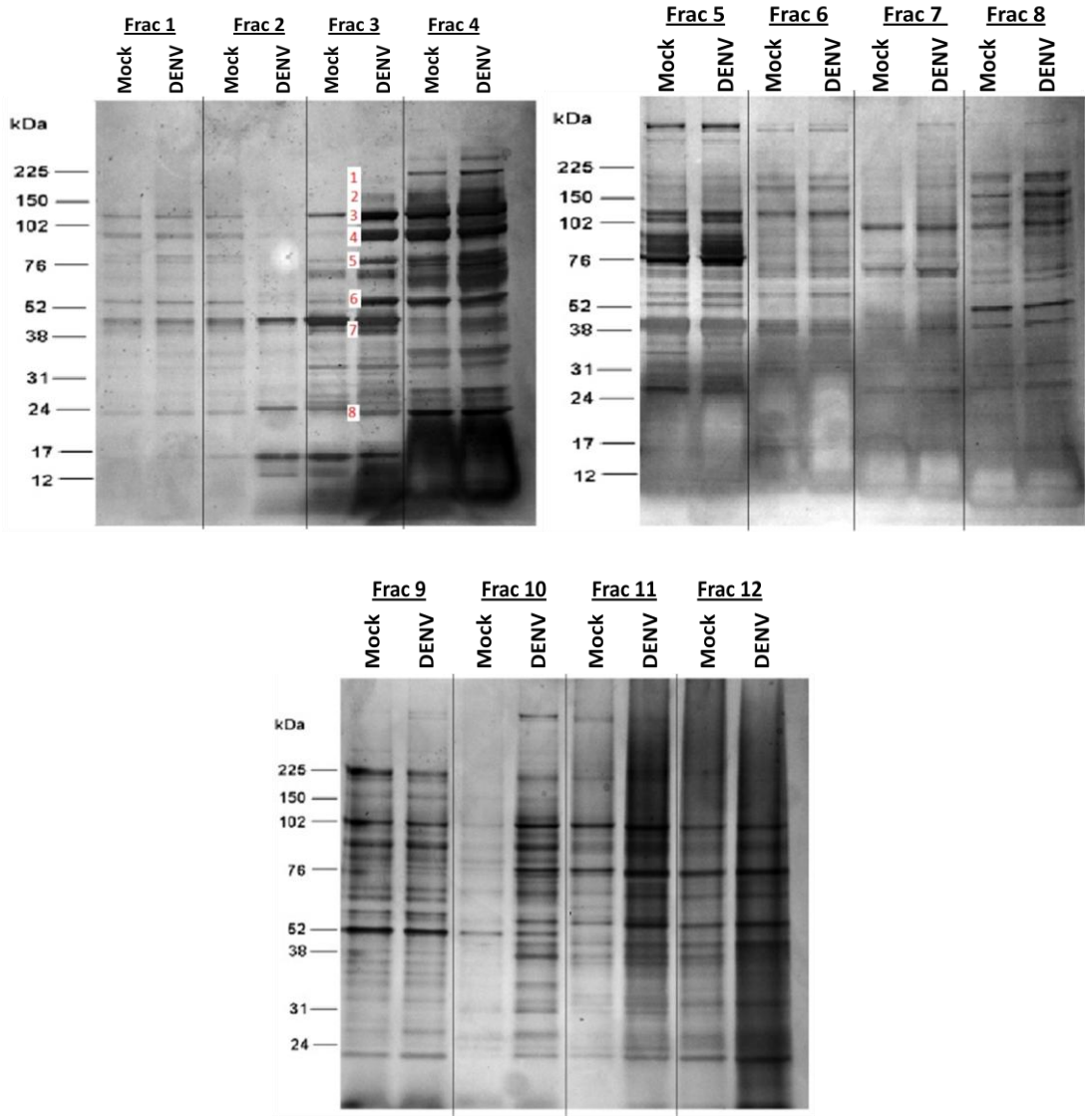
Fig. 11: ***Comparison of phospho-proteome of U937 cells stimulated with Mock or DENV***
U937 cells were stimulated with Mock or DENV for 10 min, lysed and subsequently diluted to 0.1mg/ml before being passed through phospho-protein enrichment columns. Eluates containing phosphorylated proteins only were then subject to iso-electric focussing and subsequently each fraction was separated by SDS-PAGE and silver stained to identify the phosphorylated proteins present. The asterisks indicate the most prominent differences in phosphorylation levels. 8 representative fractions from a total of 12 are shown. pH ranges from 3-10 across the 12 fractions.

As seen in Fig 11, the desired result was achieved using U937 cells, illustrated by the number of differences highlighted by asterisks on the gel. These preliminary experiments were carried out to ensure infection conditions, phospho-proteome isolation and subsequent fractionation resulted in clearly detectable levels of protein abundance when gels were stained and analysed. Only once the techniques were confirmed to work were experiments performed on all four conditions simultaneously using primary dendritic cells.

In light of this optimisation of chosen experimental methods, the project was then taken forward onto primary monocyte derived dendritic cells.

A total of 8 blood donors were used and from each of these, dendritic cells were generated and split into two plates (one stimulated with Mock and the other with DENV) before whole cell lysate from all Mock and all DENV stimulated cells was pooled and phospho-proteome isolation was carried out. The eluate from phospho-enrichment

columns was then subject to iso-electric focussing. This method resulted in a large enough protein mass to detect changes easily and overcame the problem of donor variation by pooling samples from many donors thereby diluting out individual differences.



Mock = Mock supernatant DENV = Dengue Virus 1-12 = Fraction number

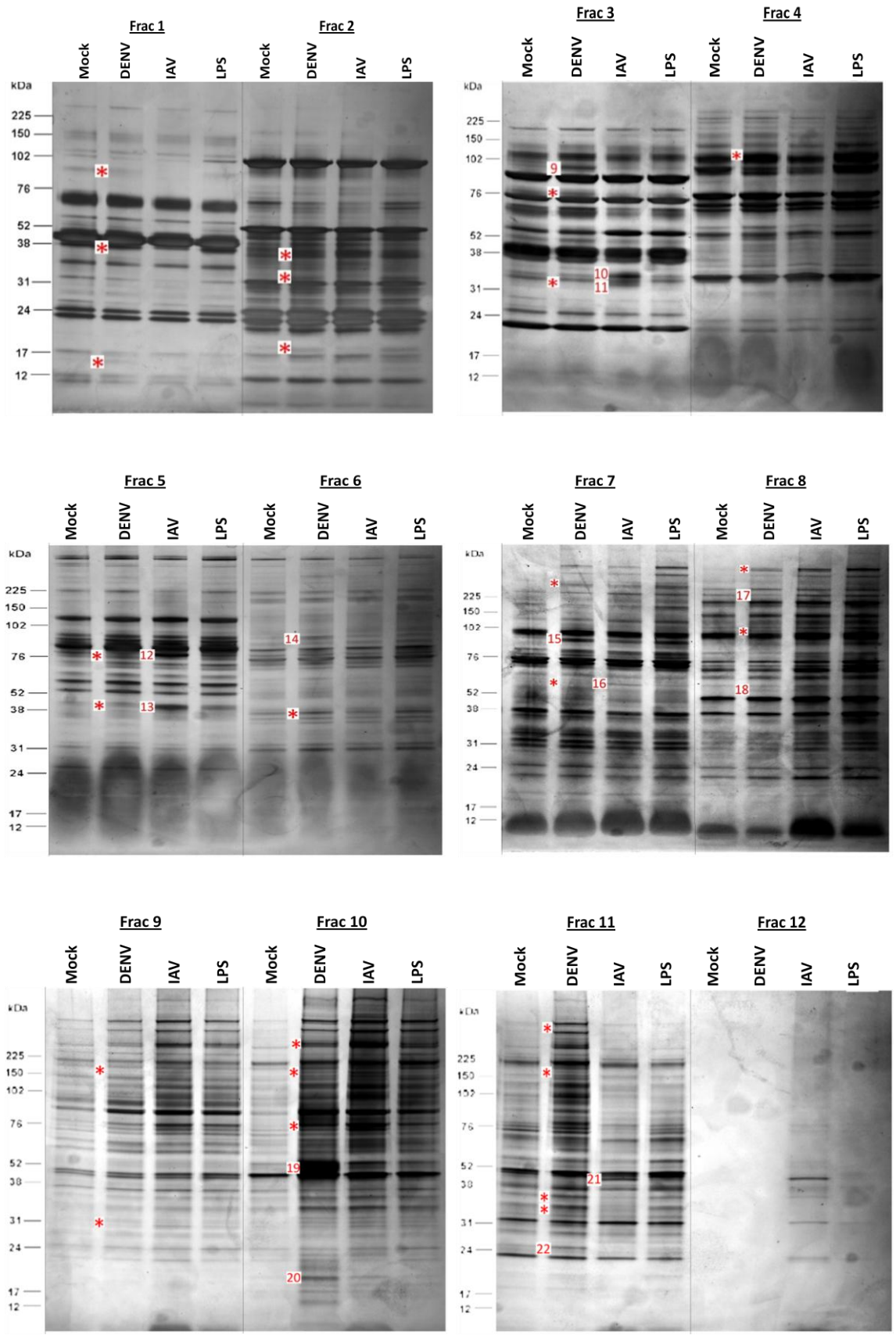
Fig. 12: ***Comparison of phospho-proteome of MDDC following stimulation with Mock or DENV***
Dendritic cells were stimulated with Mock or DENV for 10 min, lysed and subsequently diluted to 0.1mg/ml before being passed through phospho-protein enrichment columns. Eluates containing phosphorylated proteins only were then subject to iso-electric focussing and subsequently each fraction was run on an SDS-PAGE gel which was silver stained to identify the phosphorylated proteins present. The numbered bands show the most prominent differences in phosphorylation levels and these were cut from the gel for identification by mass spectrometry, proteins cut from the gel were those which lay to the right of the indicated number. pH ranges from 3-10 across the 12 fractions.

As seen in Fig 12, a vast number of differences were observed in the phospho-proteome of dendritic cells stimulated with DENV compared to those stimulated with Mock supernantant.

Having optimised the technique of phospho-proteome analysis in dendritic cells using Mock and DENV stimulation only, the experiment was repeated to include IAV and LPS stimulations. IAV was used as this virus has an overlapping target cell and receptor usage profile with DENV but does not illicit the severe immuno-pathology attributed to severe DENV infection.

LPS stimulation was included for comparison with DENV as a positive control of immune activation. Also, as LPS triggers a different innate immune response pathway (through a TLR attributed to recognising bacterial infection) it is useful to compare and identify a protein which is differentially activated/deactivated in response to DENV infection compared to both IAV and LPS.

This analysis of the phospho-proteome of dendritic cells 10 min post stimulation illustrated an array of protein bands representing differential phosphorylation (see Fig. 13).



(Figure legend overleaf)

Fig. 13: ***Comparison of phospho-proteome of MDDC following stimulation with Mock, DENV, IAV or LPS***
MDDC were stimulated with each of the 4 conditions for 10 min, lysed and WCL passed through phospho-purification columns. Proteins were then separated by OFF-GEL iso-electric focusing and an aliquot from each chamber (Frac 1-12) was run on an SDS-PAGE gel. These gels were then silver stained, shown above. Asterisks highlight the most prominent differences and numbered proteins indicate those cut from the gel for identification by mass spectrometry, proteins cut lay to the right of each number shown. pH ranges from 3-10 across the 12 fractions.

Proteins showing the largest difference in phosphorylation levels were considered most likely to go on to have recognisable effect on downstream activity. Aliquots of the fractions showing stark differences in DENV band profiles compared to other conditions underwent detailed analysis. This was alongside analysis of excised bands that showed a stark difference between abundance of phosphorylated protein in DENV stimulated cells compared to Mock, IAV and LPS stimulated cells.

The proteins showing bands present in DENV but not in other conditions or vice versa (see Fig. 14 A) below) represent the best chance of identifying a protein which has a key role in the outcome of infection as its phosphorylation will either be completely abolished or uniquely induced in response to DENV stimulation.

However, proteins differentially phosphorylated in response to DENV compared to one or two other conditions only were also taken forward for investigation (see Fig. 14 B overleaf) to ensure significant proteins are not overlooked at the analysis stage. It may be that although they are not uniquely modified in response to DENV, they still play a vital part in the eventual immuno-pathology of infection.

Bands in fraction 12 of this experiment did not stain clearly, most likely due to insufficient sample recovery from chamber 12 resulting in low protein abundance and undetectable levels of protein in the sample loaded into the gel. This is unlikely to have affected the subsequent analysis as proteins in the outermost chambers did not tend to show such stark differences as those at mid-point along the gel strip.

Band profiles of proteins uniquely modified by DENV stimulation and those modified by DENV differently to one or more other conditions

A) Uniquely different (e.g. Band 9)

B) Not uniquely different (e.g. Band 15)

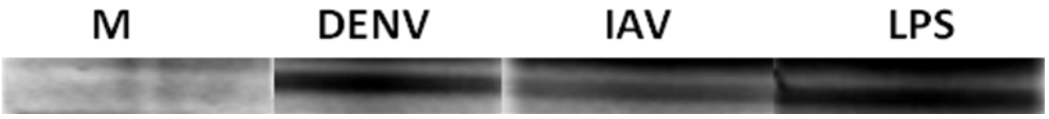


Fig. 14: ***Band profile examples of proteins taken forward for identification and validation***
A) A protein identified by silver stain as uniquely modified in response to DENV stimulation as only the DENV lane illustrates phosphorylated protein **B)** A protein identified by silver stain to be modified differently in DENV stimulation compared to Mock and IAV but not LPS, this protein was still taken forward as the difference between DENV and Mock and DENV and IAV could imply a role in severe immuno-pathology.
In total, 22 bands were excised and sent for mass spectrometry analysis and protein identification.

All results gained so far have shown promising differences in the phospho-proteome of cells stimulated with DENV as compared to those stimulated in other conditions, however up to this point results have simply shown that certain proteins show different levels of phosphorylation. In order to develop the project from here, specific proteins needed to be identified in order to ascertain if they have a role in the immune response to DENV.

Mass spectrometry analysis of specific fractions from iso-electric focussing chambers chosen by the number of differentially phosphorylated proteins identified within them by silver stain

The results of the silver staining were analysed and the fractions showing the most promising differential profiles from one condition to the next were chosen to be sent for mass spectrometry analysis. This is a sensitive protein detection method which serves to identify each protein present in each sample. As only phosphorylated proteins were isolated by the phospho-columns, any relative difference in protein abundance detected in DENV compared to other conditions can be attributed to different levels of phosphorylation of the identified protein within the cell lysate. This method of detection will result in a list of proteins identified in each sample which can be utilised to identify those showing different levels of phosphorylation in the cell after 10 min DENV stimulation. They could also illustrate proteins not necessarily phosphorylated themselves, but held in association with other phosphorylated proteins and subsequently trapped by phospho-enrichment columns.

Table 1: ***Interesting proteins identified from mass spectrometry analysis***
From initial protein lists identifying all proteins within gel pieces and selected IEF fractions, hits were narrowed down as such: proteins with a protein score below 20 were disregarded due to probability of false hits, proteins with the largest difference in score between DENV and any other condition were listed initially and subsequently those that could potentially be involved in immuno-pathology were selected as potential candidates for further study. Protein score provides an indication of relative abundance and those highlighted in red were subsequently validated by western blot.

Protein	Accession No	Protein score			
		Mock	DENV	Flu	LPS
Calmodulin	P62158	672	0	N/A	N/A
Serine/threonine-protein kinase MARK1	Q9P0L2	24	0	N/A	N/A
Ena/VASP-like protein	Q9UI08	96	47	142	253
Protein disulfide-isomerase A4 (ERp72)	P13667	30	0	360	41
High mobility group protein B1	P09429	367	142	91	428
Rho GDP-dissociation inhibitor 1	P52565	21 195	161 251	N/A 162	N/A 161
Calumenin	O43852	125	54	N/A	N/A
Endoplasmin	P14625	174 199	584 177	N/A 122	N/A 200
Protein S100-A9	P06702	45	25	39	67
Antileukoproteinase	P03973	0	153	0	0
Lactotransferrin	P02788	0	122	0	0
Charged multivesicular body protein 4b	Q9H444	0	102	N/A	N/A
Calpastatin	P20810	138 0	0 57	126 N/A	77 N/A
Docking protein 2	O60496	0	44	0	0
BH3-interacting domain death agonist	P55957	0	39	0	0
EF-hand domain-containing protein D2	Q96C19	65 0	129 34	37 N/A	46 N/A
Calcineurin subunit B type 2	Q96LZ3	0	27	0	0
Calcium-regulated heat stable protein 1	Q9Y2V2	0	27	0	0
Serine/threonine-protein kinase PAK 2	Q13177	137	216	121	111
78 kDa glucose-regulated protein	P11021	137 1143	1039 1788	N/A 1708	N/A 1416
Stathmin	P16949	0	89	52	149
Protein disulfide-isomerase	P07237	0 37	0 734	574 N/A	242 N/A
Calreticulin	P27797	364	481	N/A	N/A
Plastin-2	P13796	345	457	381	360
Protein disulfide-isomerase A3	P30101	261	310	280	354
Serine/threonine-protein kinase PAK 1	Q13153	26	46	0	0
Protein disulfide-isomerase A6	Q15084	0 419	64 770	N/A 571	N/A 736
Vimentin;	P08670	95	152	N/A	N/A
Stathmin-2	Q93045	0	79	0	0

A list of over 200 proteins was generated from mass spectrometry analysis (see Appendix for results tables) and this table shows a selection of these proteins, chosen by the magnitude of difference between protein scores in DENV stimulated samples compared to those in Mock, IAV or LPS stimulated samples. The protein score is the negative logarithmic value of the probability that the peptide matches are random and therefore is an indication of the abundance of each protein within the sample.

Proteins were also screened for potential involvement in signalling pathways or immune responses by reviewing current literature regarding their known functions. Proteins which were chosen and subsequently went on to have their differential phosphorylation profile validated by western blot are highlighted in red (see Table 1).

All the proteins with multiple hits (i.e. seen in samples from the Mock and DENV only AND in the Mock, DENV, IAV and LPS experiments) were detected in the same numbered chamber of IEF gels each time which increases the confidence that the protein was identified correctly.

SINQ analysis of data returned from mass spectrometry

The raw results were entered into a computational analysis method called SINQ, which calculates the ratio of protein abundance in one condition compared to another (Trudgian et al., 2011). This method of analysis can be used to show the ratio of difference in level of phosphorylated protein abundance in cells stimulated with DENV compared to Mock supernatant, cells stimulated with IAV compared to Mock supernatant and cells stimulated with LPS compared to Mock supernatant (see Table 2). The ratio allows a gauge of the level of phosphorylation seen in one protein compared to the level of phosphorylation seen in another protein in response to DENV stimulation, this can

then be compared to the differential phosphorylation level of these proteins in response to the other stimuli. Proteins can then be identified based on the ratio of differential phosphorylation they undergo in response to DENV, with the hypothesis that a larger difference in phosphorylation will lead to a more pronounced effect on the immune response and a higher chance that this differential phosphorylation will elicit a difference in immune responses. It will also be easier to detect and study as a small effect will likely be difficult to replicate consistently and study effectively.

Table 2: ***SINQ analysis of mass spectrometry results***
The results from mass spectrometry were subject to SINQ analysis, showing ratios of protein phosphorylation in Mock stimulated cells compared to DENV, IAV or LPS stimulated cells. The proteins with the highest ratio of difference between conditions were listed here as potential candidates for further study. Some proteins were detected in samples from initial experiments with Mock and DENV comparison only and therefore no IAV/Mock or LPS/Mock results were shown for these.

Description	Ratio DENV/Mock	Ratio IAV/Mock	Ratio LPS/Mock
Anti-leukoproteinase	DENV only		
40S ribosomal protein SA	5.82	Mock only	Mock only
Protein disulfide-isomerase A4	Mock only	80.589	4.39
Isoform 2 of Elongation factor 1-delta	3.31	12.57	6.44
Proteasomal ubiquitin receptor ADRM1	0.65	4.63	Mock only
Calcyclin-binding protein	DENV only		LPS only
Serine-threonine kinase receptor-associated protein	DENV only		
Sorting nexin-2	1.92	Mock only	Mock only
Immunoglobulin J chain	DENV only		
Ig alpha-1 chain C region	DENV only		
Lactotransferrin	DENV only		
Tropomyosin alpha-4 chain	2.16	Mock only	Mock only
Isoform 2 of Tropomyosin beta chain	5.40	Mock only	Mock only
Protein disulfide-isomerase	41.57	54.24	14.14
Vimentin	3.31	Mock only	Mock only
X-ray repair cross-complementing protein 5	DENV only		LPS only
Endoplasmin	4.04	0.38	0.35
Heat shock protein HSP 90-beta	2.40	4.52	2.58
Isoform 5 of Calpastatin	0.47	3.19	16.06
Proteasome subunit alpha type-5	DENV only		
Isoform 1 of Ena/VASP-like protein	0.20	2.30	11.75
Charged multivesicular body protein 4b	DENV only		
Eukaryotic translation initiation factor 2 subunit 1	5.66	7.15	15.13
Peroxiredoxin-4	DENV only		
Switch-associated protein 70	0.90	4.33	2.78
BH3-interacting domain death agonist	DENV only		
Mucin-5B	DENV only		
Reticulocalbin-1	DENV only		
Splicing factor 3B subunit 5	DENV only		
Docking protein 2	DENV only	IAV only	

The proteins listed here (Table 2) represent those with either unique phosphorylation profiles in response to DENV compared to the other conditions, high ratios of difference between various stimuli or interesting roles within the cell pertaining to signalling pathways and immune responses.

Mass spectrometry of bands cut from silver stained SDS-PAGE gels showing highest levels of difference in DENV as compared to Mock or IAV

As mentioned earlier, specific numbered bands were sent for mass spectrometry analysis (seen in Figs. 12 and 13) in order to identify those proteins that showed the most striking differential phosphorylation profile. Identified proteins are illustrated in Table 3.

Table 3. ***Proteins identified by mass spectrometry of gel pieces cut from silver stained gels***
Specific bands showing the largest difference in phosphorylation between DENV and each other condition were cut from silver stained gels, de-stained and digested. The samples were analysed by mass spectrometry and individual proteins identified for validation and potential further investigation

Gel Band Number	Molecular Weight (kDa)		Protein	Accession Number	Protein Score
	Gel	Actual			
1	200	104	Protein Niban	Q9BZQ8	600
2	150	125	Importin-5	O00410	558
3	120	93	Endoplasmin	P14625	5228
4	80	83	HSP-90 β	P08238	2658
5	75	80	Calpain-2	P17655	237
6	55	57	PDI	P07237	2697
7	40	42	Protein phosphatase 1 regulatory subunit	Q15435	586
8	25	28	14-3-3 protein β/α	P31946	1332
9	90	90	TER ATPase	P55072	782
10	35	47	ER resident protein 44	Q9BS26	110
11	30	33	Protein SET	Q01105	316
12	75	73	PDI A4	P13667	1234
13	40	55	Coronin 1β	Q9BR76	329
14	80	83	HSP- 90 β	P08238	846
15	80	83	HSP-90 β	P08238	921
16	55	61	EH domain-containing protein 4	Q9H223	574
17	225	312	E3 ubiquitin-protein ligase	O95071	24
18	50	122	POTE ankyrin domain family member E	Q6S8J3	63
19	45	53	VEMGP	Q8TDL5	200
20	20	26	Proteasome subunit β Type 1	P20618	1091
21	40	59	ATP synthase subunit alpha	P25705	902
22	25	26	V-type proton ATPase subunit E 1	P36543	59

As seen in Table 3, proteins were identified by mass spectrometry and the protein believed to be contained within the band was ascertained by protein score (higher score indicates higher abundance within the gel band increasing confidence in the result) and by correlating their expected molecular weight to the weight of the band seen on the gel. Many proteins were identified and some were chosen for further validation (indicated in red). Also, as many of the identified proteins have a role in signalling pathways this illustrates that even after 10 min stimulation the cell is rapidly altering in anticipation for viral infection. To identify a protein involved in this process which is regulated differently in response to DENV compared to IAV or Mock is the aim of further study.

Results from both arms of mass spectrometry carried out returned promising candidates for further investigation with a long list of over 200 proteins indicating differential phosphorylation in response to DENV stimulation compared to other viral infections.

From these proteins, the most likely candidates to alter the course of infection and immuno-pathology were chosen for validation by western blot (see Fig. 15).

Validation by western blot of proteins identified by mass spectrometry to be differentially phosphorylated in response to Mock, DENV, IAV or LPS

Proteins identified as interesting candidates based on the criteria set out previously in this chapter were subsequently validated for differential phosphorylation status by western blot (see Fig 15). This was carried out by stimulating dendritic cells for 10 min with either Mock, DENV, IAV or LPS and subsequently detecting phosphorylation levels using antibodies raised to specifically target each protein of interest. This validation process was carried out using whole cell lysate (WCL) samples alongside phospho-eluate samples after iso-electric focussing.

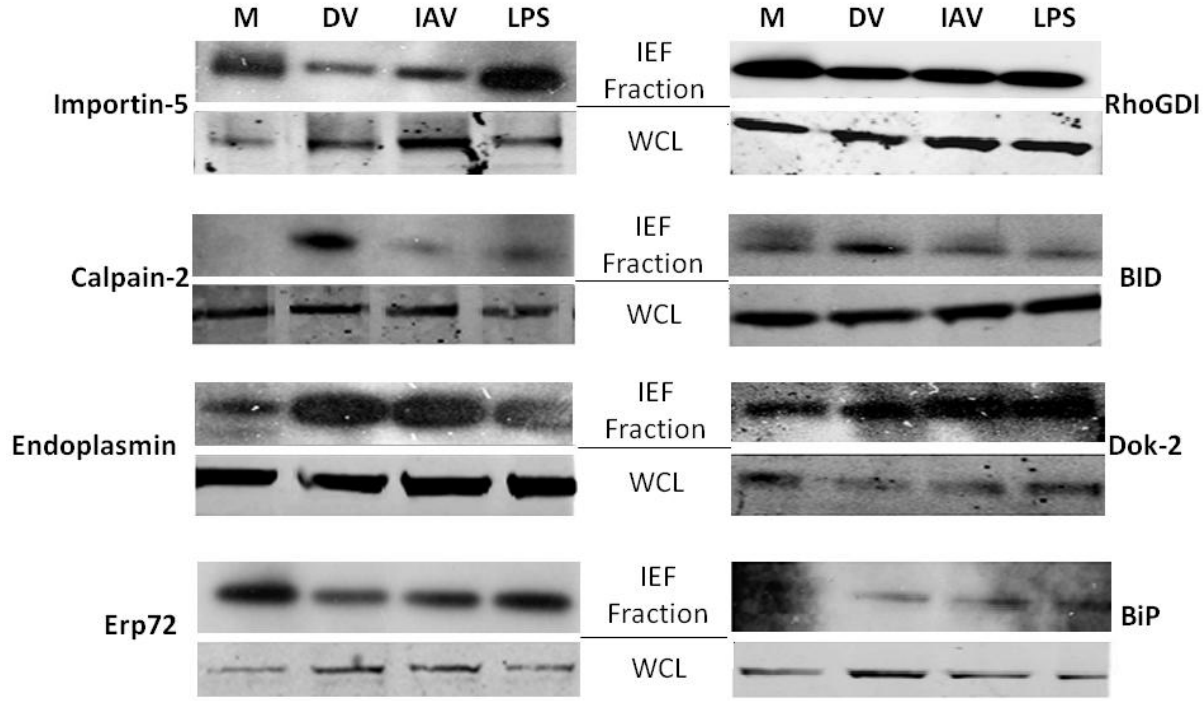


Fig. 15: ***Validation of detection of differentially phosphorylated proteins identified by mass spectrometry*** MDDC were stimulated for 10 min with Mock, DENV, IAV or LPS. Cells were lysed and phosphorylated proteins were isolated by phospho-enrichment columns. Eluate from the columns was subject to iso-electric focussing and both whole cell lysate and fractions from IEF chambers were run on separate SDS-PAGE gels, protein bands were detected using specific primary antibodies and HRP-conjugated secondary antibodies.

The expectation/assumption was that levels of protein would be seen as comparable across conditions in whole cell lysate samples as all protein within the cell is detected, not just phosphorylated protein, and there is unlikely to be protein synthesis or degradation within 10 min of stimulation. However, after the isolation of the phospho-proteome and subsequent sample simplification using iso-electric focussing the differential phosphorylation profile of each protein can be highlighted by band intensity. As seen in Fig 15 above, the differential phosphorylation status of the chosen proteins under various stimuli was confirmed and each WCL panel showed either no fluctuation or fluctuation in the opposite direction to that in the corresponding IEF panel. Whilst, contrary to expectation, protein levels were seen to change slightly even after just 10 min stimulation, for this study the fact that each protein chosen showed no change or a fluctuation in the opposite direction implies the differential phosphorylation level would be even greater than inferred by the gels and therefore proteins were still taken forward as candidates for further investigation. It would be interesting for further study to analyse each individual IEF fraction for differences in phosphorylation levels for each protein as it may be that differential phosphorylation would be detected in fractions other than those identified from the initial experiment.

Whole cell lysate was utilised as a loading control rather than β-actin, as β-actin and many other standard housekeeping proteins are not constitutively phosphorylated proteins and are therefore not present in eluate from the phospho-purification columns.

Importin-5, RhoGDI and Erp72 showed reduction in phosphorylation level in response to DENV stimulation whereas Calpain-2, Dok-2, Endoplasmin and BID showed increased phosphorylation levels. BiP showed a slight increase in phosphorylation level in response to DENV stimulation as compared to other stimuli tested. All but Importin-5

and Rho-GDI showed the same trend in phosphorylation level in both mass spectrometry and western blot analysis.

Further validation of differential phosphorylation of proteins identified to potentially play a role in immune response to DENV

Calpain-2

From the proteins whose differential phosphorylation was successfully validated by western blot, Calpain-2 showed the most striking difference in level of phosphorylation in DENV stimulated cells as compared to all three other conditions.

There are many members of the Calpain family, approx 14 in total (Storr et al., 2011), and all are calcium-activated cysteine proteases. The most well studied players in the Calpain system are μ-Calpain, m-Calpain (Calpain-2) and Calpastatin. Calpain-2 is ubiquitously expressed in mammalian cells and is generally found in close proximity to membranes in the cytoplasm. It is comprised of an active and regulatory subunit (approx 80kDa and 28 kDa, respectively) and has been seen to act in a proteolytic capacity on a number of substrates within the cell, hence its involvement in many cellular pathways.

This protein fits with the hypothesis that very early effects could play a role in the immuno-pathology given that it is affected by calcium signalling which can take place very soon after recognition of virus (Berridge et al., 2003; Zhou et al., 2009). This prompted further study as it also has known functions in cellular processes including apoptosis regulation, calcium signalling and regulation of cell motility. For example, Calpains have a controversial role in apoptosis with reported involvement in different capacities including initiation or inhibition by cleavage of apoptosis related proteins thereby either activating or inactivating them (Ozaki et al., 2009). Calpains can also have

a direct effect on the process of apoptosis via direct cleavage of cytoskeletal proteins during the event (Goll et al., 2003). With regard to DENV infection, apoptosis could theoretically contribute to the spread of virus as vesicles from apoptosing cells which contain virus could be engulfed by other innate immune cells, thereby providing a pathway for the virus to enter surrounding target cells.

Calpains have also been seen to play a role in cleavage of focal adhesions at the leading end of cells along with the trailing end, facilitating cell motility (Goll et al., 2003), and in podosome activity which is involved in dendritic cell migration (Calle et al., 2006). Cell motility could play a role in DENV infection as the ability of infected dendritic cells to migrate provides a method for the virus to spread around the body and take hold.

Signalling has also been seen to be affected by Calpains as they are calcium dependent proteases with many substrates being involved in signalling events (such as kinases and phosphotases) (Goll et al., 2003), this suggests that Calpain-2 may have a role in shaping the signalling events which occur in response to recognition of DENV.

As Calpains cleave proteins and often leave large catalytic subunits, it is likely these cleavages have a role in signalling pathways and protein activation rather than degradation (which would likely end up in smaller protein segments post-cleavage).

These functions have the potential to shape the immune response to infection and if altered by differential phosphorylation of Calpain-2 in response to DENV, this could highlight Calpain-2 as one of the proteins involved in initiating severe immuno-pathology.

Phosphorylation has been described to play a role in Calpain-2 regulation and therefore it is perfectly plausible that this method of activation is altered upon DENV recognition, leading to differentially regulated responses (Glading et al., 2004; Glading et al., 2001). It is also possible that phosphorylation results in de-activation of Calpain, a factor which would be interesting for further study aiming to pinpoint the exact effect phosphorylation may be having in this experimental setup.

Initially, validation was repeated using a fluorescent detection system for improved sensitivity. In this method, DC were stimulated for 10 min and subsequently the phosphorylated proteins were isolated and a western blot was carried out using specific antibodies for each target protein. These were detected using fluorescently labelled secondary antibodies and fluorescence intensity showed the relative abundance of each phosphorylated protein within the cells (see Figs. 16, 17 and 18).

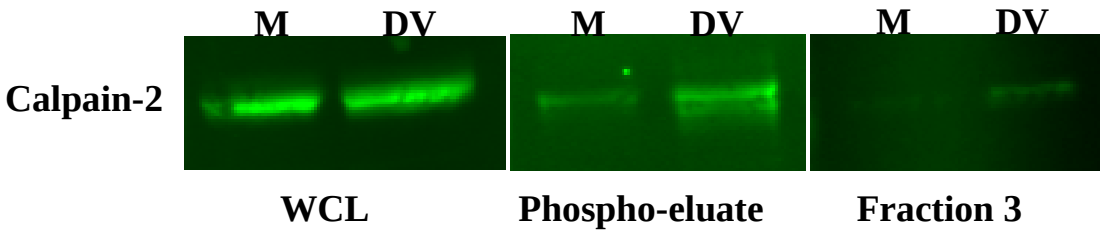


Fig. 16: ***Validation of Calpain-2 by LiCor western blot analysis***
Dendritic cells were stimulated with Mock or DENV for 10 min, lysed and subsequently diluted to 0.1mg/ml before being passed through phospho-protein enrichment columns. Eluates containing phosphorylated proteins only were then subject to iso-electric focussing and an aliquot from each stage of experimentation was used in western blots as shown. Specific anti-Calpain-2 antibodies were used for detection of proteins and fluorescently tagged anti-rabbit secondary antibodies were used for illustration on membranes. The fraction chosen for analysis corresponds to the fraction from IEF in which Calpain-2 was detected by mass spectrometry.

Again, it is seen that there are no differential protein expression levels of Calpain-2 detectable in whole cell lysate samples as expected due to the short stimulation time of 10 minutes (see Fig. 16). However, when the phosphorylation status of the protein is analysed Calpain-2 is again shown to be more highly phosphorylated in DENV stimulated cells. The double band is evident in DENV samples but not Mock and represents two different isoforms of Calpain-2 (one of ~80kDa and one of 71kDa). It could be that DENV encounter leads to phosphorylation of the smaller isoform which seems not to be present constitutively.

Further investigation of Calpain-2 was carried out in order to decipher its role in viral infection and the cellular processes affected by this differential phosphorylation status. In order to do this, functional assays were carried out after infection periods ranging from hours to days rather than the initial 10 min stimulation. This ensures that the downstream effects of initial phosphorylation are given time to manifest before sample collection and analysis (see Chapter 4).

BID

Calpain-2 is involved in apoptosis, a process that could have an effect on viral infection levels. Another protein of potential interest identified was BID, a pro-apoptotic factor affecting the same pathway of apoptosis as Calpain-2. Western blots also showed that BID is more highly phosphorylated in response to DENV stimulation than Mock stimulation (see Fig. 17). Phosphorylation of BID activates the protein and therefore the results of the western blot validation indicate a higher level of activity in response to DENV and subsequently higher levels of apoptosis in these cells.

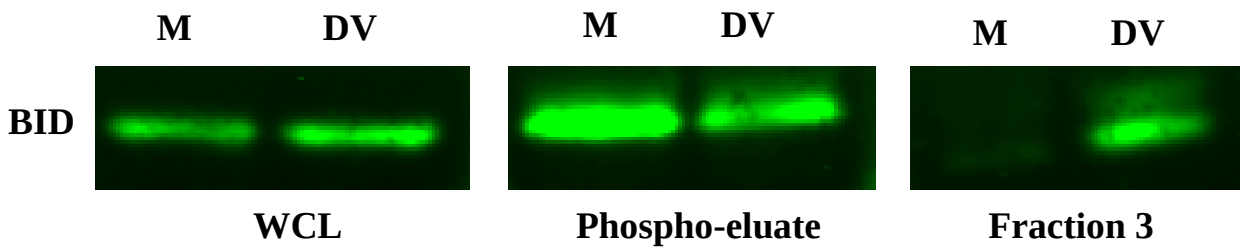


Fig. 17: ***Validation of BID by LiCor western blot analysis***
Dendritic cells were stimulated with Mock or DENV for 10 min, lysed and subsequently diluted to 0.1mg/ml before being passed through phospho-protein enrichment columns. Eluates containing phosphorylated proteins only were then subject to iso-electric focussing and an aliquot from each stage of experimentation was used in western blots as shown. Specific anti-BID antibodies were used for detection of proteins and fluorescently tagged anti-rabbit secondary antibodies were used for illustration on membranes. The fraction chosen for analysis corresponds to the fraction from IEF in which BID was detected by mass spectrometry.

As Calpain-2 and BID are both known to have a role in apoptosis it is interesting to observe that they both show a higher level of phosphorylation in response to DENV stimulation, presumably leading to a higher level of apoptosis in cells infected with DENV. The identification of two proteins involved in the same pathway and showing the same effect in response to DENV gives an increased level of confidence in the result obtained and re-enforces the theory that the level of apoptosis could indeed be regulated differently in DENV infected cells as compared to IAV and LPS stimulated cells.

Dok-2

Dok-2 is enzymatically inert and has a function as an adaptor protein or a scaffolding protein, aiding cellular processes such as formation of signalling complexes. The protein is predominantly expressed in cells of the immune system and is known to be phosphorylated on various positions, leading to interaction with molecules such as Ras-GAP and EGFR or TEK/TIE2 (Mashima et al., 2009). This molecule would be a good candidate for follow up work as it is involved in signalling and could influence the actions of dendritic cells in response to infection, possibly leading to the severe immuno-pathology seen in dengue patients.

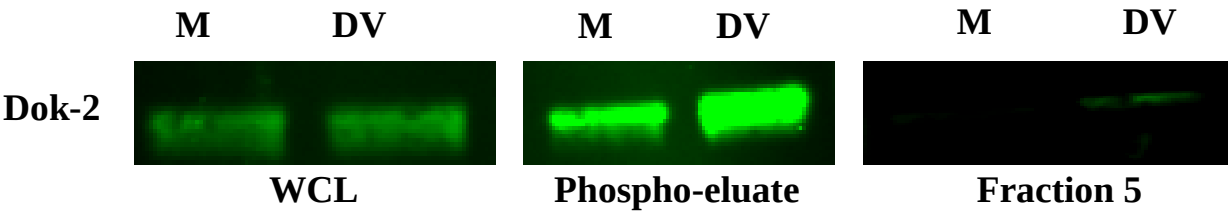


Fig. 18: ***Validation of Dok-2 by LiCor western blot analysis***
Dendritic cells were stimulated with Mock or DENV for 10 min, lysed and subsequently diluted to 0.1mg/ml before being passed through phospho-protein enrichment columns. Eluates containing phosphorylated proteins only were then subject to iso-electric focussing and an aliquot from each stage of experimentation was used in western blots as shown. Specific anti Dok-2 antibodies were used for detection of proteins and fluorescently tagged anti-rabbit secondary antibodies were used for illustration on membranes. The fraction chosen for analysis corresponds to the fraction from IEF in which Dok-2 was detected by mass spectrometry.

As seen in Fig 18 above, Dok-2 again showed a higher level of phosphorylation in DENV stimulated cells as compared to Mock stimulated cells. This confirms the earlier result and enforces the original findings, illustrating its potential as a molecule for further study.

Potentially novel phosphorylation site found on Calpain-2

Further study of mass spectrometry data pertaining to Calpain-2 detection revealed a potentially novel phosphorylation site at Ser68 (see Fig 19).

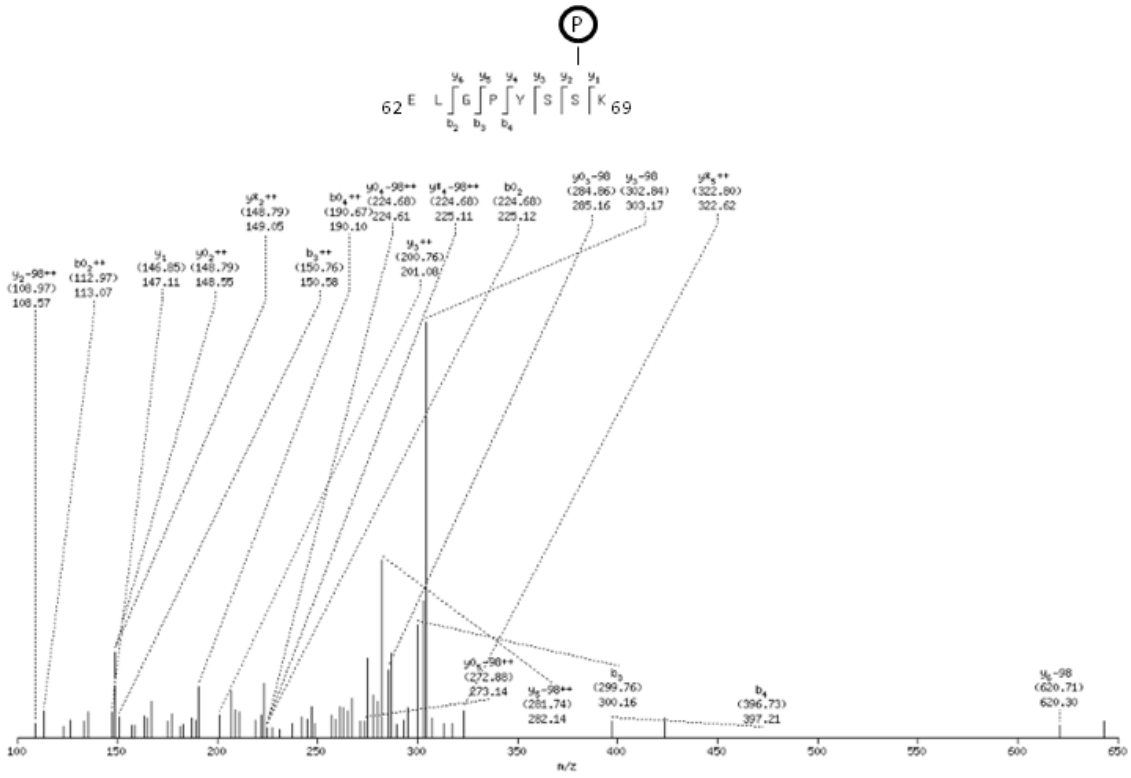


Fig. 19: ***Phosphorylation of Calpain-2 Ser67/68 upon DENV exposure***
MS/MS spectrum of the human Calpain-2 (Sprot Nr P17622) derived peptide 62-69 ELGPYSSpK containing a phosphorylation at Ser68. B and Y fragment ions are indicated; observed masses (in brackets) and theoretical masses. Evidence for phosphorylation is also provided by a loss of -98Da.

This is not one of the six phosphorylation sites reported in current literature and therefore could represent a novel phosphorylation event contributing to differential activation of Calpain-2 upon recognition of DENV. This differential phosphorylation event could potentially lead to altered regulation of protein function potentially leading to variations in immune response to DENV compared to other stimuli.

Analysis of phosphorylation levels of known signalling molecules in response to DENV

In addition to the previous wide-ranging scope of results obtained from mass spectrometry analysis, another approach was taken to identify potential proteins involved in the severe immuno-pathology seen in dengue patients. This involved utilising antibodies targeting a panel of innate immune signalling proteins in order to ascertain their level of involvement in early signalling events. The aim of this approach was to identify pathways which are triggered upon DENV recognition, possibly leading to the discovery of a differentially activated or modulated pathway in response to DENV recognition. In order to analyse these proteins, dendritic cells were infected for 10 min in order to capture very early signalling events, cells were then lysed and whole cell lysate was passed through phospho-enrichment columns to capture phosphorylated proteins. Eluates from these columns were loaded for western blotting and antibodies targeting specific signalling molecules were used for detection of protein bands (see Fig. 20). The results demonstrate the level of phosphorylation seen for each protein detected and as innate immune signalling pathways rely heavily on phosphorylation as a method of activation and inhibition as well as passing chemical ‘messages’ along prescribed pathways, these results can illustrate the activation and modulation of various pathways.

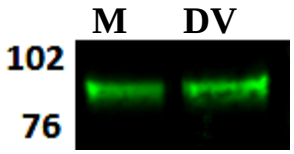
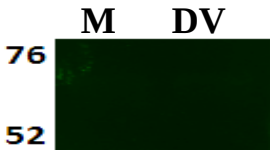
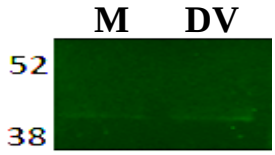
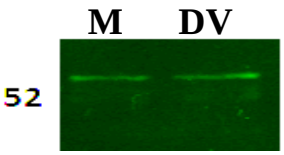
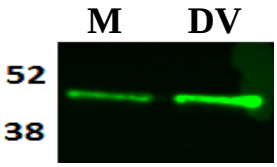
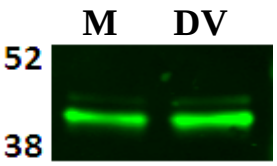
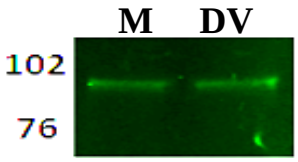
<table>
<tr><th colspan="2">Signalling proteins – Phospho-enriched samples – 10 min infection – DC</th></tr>
<tr><th>IKKb – 87Kb</th><th>IRAK-M 68kB</th></tr>
<tr><td> 
</td><td> 
</td></tr>
<tr><th>IkB epsilon – 40kB</th><th>MAPKAPK-2 (49kB)</th></tr>
<tr><td> 
</td><td> 
</td></tr>
<tr><th>MEK-1/2 (45kB)</th><th>P-p44-42 MAPK (44 or 42kB)</th></tr>
<tr><td> 
</td><td> 
</td></tr>
<tr><th>STAT 3 (79/86kB)</th><th></th></tr>
<tr><td> 
</td><td>LEGEND
M – Mock
DV – Dengue Virus</td></tr>
</table>

Fig. 20: ***Phosphorylation of known signalling molecules after 10 min stimulation with DENV or Mock***

DC were stimulated with DENV or Mock SN for 10 min, cells were lysed, whole cell lysate run through a phospho-enrichment column and subsequently eluates were run on an SDS-PAGE gel in order to detect levels of phosphorylated protein

A panel of eighteen antibodies was used for screening and from these, six of the proteins were successfully detected in the phospho-enriched samples. It was unsurprising to find a low success rate with this method as the abundance of signalling molecules as a proportion of whole cell lysate is likely to be very low, this presents a challenge as isolating phosphorylated proteins only will further reduce the level of protein within samples. From the six detectable proteins, two showed a differential level of phosphorylation between DENV and Mock stimulated cells as seen by eye. These were MEK 1/2 and IKK-β.

IKK-β is involved in the NF-kB pathway and therefore is expected to be phosphorylated in response to DENV (Thompson et al., 2011), for this reason it was deemed that this finding does not represent a novel mechanism.

MEK1 regulates ERK which plays a role in proliferation, differentiation and pro-inflammatory responses (Gaur et al., 2011). This could be important for severe immuno-pathology as proliferation and differentiation of dendritic cells can cause a more intense immune response and also more cells are available for the virus to infect, leading to more infected cells that can migrate to lymph nodes and disseminate the virus throughout the body, leading to a more severe immune response in order to clear infection.

Many other signalling proteins were also tested but no band was visible for these: IRF3, IRF4, IRF7, TRAF3, TAB-1, STAT6, STAT1, Rap 1A-1B, pMEK1/2, NFKB and MDA5. The lack of ability to detect phosphorylation of these proteins may be due to low abundance of each protein within the DC as this method may then not be sensitive enough to detect their presence.

The low hit rate and protein concentrations proved difficult hurdles to overcome along with the redundancy in signalling pathways analysed. The levels of difference were

not as striking as those seen with the phospho-proteomics approach and the protein hits provided by the mass spectrometry analysis were more likely to result in novel findings as the signalling molecules identified here are already well studied as subjects of immune responses to viral stimulation.

The aim of this approach was to identify a certain immune signalling pathway in which many proteins were differentially regulated in response to DENV stimulation. Upon analysis of the molecules found to be phosphorylated in response to DENV, no striking differences were found in pathways triggered by DENV compared to those triggered in response to other viral infections.

As the results obtained from this line of investigation were not DENV specific and merely confirmed what other groups have seen to be the case in response to other viral infections (Zhao et al., 2013) and given this study centres on identifying an immune response mechanism unique to DENV stimulation, this method of identification was not continued.

Summary

From this study so far I have ascertained that the phosphorylation profile of cells stimulated with DENV are different in comparison to Mock stimulated and IAV stimulated cells, and that specific proteins are differentially phosphorylated in response to cells recognising DENV presence. Certain proteins were validated to have a differential phosphorylation profile in stimulated and non-stimulated cells, these are candidates for further study into the mechanism of triggering the severe immune response sometimes seen in dengue patients. Finding out their role in the infection process could be invaluable in the current struggle to treat the infection before severe symptoms appear and to prevent the infection taking hold. If the function of these proteins can be identified then we can use the knowledge to find out how to inhibit/enhance their function in order to reduce disease severity. The next chapters will focus on the function of identified proteins in the immune response to viral stimulation. If the triggers of severe immuno-pathology are known, prevention and treatment methods can be specifically tailored to prevent these mechanisms from occurring upon infection.

I chose to analyse the effect on viral titre to ensure that the protein has a role in the pathogenesis of the virus and interferon stimulated gene (ISG) levels to pinpoint the stage of cellular immune responses the protein has an effect on. It could also be a cellular function like apoptosis that the protein is involved in which leads to severe disease by aiding, for example, viral spread in the host.

CHAPTER FOUR - FURTHER ANALYSIS OF PROTEINS IDENTIFIED TO BE DIFFERENTIALLY PHOSPHORYLATED IN RESPONSE TO DENV COMPARED TO OTHER STIMULI

Introduction

Having identified and confirmed differential levels of phosphorylation in candidate proteins of interest from the analysis of mass spectrometry data and subsequent western blot validation, the best candidates were taken forward for further study.

The proteins selected for follow-up investigation were Calpain-2 and Importin-5. As seen in previous figures, Calpain-2 is more highly phosphorylated in response to DENV stimulation as compared to Mock stimulation. Importin-5 on the other hand, shows a variable level of phosphorylation in response to DENV stimulation as compared to Mock.

The role of Calpain-2 was studied by ascertaining the functional levels of various cellular processes related to immune responses (such as interferon response pathways) in cells which contained a knocked down level of Calpain-2 protein.

A broader approach was adopted with Importin-5, as described in Chapter 5, given the function of the protein is less well understood making its role in infection harder to pinpoint.

Silencing of Calpain-2 for further investigation of its role in DENV infection

Silencing of target proteins is a widely accepted method of studying the effect of proteins on the process of infection in order to ascertain their function and influence on viral pathogenesis. In this study, Calpain-2 was silenced in primary dendritic cells and experiments were carried out to decipher the extent of its effect. Studying cellular processes known to involve proteins of interest in the absence of the protein gives an understanding of its role within the cell. For example, if the protein is knocked down and viral titre is subsequently reduced, this indicates that the virus requires the protein/activated protein in order to effectively infect the cell and produce infectious progeny. Also, if the response of the cell to viral infection is lessened in the absence of the protein, this indicates that the protein may have a role in the establishment of an antiviral state within the cell, perhaps through signalling pathways or by reduction in proteolytic targeting and cleavage of proteins which inhibit such a state being established in the cell during standard conditions.

In order to knockdown Calpain-2 within the cell, a passive transfection system called Accell was utilised which delivers a pool of 4 protein specific siRNA molecules and results in interference with translation by breakdown of the mRNA (Agrawal et al., 2003) corresponding to the gene of interest. This interference results in a lower level of gene translation resulting in less protein present within the cell. This system was chosen as the alternative methods of siRNA delivery were not deemed suitable for this study. Lentiviral vector delivery was immediately ruled out as it is the response of the cell to a viral stimulus that is being analysed, therefore adding a viral backbone into the cell could

trigger a response in addition to the stimulus of interest. This may result in a recorded response which is not in fact attributed to DENV stimulation, but to lentiviral stimulation.

Electroporation was also considered as a means of siRNA delivery, however a high percentage of cells were killed in the process of transfection (Lepik et al., 2003). As primary dendritic cells were utilised for this part of the study, cell number was a limiting factor and therefore the method was not utilised. Also, the pulse of electricity used to shock the cells into uptake of siRNA (giving temporary permeability) would lead to a state of 'shock' within the cell, leading to triggering of many pathways which crosstalk with immune response pathways. This general immune activation may mask the effect of DENV stimulation on cells with knocked down levels of Calpain-2 as the cellular response to shock may be recorded as a response to DENV. Controls can be used to increase confidence in the results using these systems, however in this study passive transfection was chosen as it exerts the least stress on the cell and therefore is the closest mimic of *in-vivo* stimulation with DENV.

To optimise the protein silencing technique, varying concentrations of FCS (optimal = 2%), varying number of cells (optimal = 2million) and changing Accell delivery media for regular R10 after 72 hr (no difference compared to leaving Accell delivery media on) were analysed.

Utilising these optimal conditions Calpain-2 was silenced in dendritic cells and a representative western blot is shown below (Fig. 21).

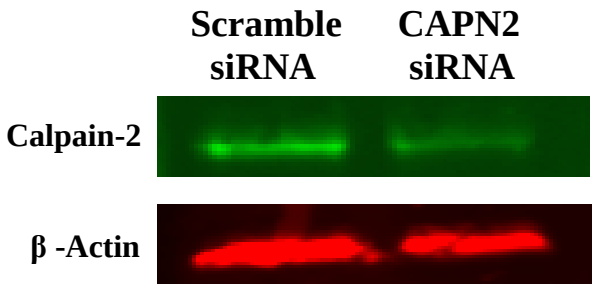


Fig. 21: ***Silencing of Calpain-2 gene in primary dendritic cells***
Dendritic cells were isolated from PBMC and incubated for 72-96 hr with a pool of 4 Calpain-2 specific siRNA molecules. Cells were then lysed and whole cell lysate was subject to western blot. Calpain-2 was detected by anti-CAPN2 primary antibody and anti-rabbit, fluorescently tagged LiCor secondary antibody was used to detect the bands with the LiCor Odyssey system. β-actin was used as a loading control.

The intensity measurement obtained from LiCor software shows that knockdown was not 100% efficient. Band intensity recorded for samples (see Fig. 21) are 1.72 with no siRNA added and with siRNA added to wells, Calpain-2 detection levels dropped to 0.34. This shows approximately 80% knockdown in cells. The differential band intensity between knockdown and wild type cells can be identified by eye however this method of establishing levels of knockdown does not provide an accurate result. To obtain a quantitative readout of siRNA effect, Odyssey software can be utilised to draw boxes surrounding each band and the intensity of fluorescence within each box is assessed by the program. This method was utilised to establish that the difference in intensity of bands representing Calpain-2 protein between cells incubated with and without specific siRNA for 72 hr is on average 40% (see Fig. 22 below).

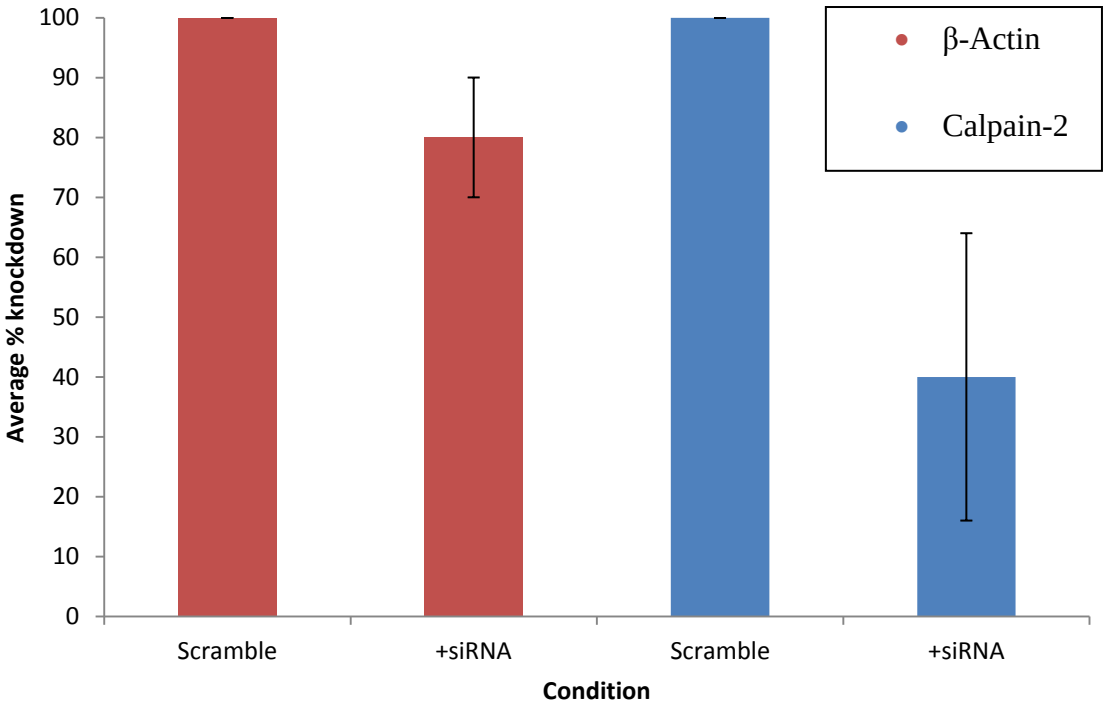


Fig. 22: ***Average percentage knockdown of Calpain-2 after incubation with siRNA***
Intensity of bands observed from western blot was measured using LiCor Odyssey software. Boxes of equal size were drawn surrounding each band and the fluorescence intensity of Calpain-2 was measured. Representative bar graph shows average level of knockdown.

Ideally, cells would be analysed under conditions of 100% knockdown of Calpain-2 (or the highest percentage of knockdown which does not result in adverse affects on regular cellular function) so as to avoid any false positive results, however the level of knockdown observed resulted in clear differences in functional assays carried out on cells incubated with Calpain-2 targeting siRNA compared to scrambled siRNA control prior to stimulation with Mock or DENV. Therefore, it is plausible that the observed effect of knockdown was due to the reduced function of this protein in cells in which targeting siRNA was added. This is implied by the detection of effects on cellular function following only a low percentage of protein knockdown, leading to the hypothesis that an even greater result would be observed following a higher percentage knockdown of Calpain-2. Therefore, the data obtained is likely to be an underestimation of the full effect of the protein on the cellular function being analysed. This increases the

confidence that the results of this study are representative of those which occur in cells upon DENV encounter *in vivo*.

Once the silencing technique had been optimised and conditions of silencing were determined, the siRNA was added as detailed in the materials and methods and subsequently the cells were infected with DENV for 48 hr, 72 hr or 5 days. This increase from 10 min stimulation to longer term infection allows the downstream effects of initial signalling pathways to play out, showing the effect of differential pathway regulation at early time-points on the eventual outcome of infection.

Dendritic cell morphology post siRNA treatment

Dendritic cells were isolated and incubated with siRNA for 96 hr and at this time point I took a digital image of cell morphology (see Fig. 23) to ensure addition of siRNA did not cause cell death and therefore skew results thought to be due to viral infection.

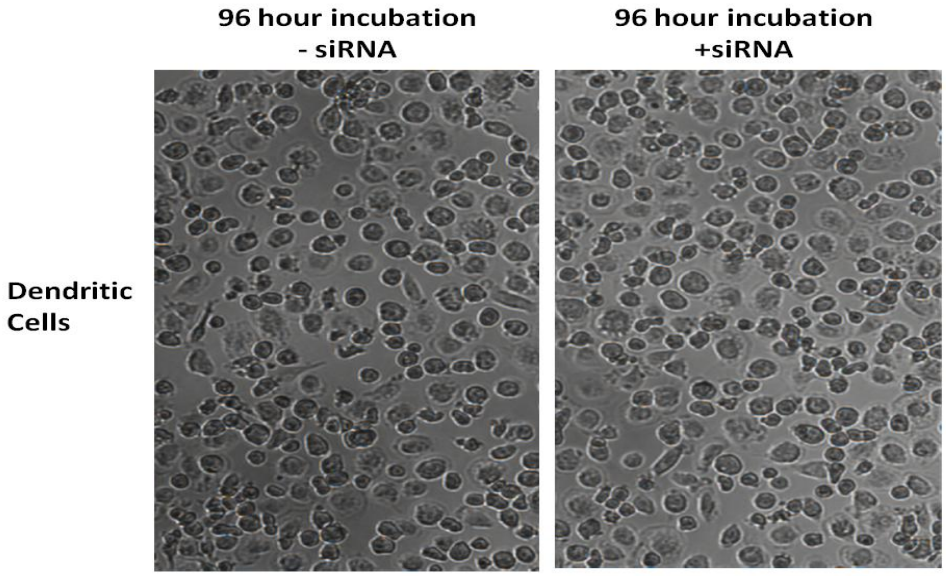


Fig. 23: ***Primary dendritic cells incubated for 96 hr with or without siRNA added***
Dendritic cells were incubated in Accell media with and without siRNA molecules for 96 hr to observe the effect of siRNA addition on cell morphology.

As seen in Fig 23, the addition of siRNA to the cell culture medium has minimal effect on visually detectable parameters of immature dendritic cells. This ensures that the effect seen in functional assays is indeed due to the lower levels of Calpain-2 within the cell, rather than a lower level of sample due to excess cell death in response to the presence of siRNA.

Measurement of viral titre post-dengue virus infection in dendritic cells with knocked down levels of Calpain-2

The first parameter measured was viral titre, in order to ascertain if the protein has any effect on downstream viral infection. This was investigated as it was interesting to ascertain if the protein of interest had a measurable effect on the ability of the virus to produce infectious progeny. This would indicate a significant role for the protein in viral pathogenesis and confirm that the protein is likely to have a measurable effect on the outcome of infection.

To investigate the effect of silencing on viral titre I used the focus forming unit (FFU) assay to measure the number of infectious viral particles/ml of sample in each condition. The method of viral titre calculation is illustrated below (see Fig. 24).

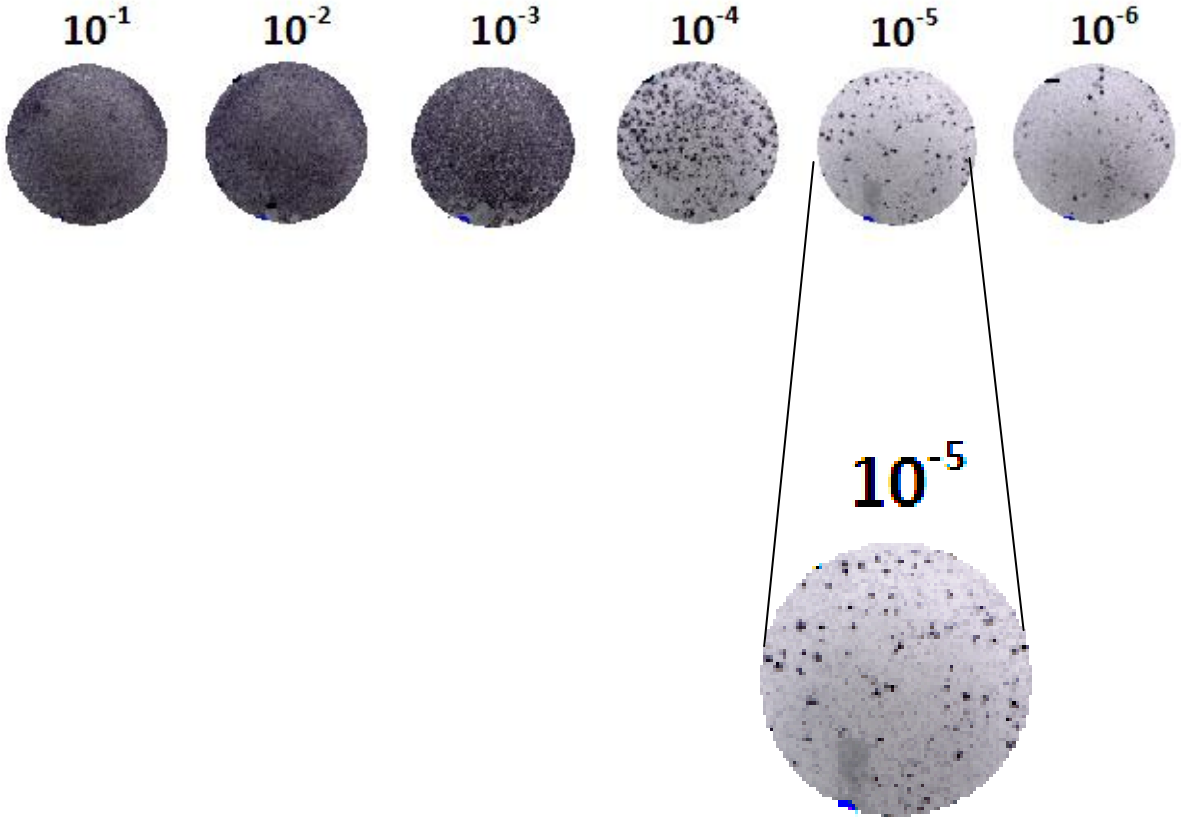


Fig. 24: ***Illustration of FFU assay titration***
Virus containing samples were diluted 1/10 in low serum media and serial dilution was carried out to obtain a titration of viral concentration. Diluted samples were added to a Vero cell monolayer and incubated for 4-5 days beneath semi-solid Avicel solution. Foci were detected using anti-DENV primary antibodies and HRP-conjugated secondary antibodies. HRP was detected using DAB substrate and virally infected cells are represented by a stained dot. Wells with a countable distribution of foci were analysed (as represented by 10^5 dilution here) and the number of foci along with the dilution factor of the sample within that well can be used to ascertain viral concentration in the stock sample.

The focus forming unit assay was carried out by serially diluting samples containing unknown viral concentration and adding a specified volume over a monolayer of Vero cells which are permissible to DENV infection. During initial infection time, the virus was recognised by cells, captured at the cell surface and internalised. As overlay medium sets into a lattice formation, virus particles are immobilised at the cell surface.

This immobilisation traps virus particles at their location, inhibiting movement throughout the inoculation/Avicel solution, ensuring each virus particle that successfully infected a cell is capable of generating one focus only. Virus progeny are released from

the cell and exposed only to neighbouring cells for subsequent infection as the lattice structure renders the progeny unable to disperse throughout the suspension and cause infection of many cells in other areas of the monolayer. Foci are detected by staining which was carried out after washing away Avicel/inoculum overlay therefore only viral particles within permeable infected cells are detected. In principle, each focus represents an initial infectious virus particle therefore the number of foci represents the number of infectious viral particles within the original sample added (regardless of the variable number of progeny produced by infected cells). It is then possible to calculate the number of infectious virus particles contained per millilitre of sample and this is known as the viral titre.

Foci counts from experiments comparing infection of DC with or without pre-incubation with Calpain-2 siRNA are presented below (see Table 4).

Table 4 (overleaf): ***Foci counts post-infection in dendritic cells pre-incubated with or without Calpain-2 specific siRNA or scrambled siRNA control***

Cells were treated with either scrambled siRNA or siRNA targeting Calpain-2 and subsequently infected with DENV. Foci counts were measured using FFU assays and results are displayed below, illustrating the difference in viral titre in cells with normal levels of Calpain-2 and cells with knocked down levels of Calpain-2. Average results were calculated including each individual result shown. C = Control (i.e. -siRNA/Scramble).

Replication Number	Foci Counts					
	Mock -siRNA	**Mock Scramble**	**Mock +siRNA**	**DENV -siRNA**	**DENV Scramble**	**DENV +siRNA**
1	0		0	$2.6x10^3$		$1.0x10^3$
2	0		0	$2.0x10^3$		$2.3x10^3$
3	0		0	$2.1x10^3$		$8.0x10^2$
			Average	$2.2x10^3$		$1.4x10^3$
			Average % of C			**61%**
1		0	0		$3.8x10^2$	$2.3x10^2$
2		0	0		$4.9x10^2$	$2.4x10^2$
3		0	0		$2.4x10^2$	$2.3x10^2$
			Average		$3.7x10^2$	$2.3x10^2$
			Average % of C			**63%**
1		0	0		$3.8x10^3$	$1.6x10^3$
2		0	0		$5.1x10^3$	$2.0x10^3$
3		0	0		$1.2x10^4$	$1.1x10^4$
			Average		$7.0x10^3$	$4.8x10^3$
			Average % of C			**70%**
1		0	0		$6.7x10^3$	$2.0x10^3$
2		0	0		$1.3x10^4$	$1.0x10^4$
3		0	0		$8.0x10^3$	$3.0x10^3$
			Average		$9.2x10^3$	$5.0x10^3$
			Average % of C			**54%**
1	0		0	$5.6x10^3$		$5.8x10^2$
2	0		0	$5.3x10^3$		$4.7x10^2$
3	0		0	$6.0x10^3$		$2.5x10^2$
			Average	$5.6x10^3$		$4.3x10^2$
			Average % of C			**8%**
1	0		0	$3.4x10^4$		$2.8x10^3$
2	0		0	$2.9x10^4$		$3.9x10^3$
3	0		0	$2.1x10^4$		$3.4x10^3$
			Average	$2.8x10^4$		$3.3x10^3$
			Average % of C			**12%**
					No siRNA	**With siRNA**
			Overall Average		$8.7x10^3$	$2.5x10^3$
			Overall Average % of C			**45%**

Each individual experiment was carried out on dendritic cells generated from a different blood donor and therefore direct comparison cannot be drawn between each experiment. This is due to the fact that blood from each donor will vary and the dendritic cells generated will elicit a differing level of response in each case. Donor to donor variation presents a significant obstacle in analysing results obtained from primary cells, however in the experiments carried out during this study the trend of difference in viral titre produced by cells in which Calpain-2 was knocked down before infection and cells in which a non-targeting siRNA was used, consistently shows the same effect.

Individual representation of each experiment completed is shown here to illustrate the trend for reduced viral titres produced by cells pre-incubated with Calpain-2 targeting siRNA (see Fig. 25).

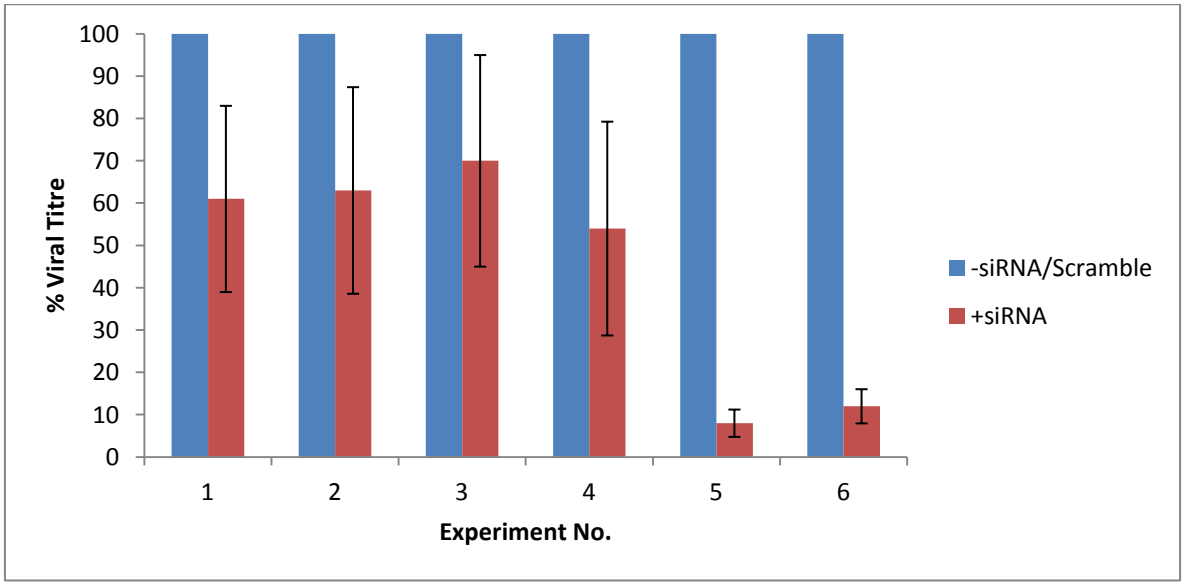


Fig. 25: ***Graph showing foci count in dendritic cells treated with Calpain-2 targeting siRNA (+siRNA) compared to scrambled siRNA control (-siRNA)***
Foci counts were seen to be reduced in cells with lower levels of Calpain-2 as compared to cells with wild type levels of Calpain-2 (titres from cells without siRNA set to 100%). Despite donor to donor variation, levels of foci produced post-DENV infection were consistently lower in cells with less Calpain-2 present.

As seen in Fig. 25, all repetitions showed lower foci counts as a result of infection of cells pre-treated with Calpain-2 targeting siRNA as compared to cells treated with scrambled siRNA. Experiments 5 and 6 showed especially marked reduction in foci count upon knockdown of Calpain-2 with foci numbers falling to just 8% and 12%, respectively, of that measured in normal conditions. To illustrate the trend observed, average values were taken from silenced cells and from wild-type cells infected with DENV and compared in Fig. 26 below.

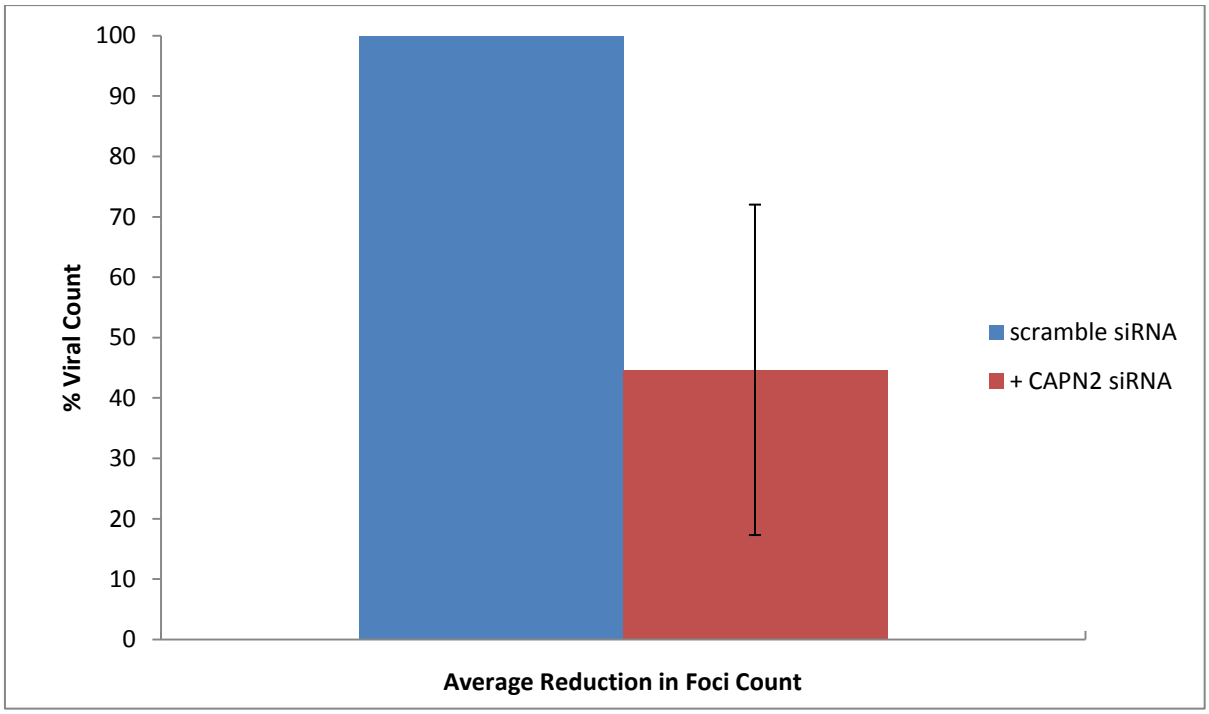


Fig. 26: ***Average reduction in foci number post-infection with DENV in cells with and without Calpain-2 targeting siRNA***
The average foci count from each repetition of this experiment shows overall reduction in viral replication in cells pre-treated with Calpain-2 siRNA compared to cells pre-treated with scrambled siRNA is 45%.

It can be seen from Fig. 26 that in the presence of Calpain-2 viral titre is consistently higher than in cells pre-incubated with siRNA targeting Calpain-2. The number of infectious viral progeny produced by cells in which Calpain-2 has been knocked down is just 45% of the number produced by cells with normal levels of

Calpain-2 present (representing a 55% reduction in the number of infectious viral progeny detected).

In order to ascertain the confidence in the results obtained from the FFU assays, statistical analysis was carried out to illustrate the significance of any difference in measured viral titre.

	Viral Titre -siRNA	**Viral Titre +siRNA**	
Mean	8711.67	2372	
SD	9972.24	2350.84	**p-value**
Sample size	6	6	0.0619

Table 5: ***Paired t test analysis of viral titres in cells with and without Calpain-2 targeting siRNA***
Statistical analysis revealed that, on average, the values obtained from measuerments of viral titre were not significantly different in cells infected with DENV post-incubation with and without Calpain-2 targeting siRNA (source: Graphpad)

The paired t-test was the chosen statistical test as the results obtained were from different assays over time. This means that the results of Mock and DENV infected samples should be compared to each other within individual experiments only in order to avoid interference from assay to assay variability. The two-tailed P value for this data set was 0.0619. This is just above the threshold of significance which is set at 0.05.

This statistical analysis suggests that the result obtained in various experiments may not be due to the fact that Calpain-2 was knocked down but rather due to random experimental variation. Given the overriding trend for viral titre to be reduced, sometimes to as little as 8% of standard conditions it would be vital to carry out further experiments

in order to increase the value of n, thereby gaining a more accurate picture of statistical significance.

When analysis was carried out on individual experiments significance was reached in three out of six donors as illustrated in the table below.

Exp. No.	p-value	Significance
1	0.279	X
2	0.189	X
3	0.075	X
4	0.021	✔
5	0.003	✔
6	0.025	✔

Table 6: ***Two-tailed, paired T-test analysis of each individual experimental repetition assessing viral titre in cells with and without Calpain-2 targeting siRNA***
Statistical analysis revealed that the values obtained from measuerments of viral titre were significantly different in cells infected with DENV post-incubation with and without Calpain-2 targeting siRNA in three out of six experimental repetitions

This individual analysis highlights that the results obtained are seen to be statistically significant in three experimental repeats with experimental variation, donor to donor variability and variation in levels of Calpain-2 silencing possibly accounting for the non-significance of the results from earlier experiments. Overall, the observed p-values from latter repetitions (see Table 6) taken with the proximity of the average significance to the threshold of 0.05 gives confidence to the conclusion that Calpain-2 silencing does indeed significantly reduce viral titre in primary dendritic cells.

Furthermore, the figures above (Figs. 25 and 26) show a consistently downward trend in viral titre from cells containing a lower level of Calpain-2 as compared to wild type controls. This illustrates that in the absence of this protein, the cell is less capable of producing the same level of infectious progeny virions as cells containing Calpain-2.

There are a multitude of pathways and processes within the cell that are affected by Calpain-2, therefore further study was undertaken to ascertain the specific mechanism which is responsible for lower viral titres.

Role of Calpain-2 and BID in apoptosis pathway axis

Apoptosis is one such pathway and investigation of the key players in this process revealed that two proteins detected in this study to be differentially phosphorylated in response to DENV stimulation were involved in the apoptosis pathway axis (see Fig. 27).

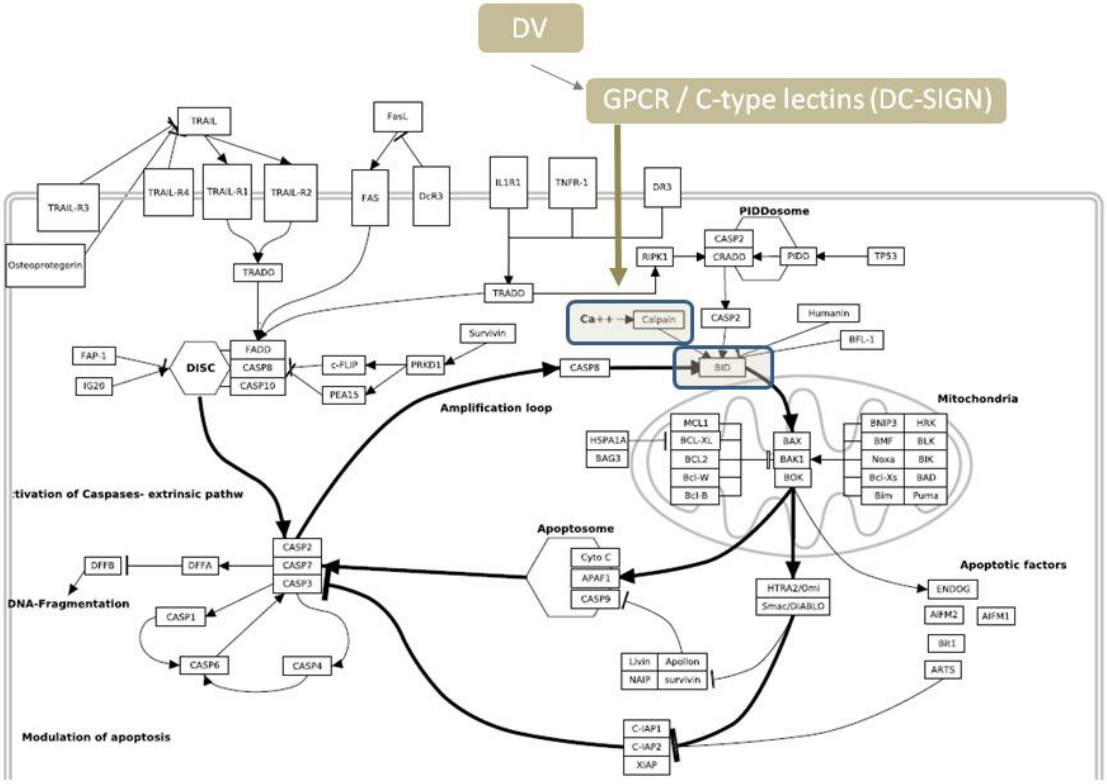


Fig. 27: Illustration of proteins involved in apoptosis pathway includes Calpain-2 and BID
Diagram of apoptotic pathway seen to involve Calpain-2 and BID, both proteins shown in this study to be differentially phosphorylated in response to DENV compared to Mock, IAV and LPS. Modification of the regulation of this apoptotic pathway could have an effect on viral infectivity, ultimately leading to altered immune response levels. (Source: KEGG PATHWAY Database)

This data indicates that DENV binding to DC within the first few minutes could trigger and modulate apoptotic pathways through a Calpain-2 and BID dependent process, possibly by binding to a G-protein coupled receptor and calcium flux (see Fig. 27) in a similar fashion that has been observed for HCV (Simonin et al., 2009) and Enterovirus 71 (Lu et al., 2013).

Measurement of interferon stimulated gene transcription (Mx1) in dendritic cells with knocked down levels of Calpain-2 at 72 hours post-dengue virus infection

As previously described, along with apoptosis Calpain-2 has a number of functions within the cell. However as the initial difference in phosphorylation level was detected after 10 min stimulation, outcomes which are affected by events occurring very early in the process of infection were pinpointed. Calpain-2 has a role in many cell signalling pathways within the cell. Signalling pathways induced upon stimulation of dendritic cells with virus largely focus on the induction of an antiviral state within the cell by up-regulating transcription of genes which work to inhibit viral pathogenesis.

Many of these genes are stimulated in response to interferon production through the up-regulation of interferon stimulated genes. There are a large number of these genes described in the literature but for this study, Mx1 was chosen as the ISG of interest. This is an antiviral ISG and so contributes to the establishment of an anti-viral state within the cell. The hypothesis was that Calpain-2 has a role in the pathway leading to stimulation of Mx1 and when silenced, the expectation was that the pathway is down-regulated/inhibited. This could be a potential indication that induction of this pathway by

Calpain-2 phosphorylation represents a mechanism by which the cell protects itself to inhibit the spread of virus.

In order to ascertain the optimal duration of viral stimulation required to induce up-regulation of Mx1, a time course experiment of ISG levels within the cell in response to viral stimulation (no silencing) was carried out (see Fig. 28).

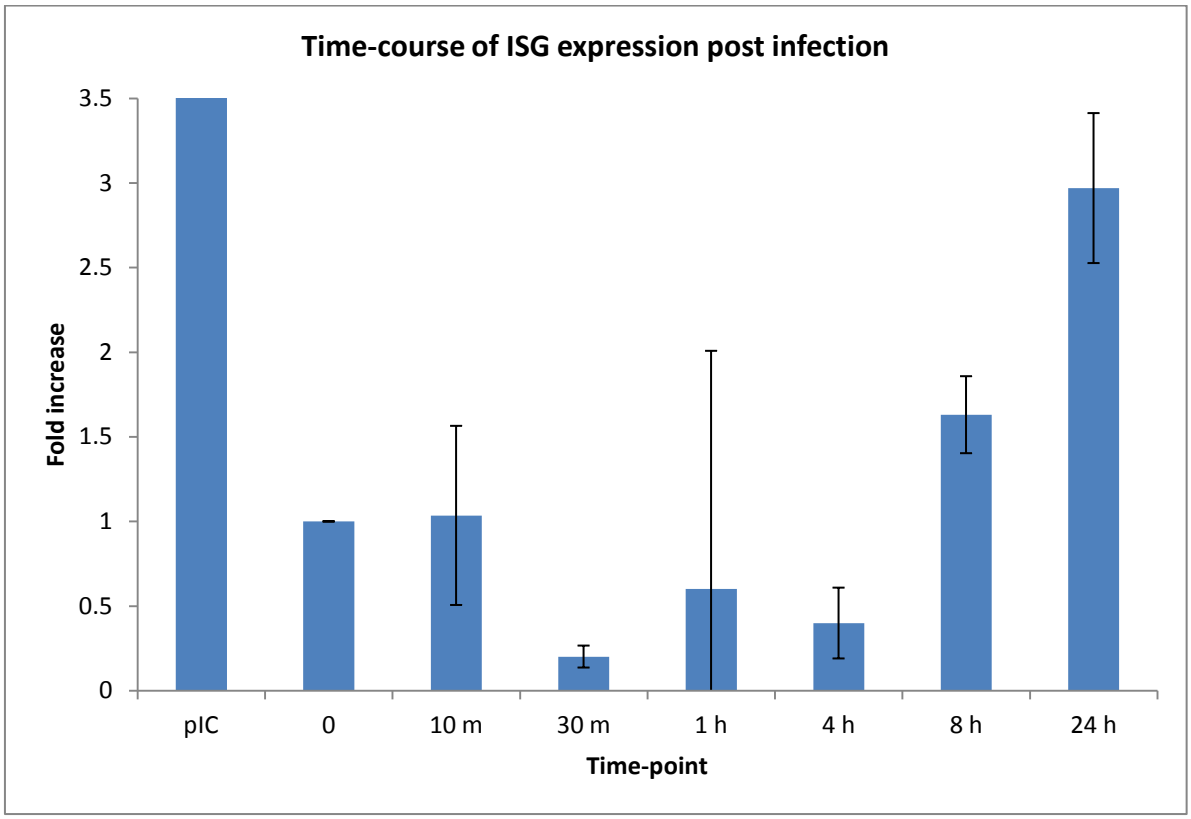


Fig. 28: ***Level of ISG (Mx1) expression over time post-DENV infection***
Dendritic cells were infected with DENV and at each time point, RNA was extracted and qPCR was performed to ascertain the level of Mx1 mRNA present in the cells. Results are represented as the fold change of Mx1 mRNA over time as compared to time point 0. pIC was used as a positive control of ISG up-regulation.

This showed that ISG levels increase (after an initial dip) up to the 24 hr measured during the time-course. It was presumed that this increase would continue at least up to 72 hr and therefore, experiments including silencing of Calpain-2 were terminated at 72 hr post infection. The aim of selecting this time point was it would provide the highest possible level of readout before the level of Mx1 began to decline back to steady state

levels. The fluctuation at the 30 min, 1 hr and 4 hr time points can be explained by inconsistent experimental technique as the 18s housekeeping gene values were not consistent across replicates and samples, a problem which was rectified for all further experiments.

To ascertain if Calpain-2 has a role in Mx1 production within the cell, DC were incubated with Calpain-2 targeting siRNA (or scrambled siRNA) and subsequently infected with DENV for 72 hr. Levels of Mx1 mRNA were measured by qPCR and analysed to show fold-change in expression level of Mx1 in DENV stimulated cells as compared to Mock (see Fig. 29). This will illustrate any differential change in Mx1 levels which may occur in cells with standard compared to knocked-down levels of Calpain-2, post-DENV infection.

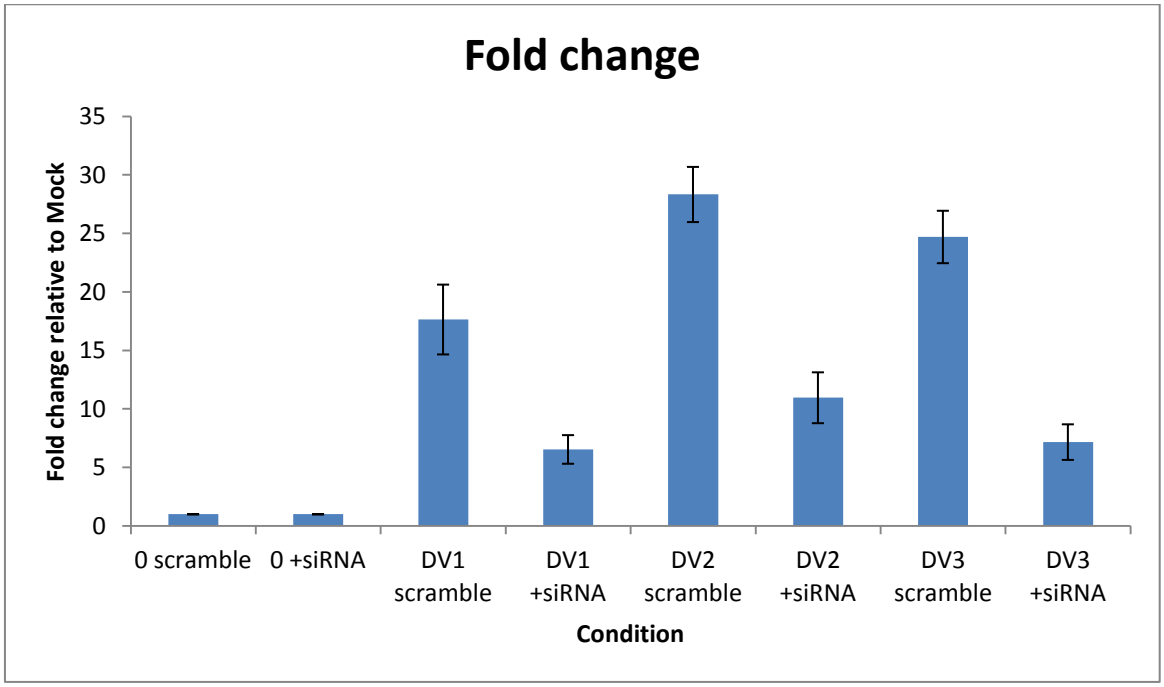


Fig. 29: ***Fold change of ISG expression after DENV infection in cells with and without Calpain-2 siRNA***
Dendritic cells were incubated with scrambled siRNA or CAPN2 specific siRNA for 72 hr, cells were then infected with DENV for 72 hr and at this time point, RNA was extracted from WCL and qPCR was utilised to measure level of Mx1 mRNA expression in cells containing wild-type levels of Calpain-2 compared to cells containing knocked-down levels of Calpain-2. Results are represented by the fold-change in Mx1 mRNA expression levels in DENV stimulated compared to Mock stimulated cells.
Scramble – siRNA with no specific target within the cell, DV – Dengue Virus, 1/2/3 – Assay number

As seen in Fig 29, the level of Mx1 was reduced as a result of Calpain-2 protein knockdown, indicating that Calpain-2 has a role in the pathway which induces its transcription.

Summary

It has been shown through these functional assays that Calpain-2, a protein identified to be differentially phosphorylated in response to DENV stimulation compared to Mock, Influenza and LPS stimulation, has a measurable role in DENV infection. Reduction in the level of Calpain-2 protein in dendritic cells results in lower levels of infectious progeny produced upon infection. Also, lower levels of Calpain-2 lead to lower levels of Mx1 (interferon stimulated gene) mRNA production showing that this protein has a role in immune signalling pathways which lead to ISG production.

It is interesting to ascertain the effect of proteins on immune responses to infection in terms of innate signalling events, but there are also several other ways in which cellular proteins can alter the outcome of infection at later points in the infection process. As seen in Fig 15, Importin-5 was also identified as being differentially phosphorylated in response to DENV stimulation. This protein is described to have an effect on DENV infection by facilitating entry of DENV proteins into the nucleus for reasons as yet undiscovered. It would be interesting to investigate the interaction partners and phosphorylation status of this protein in order to gain an insight into the effects it has on the progress of DENV infection.

CHAPTER FIVE - FURTHER INVESTIGATION OF THE ROLE OF IMPORTIN-5 IN DENV INFECTION

Introduction

In addition to identifying proteins with a potential role in influencing early events in DENV infection, the analysis of the cellular phospho-proteome of dendritic cells highlighted a protein (Importin-5) which may be involved in later events in the process of infection.

Importin-5 was shown by mass spectrometry analysis to be less phosphorylated in Mock stimulated samples compared with DENV stimulated samples after 10 min incubation. Subsequent western blot validation showed a higher level of phosphorylation in Mock stimulated, rather than DENV stimulated cells giving a contradicting result. This may be due to the nature of this post-translational modification which could result in transient phosphorylation which may or may not be identified in individual snap-shot moments at cell lysis. Nonetheless, it was taken as an interesting candidate for further investigation as it has been shown to function as a carrier of DENV NS5 protein into the cellular nucleus. Given Importin-5 is a nuclear import protein known to be involved in import of NS5 to the nucleus during DENV infection (Pryor et al., 2007) (though the reason for this process remains unknown as replication of DENV occurs in the cytoplasm only), its role in the early stages of infection was studied.

Importin-5 is a 125 kDa member of the karyopherin family of transport proteins and is responsible for movement of target molecules from the cytoplasm to the nucleus (see Fig. 30). Whilst small proteins are able to diffuse freely though the nuclear membrane, larger molecules (over approx. 70kDa) require assistance from specialised

transport proteins in an energy dependent process. Importin-5 recognises a specific NLS on target proteins (Rout et al., 1997) and the Importin-5/substrate complex is then docked to the nuclear pore on the cytoplasmic side, allowing Importin-5 and its substrate to enter the nucleus. Importin-5 is then cycled back into the cytoplasm for association with the next target protein.

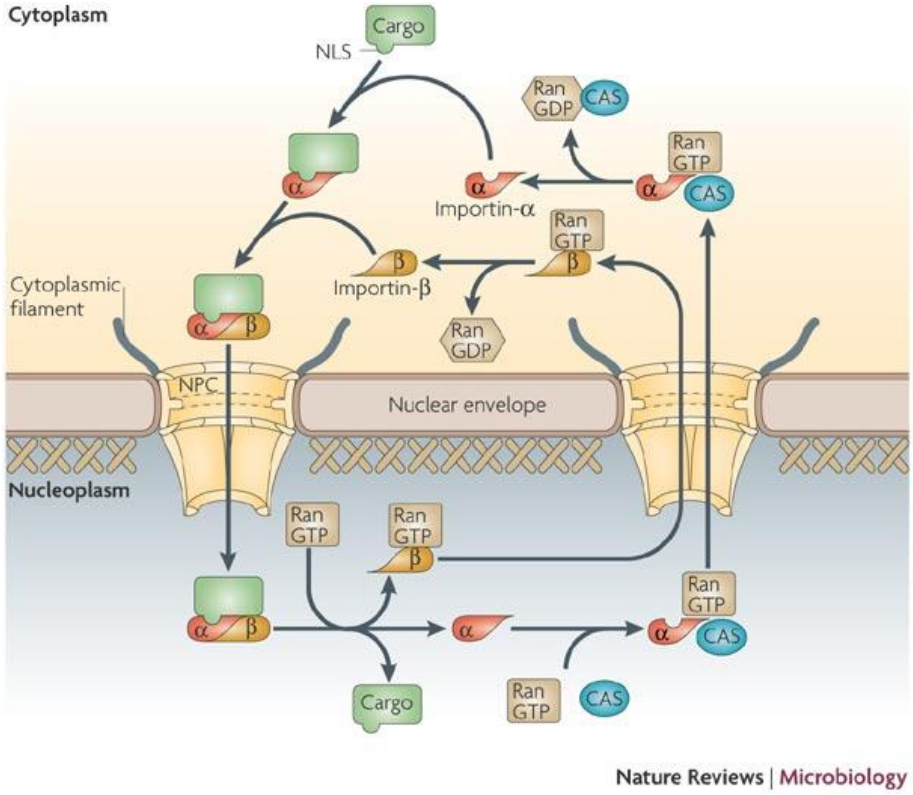


Fig. 30: ***Method of import of Imp-5 substrates into the nucleus***
Importin binds to its substrate protein and this complex docks to the nuclear pore complex, allowing entry into the nucleus. Within the nucleus, RanGTP initiates dissociation of Importin and cargo leading to cycling of Importin back into the cytoplasm for association with another cargo protein (Suzuki and Craigie, 2007).

Importin-5 is known to import ribosomal proteins and histones into the nucleus and plays a role in facilitating transport of proteins vital for the cell replication cycle (Jäkel and Görlich, 1998).

Investigation of Importin-5 phosphorylation status and interaction partners by mass spectrometry

To investigate the involvement of Importin-5 in DENV infection, mass spectrometry was again utilised for comparison of DENV and Mock stimulated samples. On this occasion, cells were infected for 30 min rather than the 10 min stimulation initially studied. The initial difference in phosphorylation detected at 10 min indicates a differential regulation of a cellular pathway and this study aims to ascertain the downstream effect of this pathway/phosphorylation difference. As 10 min stimulation would unlikely allow time for alteration of protein effector functions, the stimulation was allowed to continue for 30 min to allow the phosphorylation pathway to be seen through to completion and for any change in protein function to occur.

Validation of immuno-precipitation technique by western blot and silver stain

Prior to mass spectrometry analysis, DENV infected cells were lysed with NP-40/Igepal CA-630 and the whole cell lysate was centrifuged. The soluble fraction was removed and Importin-5 targeting antibody was added. Any Importin-5 protein within the lysate was coated with primary antibody and subsequent addition of Protein G sepharose beads trapped Importin-5/antibody complexes to bead surfaces for pull down of proteins (and any interacting partners alongside) during the washing steps.

The aim of this investigation was to ascertain both the phosphorylation status of Importin-5 and any interaction partners pulled down alongside it. The phosphorylation status may provide an insight into whether or not it is in an activated state in response to DENV infection and which state of phosphorylation induces the activity whilst

identification of interaction partners may be beneficial in ascertaining how Importin-5 may be involved in influencing the outcome of infection.

Initially, an aliquot of the material harvested from the immuno-precipitation for mass spectrometry was used for SDS-PAGE, followed by a western blot where Importin-5 was detected using a specific anti-Importin-5 antibody (different to the antibody used during the immuno-precipitation procedure) together with a HRP-conjugated secondary. In parallel, an identical gel was subjected to silver stain procedures (see Fig. 31).

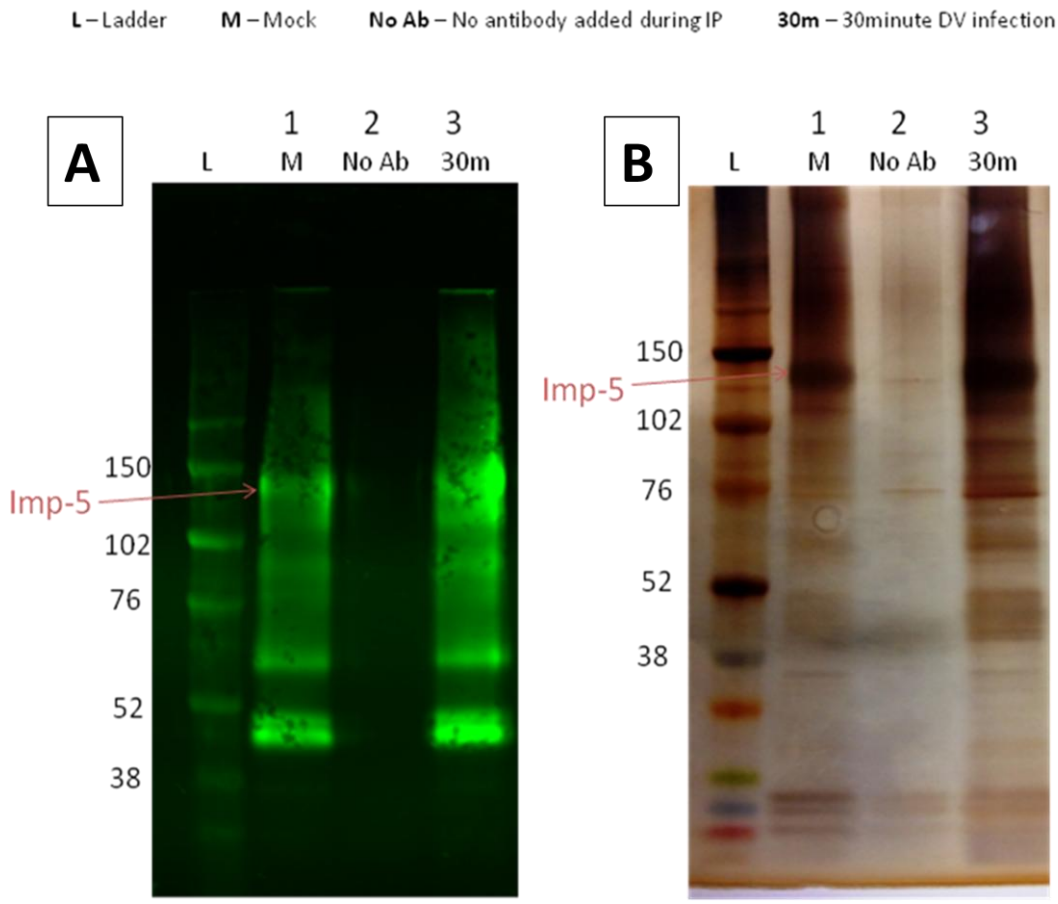


Fig. 31: ***Western blot (A) and silver stain (B) to identify Importin-5 protein levels in Mock and DENV stimulated cells after immuno-precipitation compared to immuno-precipitation with no antibody (no Ab) as a control***
Dendritic cells were infected with Mock (M) or DENV (30m) supernatant for 30 min and subsequently WCL was obtained and immuno-precipitation to pull down Importin-5 was carried out. Mock and DENV samples were compared to a sample in which only secondary antibody was used for immuno-precipitation as a negative control. Result shows Importin-5 was successfully precipitated and background levels were minimal. (Molecular weight marker= L)

These results validated that the immuno-precipitation was successful as the western blot shows the presence of Importin-5 both in the whole cell lysate (before immuno-precipitation) and in the eluate (after immuno-precipitation), but not in the control (in which no antibody was used during the immuno-precipitation) and the silver stain shows the presence of Importin-5, again in the Mock and DENV samples but not in the negative control (see Fig. 31). The silver stain also highlights the fact that there are various other proteins within the sample which could be potential interaction partners pulled down through their interaction with Importin-5 at the time of cell lysis. The band profile of Mock and DENV stimulated cells by silver stain is very similar which suggests that there are no proteins which interact with Importin-5 to a significantly higher degree after DENV stimulation, however this technique is not sensitive enough and so samples were sent for mass spectrometry analysis in order to ascertain the level of abundance more accurately and also to identify the proteins which are involved.

Mass spectrometry samples containing Importin-5 and its interaction partners pulled down by immuno-precipitation

In order to detect Importin-5 interaction partners with a more sensitive method, eluates from IP of Mock, no antibody and DENV (30m) samples were sent for mass spectrometry analysis. This method was used in order to identify the proteins pulled down with Importin-5 during IP and to ascertain whether or not any protein interactions are significantly enhanced or inhibited in response to DENV infection. The most interesting candidates are listed in Table 7 below and were identified based on expression ratio and relative abundance (ascertained semi-quantitatively).

The result obtained for Importin-5 itself showed relative protein abundance to be $7.9x10^{-6}$ in Mock stimulated cells compared to $1.17x10^{-5}$ in DENV stimulated cells after 30 min infection. This shows an increase in the level of Importin-5 detected when cells were responding to DENV infection. Also, the control sample in which no antibody was used for immuno-precipitation showed no Importin-5 detection, showing assay background levels were minimal.

Analysis of proteins pulled down in association with Importin-5

The table below (Table 7) lists the proteins detected by mass spectrometry to have been pulled down using Importin-5 specific antibodies, this suggests they are interaction partners of Importin-5 and were in complex when it was captured during the immuno-precipitation.

Table 7: ***Interesting candidates pulled down alongside Importin-5***
This table lists those proteins pulled down with Importin-5 which show a significant ratio of difference in relative abundance between Mock and DENV stimulated samples and which have a potential role in the infection process. **(**PSM – Peptide Sequence Match)

Accession Number	Protein	PSM	% Sequence Coverage	Mock	DENV	Expression Ratio (DENV/M)
P41208	Centrin-2	**3**	**25.6**		6.46E-07	-
P06748	Nucleophosmin	**6**	**14.6**	2.62E-06		-
P12268	IMP dehydrogenase 2	**6**	**14**	3.19E-07		-
Q02413	Desmoglein-1	**8**	**7**	8.21E-08	2.39E-07	2.91
Q16891	Mitochondrial inner membrane protein	**8**	**9.4**		6.75E-07	-
P41252	Isoleucine--tRNA ligase	**2**	**1.3**		8.98E-08	-
P53999	Activated RNA polymerase II transcriptional coactivator p15	**8**	**48.8**	8.23E-06	1.05E-06	0.13
P62826	GTP-binding nuclear protein Ran	**7**	**19.9**	2.91E-06	4.71E-07	0.16
Q99729	Heterogeneous nuclear ribonucleoprotein A/B	**12**	**22.6**	4.77E-06	9.58E-06	2
P12314	High affinity immunoglobulin gamma Fc receptor I	**46**	**28.9**	1.73E-05	4.60E-05	2.66
P60842	Eukaryotic initiation factor 4A-I	**4**	**10.8**	9.82E-07		-
P62873	Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	**5**	**5.9**	9.10E-07	1.94E-06	2.13
Q00610	Clathrin heavy chain 1	**16**	**8.5**	8.21E-08		-
Q01085	Nucleolysin TIAR	**8**	**10.7**	1.47E-07	7.66E-07	5.21
P37108	Signal recognition particle 14 kDa protein	**4**	**44.9**	6.33E-07		-
Q8WU79	Stromal membrane-associated protein 2	**5**	**5.8**	1.31E-07		-

This table illustrates a selection of the numerous proteins to be found in samples of Importin-5 pull down eluate. These proteins, through potential interaction with Importin-5, could have an effect on its function during infection with DENV. There are a number of proteins here which are present in amounts at least two fold different when comparing eluate obtained from DENV infected cells and from Mock infected cells, some of the most notable include: Nucleolysin TIAR, Guanine nucleotide-binding protein G (I)/G(S)/G(T) subunit beta-1 and Desmoglein-1.

By studying the known roles of possible interaction partners, potential functions can be hypothesised as to how these proteins function together to aid or inhibit viral infection. It would be interesting for further investigation to look into co-localisation of these proteins with Importin-5 within the cell to validate the result from immuno-precipitation, prior to carrying out further analysis of the function of this interaction.

There are many groups of proteins detected here which act upon similar cellular processes such as cell death and apoptosis, many that have a role in transcription or translation and also, some proteins involved in or influenced by calcium levels/signalling within the cell. This shows that Importin-5 probably has a wide ranging role involved in many processes within the cell and this range of function could be used by the virus/host to offer an advantage in either increasing or inhibiting infectivity.

A number of these co-precipitated proteins are detected only in Mock stimulated samples as compared to DENV stimulated samples or vice versa. These hits represent proteins which are likely to have the greatest impact on infection as they are exhibiting a function which is completely absent in the control condition. This indicates a response which may be unique to DENV recognition and which may play a role in subsequent severe immuno-pathology encountered with certain cases of dengue.

Analysis of phosphorylation status of proteins associated with Importin-5

In addition to analysing potential interaction partners of Importin-5 in cells infected with DENV or Mock infected, the results obtained from the mass spectrometry also indicate any post-translational modification detected on the protein itself (see Table 8). Again, the focus of this study is on phosphorylation as this was the initial modification detected on Importin-5. It is also a type of modification which is highly likely to cause an effect on protein function within the cell as a result of phosphorylation and de-

phosphorylation of proteins involved in multiple pathways triggered in the early events after DENV infection. The role of phosphorylation in nuclear import is not well understood with contradictory evidence presented by various groups, summarised well by Nardozzi et al. (Nardozzi et al., 2010). This means that it is difficult to draw solid conclusions as to the effect of phosphorylated interaction partners on the outcome of infection. The proteins pulled down in association with Importin-5 which also seem to contain phosphorylated peptides are listed below.

Table 8: ***Table of phosphorylated proteins pulled down with Importin-5***
Proteins pulled down with Importin-5 which show phosphorylation on specific residues. Relative abundance of protein in each sample is also shown. PSM – Peptide sequence match.

Protein	Description	PSM	% Coverage	Modifications	Mock	No Ab	DENV
P07305	Histone H1.0	17	28.90	Phospho (ST)	2.10E-05	1.11E-05	1.11E-05
P14866	Heterogeneous nuclear ribonucleoprotein L	11	13.90	Phospho (ST)	8.76E-07		4.98E-07
P15924	Desmoplakin	46	10.10	Phospho (ST)	2.87E-07	1.22E-06	8.45E-07
P19338	Nucleolin	18	22.40	Phospho (ST)	2.30E-06		4.88E-07
Q08211	ATP-dependent RNA helicase A	35	18.40	Phospho (ST)	1.05E-06		1.91E-06
Q5SSJ5	Heterochromatin protein 1-binding protein 3	9	9.40	Phospho (ST)	1.97E-07		1.93E-07
P08238	Heat shock protein HSP 90-beta	43	36.20	Phospho (ST)	6.94E-06	4.59E-06	1.59E-05
P07900	Heat shock protein HSP 90-alpha	30	28.40	Phospho (ST)	1.18E-06		6.46E-06
P38159	RNA-binding motif protein	8	17.60	Phospho (Y)	1.12E-06		2.73E-07
P42704	Leucine-rich PPR motif-containing protein	13	5.90	Phospho (ST)	3.11E-07		2.37E-07
P62979	Ubiquitin-40S ribosomal protein S27a	85	44.20	Phospho (Y)	1.18E-04	1.15E-04	5.42E-05
Q15233	Non-POU domain-containing octamer-binding protein	6	14.40	Phospho (ST)	8.11E-07	1.76E-06	6.24E-07
Q969Q0	60S ribosomal protein L36a-like	5	31.10	Phospho (ST)	3.93E-06		1.03E-06
P23396	40S ribosomal protein S3	27	57.60	Phospho (ST)	8.27E-06	2.60E-06	8.50E-06
Q16778	Histone H2B type 2-E	18	81.70	Phospho (ST)	7.25E-05	1.82E-05	1.64E-06
P23527	Histone H2B type 1-O	1	81.70	Phospho (ST)	5.57E-07		
P83881	60S ribosomal protein L36a	1	31.10	Phospho (ST)	1.41E-06		

Phosphorylated proteins pulled down with Importin-5 in this study highlighted certain currently known interaction partners which could lead to alteration of cellular activity in response to DENV stimulation, for example histones which are known to be transported into the nucleus by Importin.

Importin-5 itself showed possible phosphorylation sites in this experiment, however they were not detected with certainty. The protein score was very low indicating low confidence that the detection of phosphorylation was not a false positive. The putative phosphorylation site detected by mass spectrometry on this occasion is potentially a newly discovered phosphorylation site on Importin-5 as this site has not been reported to show phosphorylation. However, this further reduces the confidence in the accuracy of the result as if there was a correlation with established phosphorylation sites it would be deemed less likely to be a false positive.

CHAPTER SIX - DISCUSSION AND CONCLUSION

Overview

As dengue is widely thought as the most important emerging disease with the potential to spread further afield with its vector *Aedes* aegypti, it is imperative that we gain a better understanding of the viral infection process in order to best formulate treatments and vaccines which are currently unavailable. This study addressed specific aspects of the basic science of viral pathogenesis in order to aid better design of effective treatments and vaccines.

Experimental design

A new method of phospho-proteomics was used to investigate the level of phosphorylation of proteins involved in early events triggered by recognition of DENV at the surface of dendritic cells. This was done with the aim of finding a phosphorylation event which could influence subsequent immune responses to the virus and have an effect on severe immuno-pathology sometimes seen as a result of infection.

By isolating only the phosphorylated proteins present in the WCL of DCs after 10 min of stimulation with DENV, the relative level of phosphorylation of each protein present in cells stimulated with Mock, DENV, IAV and LPS was ascertained. The large number of proteins found to show differential phosphorylation profiles in response to DENV as compared to Mock, IAV or LPS was an expected outcome given that dendritic cells are primed to act rapidly upon recognition of foreign particles (Reis e Sousa et al., 1997) as they are the first line of defence against viral infection. One of the immediate ways to activate proteins or alter their activity in order to create anti-viral state within cells is post-translational modification such as phosphorylation. This process can quickly

activate or inactivate proteins involved in the reduction of cellular permissiveness to infection and can also have an effect on activation or inhibition of innate immune pathways. These pathways ultimately result in inducing processes such as interferon response pathways which lead to eventual clearance of virus from infected cells. As these pathways are often triggered by receptors which recognise virus at the cell surface, it is unsurprising that so many proteins are shown to be differentially phosphorylated after 10 min. At this time point, viral entry will not have occurred but virus particles will have docked into cell surface receptors and triggered the pathways downstream of these. As expected, many of the proteins seen to be phosphorylated in response to DENV stimulation are either involved in the heat shock response or are kinases which play a vital role in phosphorylation of proteins along immune signalling pathways.

In addition, effects on the general viral infection process can be seen from proteins, detected here by mass spectrometry, which are involved in numerous wide-ranging cellular pathways including signalling, apoptosis, cellular motility and chemokine/cytokine production and secretion.

Cellular processes which could be affected by differential phosphorylation of proteins identified by mass spectrometry

Signalling pathways

Signalling pathways are imperative in the fight against infection, especially when the target cells of infection are dendritic cells which instigate anti-viral responses and drive innate immunity. The ability of these cells to induce innate immune responses will shape the eventual outcome of infection as they not only serve to reduce the level of infection early on but are also responsible for inducing and shaping the subsequent

adaptive immune response. It is the combination of these that, when irregularities occur, can result in immuno-pathology as seen in cases of severe dengue infection. Therefore, the control of signalling pathways (whether it be activation or inhibition) is critical in determining how the infection will proceed. As phosphorylation is a major player in the control of these pathways, proteins seen to be differentially phosphorylated in response to DENV compared to other infections and Mock infected cells could potentially be having an effect on signalling pathways which may trigger severe immuno-pathology. Proteins identified to be differentially phosphorylated and known or suspected to play a part in immune signalling pathways were put forward as very strong candidates for follow up investigation in this study, including Protein S100-A9 and HSP90-Beta.

Apoptosis

In addition to signalling pathways, apoptosis is also a process which could alter the course of infection leading to a difference in factors such as viral load. It may be especially effective in DENV infection as when cells apoptose they break up into vesicles which are then taken up by phagocytes in order to be degraded. As cells such as macrophages, which take up these apoptotic bodies, are also target cells of DENV the virus could potentially escape the vesicle before degradation and this process could then turn into an efficient way of viral dissemination and entry into fresh target cells. This would ultimately increase viral load and potentially lead to a higher level of immune response and in turn immuno-pathology. It would be interesting in future work to assess the affect of DENV on apoptosis levels using flow cytometry to measure the level of apoptotic factors such as Caspase-3 and Cytochrome-C with and without DENV infection. This line of investigation could also be taken further to include cells with and without Calpain-2 targeting siRNA in order to ascertain the involvement of Calpain-2 in levels of apoptosis after DENV infection. Many proteins known to be involved in the

process of apoptosis were identified by mass spectrometry in this study to show a differential phosphorylation profile in response to DENV. These include BID and Calpain-2 and the identification of more than one molecule along the same apoptosis pathway lends more confidence to the fact that this pathway is indeed affected by DENV stimulation. Pathway analysis reveals that Calpain / BID / Importin are all in fact acting along the apoptosis pathway axis. Modulation of the apoptosis pathway seems to be a common mechanism targeted by many viral and other pathogens to persist and enhance the dissemination of infection. The results presented here support the notion of targeting Calpain proteases using a pharmaceutical approach in order to interfere with infectious agents such as DENV.

Cellular motility

Cellular motility is another pathway which could affect viral dissemination. For example, proteins which facilitate motility allow infected cells to travel around the body (namely to draining lymph nodes for dendritic cells) thus allowing transport of virus to new areas. This provides access to new target cells for infection, again leading to higher viral load and potentially increased immuno-pathology. Also, when infected dendritic cells migrate to lymph nodes the viral particles transported within them, when released, will be in an environment full of target cells which would be a large advantage for the virus as there will be plenty of opportunity to infect DENV naive cells and produce the maximum number of progeny possible. Proteins shown in this study to be differentially phosphorylated in response to DENV which are known to play a role in cell motility include Dok-2 and Ena/VASP like protein and these proteins could be acting to increase the level of dendritic cell motility in infected cells in order to aid viral dissemination, potentially resulting in higher levels of viremia and immuno-pathology. It has been

shown in other studies that motility of hematopoietic cells is imperative for DENV dissemination (Pham et al., 2012) and therefore up-regulating DC motility could represent a virally induced method of increasing pathogenicity.

Production and secretion of chemokines and cytokines

Another factor contributing to cellular immune responses is the production and secretion of cytokines and chemokines. These molecules play a large role in immune responses and act as a branch between innate and adaptive immunity. This is a vital link as severe immuno-pathology can also be attributed to the adaptive response (Pulendran et al., 2001). Therefore, proteins with a role in linking the two branches of the immune response and which are identified to be differentially phosphorylated in response to DENV as compared to other infections may be contributing to hyper-activation or de-regulated activation of the adaptive response. This could potentially lead to severe immuno-pathology which is not inhibited as immune responses would normally be. One such protein identified in this study is HMGB1 which acts at the interface of innate and adaptive immunity and could therefore influence the adaptive immune response initiated by recognition of DENV by target cells. Differential phosphorylation of this protein in response to DENV as compared to Mock and IAV could potentially lead to differently controlled adaptive immunity and may prove to be part of the reason why immuno-pathology is sometimes de-regulated as seen in severe dengue disease.

Proteins identified with differential phosphorylation levels in response to DENV compared to Mock, IAV or LPS and which may play a role in altering immune responses to viral stimulation

In order to ascertain which of the proteins identified to be differentially phosphorylated in response to DENV were the best candidates for further study, a method was developed to cut many of the proteins from the list returned from mass spectrometry. Initially, all proteins returned with a protein detection score below 20 were discarded as such a low score does not provide sufficient confidence that the identification was correct. Protein lists were then sorted into those showing differences in phosphorylation between DENV and other conditions and those uniquely modified in DENV compared to all other conditions. Lists were also sorted into those proteins for which phosphorylation was up-regulated more in response to DENV than other conditions and those for which phosphorylation was down-regulated more in response to DENV than other conditions. Proteins were then selected from these lists according to their potential to play a role in immuno-pathology induced by DENV. Such proteins were selected by current knowledge of their role within the cell. Proteins were selected if they were shown to play a role in cellular processes such as those previously listed including immune signalling pathways, apoptosis and cellular motility. Once the vast lists of proteins were narrowed down to potential players in immuno-pathology, these proteins were sorted into those with the highest ratio of difference between phosphorylation level in DENV and each other condition. Ratios were obtained by SINQ analysis which serves to show the ratio of difference in the abundance of phosphorylated proteins in each sample, proteins with the highest ratio of difference were considered the best candidates for further study.

Proteins showing an increase or decrease in phosphorylation only in DENV samples when compared to all three control conditions are the most interesting as these are uniquely modified in response to DENV stimulation. These proteins are not affected, or affected to a lesser extent, in response to other similar viruses (IAV) and to bacterial stimulation (LPS).

Proteins were chosen as described above as the hypothesis of the study was that DENV elicits a response which is unique to DENV recognition and therefore may be responsible for the immuno-pathology which occurs in some cases of dengue but is not seen for other similar viral infections. It may be that the differentiation in phosphorylation level leads to a de-regulated control of a pathway with the potential to shape subsequent immune responses. If the pathway is regulated differently in response to DENV as compared to other conditions there is a chance that this differential regulation is what results in uninhibited immuno-pathology leading to severe disease. In this study, interesting proteins were taken forward for further study in order to ascertain more fully their role in cellular processes in response to infection with the aim of discovering the extent of effect they have on infection levels and immune responses. With this knowledge, it may be possible to pinpoint a protein or cellular process which could be targeted by treatments for dengue patients thereby lowering the morbidity and mortality rates of dengue patients.

It was then important to validate the result obtained from mass spectrometry by other experimental means in order to ensure the hit was not a false positive and this was done with western blot as specific proteins can be validated with antibodies targeting the protein of choice. Those successfully validated were: Calpain-2, BID, Importin-5, Rho-GDI, Dok-2, ERp72, Endoplasmin and BiP.

These proteins are known to have a role in processes mentioned earlier, including apoptosis, signalling, motility and nuclear import. All of these processes within the cell can have an influence on the progress of viral infections and therefore play a role in the outcome of disease.

Calpain-2

Calpain-2 was seen to be more highly phosphorylated in response to DENV stimulation in comparison to Mock, IAV and LPS. Silver stains of fractionated phospho-eluates showed a larger band at 80kDa, corresponding to the size of Calpain-2. This was supported by western blot analysis using Calpain-2 specific antibodies which detected a higher level of phosphorylated protein in response to DENV stimulation. Calpains are involved in the apoptosis pathway of the cell and through modulating this process they have the ability to modify the outcome of viral infection. They are activated by calcium flux (Reverter et al., 2001; Strobl et al., 2000) which influences many processes within the cell and notably, many proteins returned from mass spectrometry analysis carried out in this study are also regulated by calcium flux. This suggests that the cells were indeed stimulated by virus and that this induced calcium flux leading to initiation of phosphorylation for many calcium regulated proteins. Calpains are non-lysosomal cysteine proteases and function to initiate the process of apoptosis by cleaving caspase-3 and/or AIF. They are also directly involved in the process of cell death by breaking down cellular architecture through degradation of various cellular substrates including proteins involved in membrane structure (Momeni, 2011). They reside in the cytoplasm of cells in an inactive state with activation of the pro-enzyme occurring upon sufficient increase of cellular calcium levels. There are a number of hypotheses regarding the mechanism of activation, one of which is phosphorylation at Ser 369 (Suzuki et al., 2004).

In order to shed light upon potential roles of Calpain-2 in DENV infection, it is interesting to note its function with regard to various other viral infections. Calpain-2 has been seen to have an effect in the pathogenesis of a number of other viruses, for example it has been shown that SARS-CoV replication is inhibited by Calpain-2 knockdown (Schneider et al., 2012). This is similar to the effect seen here with DENV titre when stimulation occurred post Calpain-2 knockdown. This could be an indication that these viruses both rely on Calpain-2 and viral titre may be reduced in both viral infections by a similar method. HIV-1 has also been shown to require Calpains for replication, with their role shown to be involved with NF-κB which, as a transcription factor, up-regulates transcription of viral genes leading to increased virus production (Teranishi et al., 2003). It could be that during DENV infection, NF-κB also serves as a transcriptional activator up-regulating the rate of production of viral proteins. This could explain why DENV titre was reduced when infected cells were pre-incubated with siRNA targeting Calpain-2.

In addition to SARS-CoV and HIV-1, Hepatitis C virus infection is also modulated by Calpains as illustrated by the fact that Calpain inhibitors block cleavage of specific viral proteins required for replication (Kalamvoki and Mavromara, 2004). It could be proposed that the lower DENV titre seen from cells with lower levels of Calpain-2 is a result of viral proteins requiring the proteosomal action of Calpain-2 for processing, leading to efficient progeny production. With low levels of Calpain-2 present, it may be that viral titre is reduced due to lack of polypeptide cleavage or required cleavage of specific proteins leading to proper folding or assembly of viral progeny. It may be that without these cleavage events, virus is unable to assemble into functional virions or be released from the cell in a mature form. Also, Calpain activation by Hepatitis C virus proteins is seen to inhibit the extrinsic apoptotic signalling pathway (Simonin et al., 2009). This modulation works in the opposite direction to proposed

Calpain modulation of apoptosis in DENV but shows that this pathway is often a target in viral manipulation of host cells.

I propose that calcium flux is initiated upon dendritic cell recognition of DENV leading to phosphorylation of Calpain-2 and subsequent induction of apoptosis. This theory is substantiated by the literature, which suggests calcium regulation also plays a role in Reovirus pathogenesis. Apoptosis is induced upon stimulation of cells with Reovirus and this induction is thought to be due to increased levels of Calpain in response to calcium flux (Debiasi et al., 1999).

It has also been described that calcium flux is involved in extending the survival of cells infected with West Nile Virus, possibly to allow the virus maximal time for progeny production (Scherbik and Brinton, 2010).

Calcium flux induced upon viral attachment has also been seen in other virus infections including HIV-1 (Papp and Byrn, 1995), Coxsackievirus (van Kuppeveld et al., 1997) and HSV (Cheshenko et al., 2003).

Alongside this, Echovirus research has also found Calpain-2 has a role in viral replication with knockdown of Calpains inhibiting replication, this is mirrored in this study as DENV titre is reduced when Calpain-2 is knocked down. As both viruses replicate in a similar fashion, i.e. in the cytoplasm via a polypeptide intermediate and negative strand RNA templates, it is plausible that DENV also requires Calpain for replication and it would be very interesting to investigate this possibility in future research (Upla et al., 2008).

In addition, calcium flux and Calpain-mediated activation of the apoptosis-inducing factor contribute to enterovirus-71 induced apoptosis (Lu et al., 2013). It is

thought that this mechanism can lead to increased cytopathology, an outcome which could lead to increased immuno-pathology in patients.

It has also been reported that bacterial infections can lead to increased levels of apoptosis for example, group B Streptococcus (GBS) induces disruption of the actin and microtubule cytoskeleton of macrophages leading to Calpain activation and subsequent apoptosis (Fettucciari et al., 2011). This demonstrates that apoptosis regulation is a widely used strategy by pathogens with the aim of increasing infectivity.

BID

Another protein validated here by western blot analysis was BID which, in line with Calpain-2, was also more highly phosphorylated in response to DENV stimulation as shown by mass spectrometry analysis and western blot confirmation. This protein also acts along the apoptosis pathway and the fact that two proteins working along the same pathway are modulated differently in response to DENV as compared to Mock, IAV and LPS increases the confidence in the fact that this pathway actually plays a role in the outcome of infection. The increase in apoptosis could be due to both host and virally induced effects, with host cells evolving an apoptotic response in order to clear infected cells and the virus inducing an apoptotic effect for a number of reasons including evasion of immune recognition and viral progeny dissemination.

BID is also seen to function in the necroptosis pathway, inducing another means of cell death upon DENV stimulation of DC (Cabon et al., 2012).

Importin-5

In contrast to Calpain-2 and BID, Importin-5 seems varied in its level of phosphorylation upon stimulation of dendritic cells with DENV. It may be that the

phosphorylation level is irregular and does not impact function. The literature and present knowledge as to the phosphorylation status of Importin-5 upon stimulation is limited. The validating western blot illustrated a lower level of Importin-5 phosphorylation in response to DENV stimulation however, mass spectrometry of samples sent after 10 min stimulation showed an increase in Importin-5 phosphorylation in response to DENV compared to Mock, IAV and LPS. After 30 min stimulation, it was seen that the abundance of Importin-5 within the cell was increased.

Generally, import of proteins into the nucleus of cells has many functions in cellular processes and is imperative for cellular function. Many processes rely on it and it is therefore a good target for manipulation of cellular activity (Hutchinson et al., 2011). Also, it has been reported that siRNA targeting Importin-5 reduces viral infection suggesting it has a key role in the life cycle.

Importin-5 has a known role in DENV infection (Fraser et al., 2014) whereby it is seen to import DENV NS5 into the nucleus for a reason which is as yet unknown. This was found to be imperative for DENV infection (Pryor et al., 2007) as replication is inhibited by Ivermectin (an Importin inhibitor) (Wagstaff et al., 2012). As Importin-5 has a role in DENV infection it was taken for further study in order to gain further insight into its mechanism of action in relation to DENV infection. Finding out the interaction partners of Importin-5 upon infection with DENV may shed light onto a mechanism of action that results in illustration of the reason DENV RNA polymerase is transported into the nucleus despite the virus undergoing all stages of replication within the cytoplasm. It may be that a protein is activated in response to DENV recognition and subsequently associates with Importin-5 leading to activation of Importin-5 functions. Importin-5 may also interact with a protein which has a role in DENV infection thereby altering the course of infection (Wagstaff et al., 2012).

As mentioned earlier, Ivermectin specifically inhibits Importin alpha/beta and this results in inhibition of DENV infection. Again, this suggests that DENV NS5 protein is required in the nucleus for a reason which has not yet been elucidated. This phenomenon is also evident for other viruses such as RSV and rabies. All three virus genomes are replicated within the cytoplasm but require viral polymerase in the nucleus for replication to occur. It may be that this polymerase alters host cell transcription leading to increase or decrease in a host cell protein that is vital for effective replication. Looking for interaction partners of Importin-5 may shed light onto the method of transport and any additional proteins involved in the process.

With regard to the effect of DENV NS5 transport into the nucleus, it is interesting to note the effect of Hepatitis C virus NS5A in HCV infection. The HCV protein NS5A activates NF-kB and STAT-3 gene expression by Ca2+-dependent mechanisms (Dionisio et al., 2009). I hypothesise that calcium flux occurs in response to DENV as seen by the up-regulation of phosphorylation of many proteins known to be activated by this mechanism, it may be that the calcium flux occurs and this has an effect on DENV NS5 interaction with Importin-5 leading to up-regulation of transcription of genes involved in the immune response.

As described, Importin has a role in viral pathogenesis in other viral infections (including Influenza), it is notable that Influenza is also one of the proteins whereby import of viral encoded polymerase is vital for replication (Deng et al., 2006).

Influenza viral genome replication occurs in the nucleus (unlike DENV replication) and requires that Importin-5 directly interacts with the viral polymerase to facilitate its transport into the nucleus. Importin-5 interacts with a dimer (of which NS5 is a part), and subsequently RanGTP is responsible for releasing the dimer allowing

subsequent interaction of the polymerase with substrates in the nucleus. It may then be the case that Importin-5 acts in a similar way for NS5 as it does for influenza polymerase and for this reason, interaction partners may shed light onto how and why the viral polymerase is transported into the nucleus during DENV infection.

Also, Importin-5 is known to import histones and ribosomal proteins such as ribonucleoprotein (detected by mass spectrometry analysis in this study and seen at a higher level in response to DENV compared to Mock) into the nucleus. It could be that differential import of these proteins in response to DENV stimulation leads to alteration of levels of gene transcription in the nucleus which could affect the cell's response to infection through alteration of transcription of genes which are involved in viral clearance and cellular immune responses. The higher level of Importin-5 illustrates an increased cellular capacity to transport proteins into the nucleus which could result in increased viral genome replication. Also, the increased detection of nuclear proteins involved in gene transcription shows the up-regulation in gene transcription occurring. The fact that proteins already described to interact with Importin-5 were illustrated in this study lends confidence that the interaction partners identified were truly associating with Importin-5 and not just background proteins picked up by mass spectrometry.

Rho-GDI

Another protein for which phosphorylation was differentially regulated in response to DENV compared to other conditions is RhoGDI. This protein was seen to be more phosphorylated in response to DENV than any other condition tested as analysed by mass spectrometry. However, when the result was validated by western blot, phosphorylation levels were lower in response to DENV stimulation than all other conditions. This again shows a contradictory result between mass spectrometry data and

western blotting which could be attributed to sensitivity. The validated western blot shows only a small difference in phosphorylation levels and it may be that this method was not sensitive enough to pick up the differences seen by mass spectrometry analysis.

RhoGDI as a molecule inhibits the function of the Rho family of proteins by keeping them inactive and away from the cellular membrane, where they are functionally active. Rho proteins have been reported to function in a wide range of cellular processes (Bishop and Hall, 2000).

These processes include cellular motility and signal transduction. Rho proteins cycle between active (GTP bound) and inactive (GDP bound) states and this cycling is regulated by a number of molecules including Rho-GDI (Sasaki and Takai, 1998). Ultimately, it is thought that the Rho system has an effect on the actin cytoskeleton and therefore has a role in cellular motility.

Processes like cellular motility and signalling are reduced by RhoGDI which is beneficial for the host cell as it will restrict viral dissemination. However, phosphorylation of RhoGDI allows Rac1 (and not other substrates) to leave the complex, meaning it can switch to an active state resulting in higher cell motility. It may be that DENV increases phosphorylation of RhoGDI in order to take advantage of this. As seen in this study, RhoGDI phosphorylation is evident post DENV stimulation at a higher level than other conditions and this could be a mechanism induced by the virus to increase viral progeny dissemination (especially by increased migration of infected cells to lymph nodes which would provide a generous pool of DENV naive innate cells for infection). It was also proposed that different methods of cellular activation may lead to different molecules being specifically released from RhoGDI complexes for activation. It may be that stimulation with DENV uniquely allows Rac release and subsequent

increased cellular motility compared with other viral stimuli (DerMardirossian et al., 2004). This seems a plausible explanation as PAK1 was seen in mass spectrometry to be more phosphorylated in response to DENV and therefore could be playing a role in up-regulation of Rac release in response to DENV but not Mock, IAV and LPS.

RhoGDI is also thought to play a role in cellular signalling processes by forming part of cellular signalling complexes (Olofsson, 1999).

Endoplasmin

Endoplasmin (aka GRP95) was seen to be strongly phosphorylated in response to DENV as detected by silver stain, mass spectrometry and western blot. It was however also highly up-regulated in response to IAV and LPS, nonetheless the level of up-regulation in comparison to Mock made it an interesting candidate for further investigation.

Endoplasmin is an ER chaperone protein thought to assist in building ER complexes under conditions of stress and it also has a role in the correct folding of TLR proteins in the ER (Randow and Seed, 2001). The increase in phosphorylation (if resulting in activation) in response to DENV could be readying the cell for immune responses to the virus and for more efficient antigen recognition leading to faster viral clearance. As the molecule is so well studied in the context of viral infections it is unlikely to be exerting a unique effect on cells recognising DENV, the up-regulation of phosphorylation upon recognition of DENV is likely to be seen in response to many other viral infections which lead to severe and non-severe disease. This means that this protein is unlikely to be causing the immuno-pathology seen in severe DENV infections.

Dok-2

Dok-2 is a protein which was found to be slightly more phosphorylated in response to DENV stimulation compared to Mock, IAV and LPS as seen by mass spectrometry analysis and western blot validation.

Dok-2 is involved in many immune signalling pathways including the Erk pathway (Mashima et al., 2009). Dok proteins are negative regulators of signalling (Suzu et al., 2000) and therefore could be more phosphorylated in response to infection as a means of DENV inducing negative regulation of immune responses in order to escape detection and clearance whilst replicating.

BiP

BiP is seen in this study to be more highly phosphorylated in response to DENV compared to Mock, IAV and LPS. The difference was striking upon analysis of mass spectrometry results and confirmed (though to a lesser extent) upon western blot validation.

BiP is (along with Endoplasmin) an ER chaperone with a role in correct protein folding. It has also been described as a known receptor for DENV (Jindadamrongwech et al., 2004). It therefore plays a vital role in viral pathogenesis and has been seen in other studies to be up-regulated in response to infection in both infected and bystander cells (Wati et al., 2009). This could possibly be a virally induced method increasing access points into naive cells for progeny virions. BiP is seen to complex with many ER molecular chaperones including some validated in this study, for example GRP-96 (Endoplasmin) and ERp72 (PDI-A4) (Meunier et al., 2002). This complex, utilised for correct protein folding, could be activated by phosphorylation in response to DENV

stimulation in order to ready the cell for increased viral protein production and correct folding of viral proteins to maximise progeny production. BiP has also been described to have a role in calcium homeostasis in the ER (Lièvremont et al., 1997) which is interesting as calcium flux plays a role in activation of Calpain-2, the protein investigated further in this study.

ERp72

As mentioned, BiP is found in complex with many proteins including PDI-A4 or ERp72. In this study this protein was seen to be less highly phosphorylated in DENV stimulation, it may be that this protein is active when de-phosphorylated and DENV induces this de-phosphorylation in order to allow correct folding of viral proteins leading to increased virion production. It may also be the case that ERp72 is able to complex with these proteins only when de-phosphorylated, meaning the lower level of phosphorylation detected here was induced in response to DENV recognition in order to increase correct folding of proteins post-infection.

Further investigation – Calpain-2

As Calpain-2 and Importin-5 were chosen for further study based on the information elicited from the literature about their function in cells, further experiments were carried out to investigate their role in DENV infection.

To recap, Calpain-2 is part of the apoptosis pathway along with BID, another protein validated with a differential phosphorylation status in DENV compared to Mock, IAV and LPS conditions. Therefore it is possible that these proteins have a unified effect on the outcome of viral infection. Also, Calpain-2 has numerous other roles in cellular function including cell motility, cell signalling pathways and proteolytic cleavage of

substrates. Currently, Importin-5 is not described to have an effect on immune response in general but it is reported to be involved in the process of DENV infection by carrying DENV NS5 protein into the nucleus during infection, for a reason as yet unknown.

Upon silencing of Calpain-2 in dendritic cells and subsequent infection with DENV, viral titres were lowered in comparison to cells with a normal level of Calpain-2. If viral titre was unchanged it would indicate that whilst the protein may have some effect on the processes triggered by virus recognition, it would likely be insignificant in the outcome of the viral infection. The fact that a protein is able to change the level of viral replication shows that it must be significantly modulating the host-virus interaction at some point and therefore further study is required to determine what the protein does in order to modulate the capacity of the virus to propagate. This difference in levels of viral titre could lead to discovery of a mechanism which contributes to the severe immuno-pathology observed in DENV infection as a higher level of viral replication could lead to more efficient infection and therefore higher levels of immune response and immuno-pathology. The observed reduction in viral titre could be due to Calpain-2 having an effect on signalling pathways which lead to the induction of an antiviral state within infected cells. As Calpain-2 is seen here to be a requirement for more efficient progeny production and release it may serve to inhibit the production of antiviral state by cells. It could achieve this either through direct modulation of signal transduction or by acting in its protease capacity to degrade proteins involved in antiviral responses. It could also be acting in its capacity as a protease to cleave (and potentially activate) proteins required for efficient viral progeny production/packaging processes.

It was also seen that after silencing of Calpain-2, dendritic cells infected with DENV are less capable of inducing transcription of Mx1 which is an interferon stimulated gene. Calpain-2 must therefore play a role in the induction of this ISG either

directly or by exerting an action on a protein acting along the Mx1 induction pathway. For example, as Calpain-2 is a cysteine protease it may degrade a substrate which is involved in inhibition of Mx1 transcription factors and therefore when Calpain-2 is not present the pathway will remain inhibited.

Although Calpain-2 assists in the creation of an antiviral state within the cell via the Mx1 transcription factor pathway, it was previously observed that viral titre is higher in the presence of Calpain-2. This suggests that the opposite effect would occur, i.e. if the level of Mx1 was having a direct effect on viral titre then in the presence of Calpain-2 (as it is up-regulated) viral tire would be lower rather than higher, as illustrated by the FFU assay.

It may be that Mx1 as a specific ISG is up-regulated with the aid of Calpain-2, however a larger number of other interferon stimulated genes are not. Also, another ISG may be down-regulated by Calpain-2 to a greater extent than Mx1 is up-regulated. This could lead to a net down-regulation of antiviral interferon stimulated genes resulting in the increased level of viral titre seen when Calpain-2 is present. It would be interesting to continue this line of investigation by assessing the level of a number of different ISGs in order to ascertain whether all genes are affected in the same manner by Calpain-2 presence. Other genes were tested in this study (results not shown) but the level of induction varied between experiments and did not show a specific trend of up or down regulation as a result of Calpain-2 knockdown (maybe as knockdown differed in efficiency each time and this could have had a knock on effect on the level of difference in ISG transcription between silenced and non-silenced cells). Mx1 may have been so substantially affected that the trend was always down-regulation after silencing Calpain-2 even if the degree of this differed between experiments.

It could also be the case that interferon stimulated genes are up-regulated with the aid of Calpain-2 (theoretically leading to less viral replication), however a different cellular pathway involving Calpain-2 activity is affected to a greater extent by the absence of the protein. This would result in higher replication levels, i.e. the negative effect of interferon stimulated genes on viral replication levels (which are up-regulated by Calpain-2) could be masked in terms of the effect on viral titre by a process (involving Calpain-2) which results in higher viral replication levels. This would mean that the presence of Calpain-2 results in a higher level of Mx1 production (with expected decrease in replication), but overall leads to increased viral replication via an unrelated pathway which is affected by Calpain-2 to a greater extent than Mx1 up-regulation.

One such pathway that fits this theory is apoptosis, which is up-regulated by Calpain-2 and as Calpain-2 is a calcium dependent protease, it may be that an increased calcium flux leads to phosphorylation / activation of this enzyme (as suggested for Calpain-1 which then goes on to cleave AIF during apoptosis (Norberg et al., 2008)). In the context of DENV recognition, calcium dependent signals may be triggered through interaction with a G-protein coupled receptor (GPCR), for example the interaction seen by Le Sommer et al., 2012, leading to Calpain activation/phosphorylation. However, despite the many receptors on DCs that have been associated with DENV binding subsequently leading to entry, GPCRs have not yet been directly linked to this event. Once activated, as Calpain-2 has pro-apoptotic function, its presence leads to higher levels of apoptosis and therefore increased viral dissemination resulting in higher viral titre. When cells apoptose, they are broken down into apoptotic bodies which are taken up by phagocytes, potentially providing an additional method of viral entry alongside the possibility of viral particles breaking out from apoptotic bodies within the supernatant upon whole cell lysate collection i.e. membrane lysis. This would result in more

infectious progeny detected in the whole cell lysate as it will not be spun down given its small size. This method of increased viral spread due to apoptosis could also happen *in vivo*.

Further study – Importin-5

As for the involvement of Importin-5 in DENV infection, it was seen that Importin-5 itself was detected at higher levels in samples from cells infected with DENV compared to Mock. This shows that nuclear import is probably up-regulated in response to viral infection in order for the cell to instigate up-regulation of transcription of 'anti-viral' proteins.

However, immuno-precipitation results were also utilised to ascertain potential interaction partners which may shed light onto how and why DENV NS5 is transported into the nucleus.

By studying the known roles of such proteins, mechanisms can be hypothesised regarding interaction of these proteins with Importin-5 and how they work together to alter viral infection levels.

Nucleolysin TIAR is a known RNA-binding protein thought to have functions in cellular processes including regulation of translation and apoptosis. It is seen to be pulled down in higher abundance in cells infected with DENV as compared to Mock. This protein could have an influence on the translation of various proteins post viral stimulation to aid or inhibit an anti-viral state within the cell. Also, proteins with a role in apoptosis have been explained to be crucial for successful DENV infection. Interaction of this protein with Importin-5 to allow its transport into the nucleus could add to the effect

of Calpain-2 in initiating more apoptosis within the cell once DENV is recognised at the cell surface and indeed later on in the infection process.

Desmoglein-1 is a component of desmosomes which are a type of cell-cell junction. The protein is a transmembrane, calcium binding glycoprotein and with a function in cellular adhesion it may play a role in viral dissemination *in vivo*. It was seen here to be higher in abundance in response to DENV stimulation compared to Mock and therefore may represent a host regulated mechanism to inhibit cell motility thereby reducing viral dissemination.

Isoleucine tRNA ligase is seen to be more abundant in DENV infected samples than Mock and this could suggest the cell is preparing for an increase in mRNA and protein synthesis, this could be a result of cellular or viral factors influencing the instigation of viral genome replication and protein synthesis.

Another protein detected which could have a role in viral genome production is heterogeneous nuclear ribonucleoprotein A/B. Again, it is more abundant in DENV infected cells as compared to Mock and therefore could be up-regulated in preparation for increased genome production.

High affinity immunoglobulin gamma Fc receptor-I plays a role in the activation of infected cells and as it is seen to associate with Importin-5 to a higher degree in response to DENV infection this could illustrate a method of protection instigated by cellular activity in order to minimise levels of infection.

Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1 forms part of a heterotrimeric complex which functions to transport signals from their origins at various receptors to the effector protein. It is unknown which pathways this subunit is involved in

and therefore difficult to predict its involvement with viral infection, however as it associates with Importin-5 to a higher degree in DENV infected cells it may serve to increase the volume of 'signals' transmitted throughout the cell as there will be a rise in the number of activated pathways within the cell in response to viral infection.

As many of the proteins detected in association with Importin-5 are involved in the same cellular pathways e.g. cell death and apoptosis, transcription and translation and signalling pathways, Importin-5 seems to have an effect on many processes in response to viral infection and as such it would be interesting to carry out further work pinpointing any alteration in activity in response to DENV.

Analysis of the phosphorylation status of proteins pulled down in complex with Importin-5 showed a number of hits which contained phosphorylated peptides. As mentioned previously, the effect of phosphorylation on nuclear import is a contentious issue. However, some Importin-5 interaction partners shown to be phosphorylated in response to DENV infection could play a role in cellular processes affecting the outcome of viral infection. For example, histones are known to be transported into the nucleus by Importins and have a role in chromatin regulation during apoptosis (Cheung et al., 2003). In this study, it was seen that in response to DENV (as compared to Mock) histones containing phosphorylated peptides were less abundant, this could illustrate a mechanism employed by the DCs to reduce levels of apoptosis in order to maintain the immune response, leading to higher levels of immuno-pathology. It is possible that regulation of nuclear import does indeed play a role in apoptosis events as seen by Yasuhara et al. (Yasuhara et al., 1997). As Importin-5 was seen to be up-regulated in DENV infected cells, it could be that the virus has evolved a mechanism to increase transport of apoptosis-inducing proteins into the nucleus leading to increased apoptosis and potentially increased viral dissemination.

Summary

In conclusion, it has been found that many proteins are differentially phosphorylated in response to DENV infection and these proteins have roles in varying cellular functions, most notably apoptosis, signalling and cellular motility. Many of the proteins detected are reportedly involved in the same pathways and this re-enforces the confidence that the pathways identified are truly altered in response to DENV.

Processes such as apoptosis and cellular motility can have a significant influence on the course of DENV infection by altering the rate of spread of virus and differential regulation of proteins along signalling pathways can alter infection by enhancing or inhibiting cellular immune responses.

It has been reported for the first time in this thesis that Calpain-2 is required for efficient DENV replication and it is hypothesised that in response to calcium flux induced upon recognition of DENV, Calpain-2 is activated by phosphorylation and this leads to induction of the apoptosis pathway. This results in higher levels of viral dissemination as progeny escape the cell in apoptotic bodies and are engulfed by naive APCs where they can establish infection and viral replication can begin.

It has also been shown that Importin-5 interacts with various proteins involved in viral and host transcription and Importin-5 is itself up-regulated in response to DENV infection. It may be that import of these proteins into the nucleus leads to higher levels of transcription of viral proteins leading to higher levels of viral replication. As Importin-5 is up-regulated in response to DENV, it can also be hypothesised that this leads to increased transport of DENV NS5 into the nucleus which may increase induction of the transcription of viral proteins or cellular proteins which could increase or decrease infectivity respectively.

The proteins identified in this study could potentially be targeted using pharmaceutical approaches in a bid to combat their potential role in DENV pathology. Treatments developed to inhibit the effect of these proteins on the course on infection may well lead to reduction in severe immuno-pathology, significantly reducing the burden of dengue worldwide.

APPENDIX

Sorting of mass spectrometry results into tables aiming to highlight those which could potentially have the greatest effect on viral infection and immune responses.

Lower phosphorylation level in DENV stimulation:

Not in DENV	Mock
Calmodulin; CaM	672
14-3-3 protein gamma; Protein kinase C inhibitor protein 1; KCIP-1	199
Prothymosin alpha	178
Myristoylated alanine-rich C-kinase substrate; MARCKS; Protein kinase C substrate, 80 kDa protein, light chain; 80K-L protein; PKCSL	120
Myosin light polypeptide 6; 17 kDa myosin light chain; LC17; Myosin light chain 3; MLC-3; Myosin light chain alkali 3; Myosin light chain A3; Smooth muscle and n	118
Myosin light chain 6B; Myosin light chain 1 slow-twitch muscle A isoform; MLC1sa; Smooth muscle and nonmuscle myosin light chain alkali 6B	83
Parathymosin;	72
Angiomotin-like protein 2; Leman coiled-coil protein; LCCP	46
Sodium leak channel non-selective protein; CanIon; Voltage gated channel-like protein 1	46
Angiomotin-like protein 1	46
Spectrin beta chain, brain 4; BSPECV; Beta-V spectrin; Spectrin, non-erythroid beta chain 4;	46
Vascular endothelial growth factor C; VEGF-C; Flt4 ligand; Flt4-L; Vascular endothelial growth factor-related protein; VRP	46
General transcription factor 3C polypeptide 5; TF3C-epsilon; Transcription factor IIIC 63 kDa subunit; TFIIIC 63 kDa subunit; TFIIIC63; Transcription factor IIIC subunit epsilon;	46
Macoilin; Transmembrane protein 57	46
Angiomotin;	46
SAM and SH3 domain-containing protein 1Proline-glutamate repeat-containing protein	45
Protein canopy homolog 2MIR-interacting saposin-like protein; Putative secreted protein Zsig9; Transmembrane protein 4	41
Ribonuclease inhibitor; Placental ribonuclease inhibitor; Placental RNase inhibitor; Ribonuclease/angiogenin inhibitor 1; RAI	41
Heterogeneous nuclear ribonucleoprotein K; hnRNP K; Transformation up-regulated nuclear protein; TUNP	36
Phosphatidylinositol 3,4,5-trisphosphate-dependent Rac exchanger 2 protein; P-Rex2; Short=PtdIns(3,4,5)-dependent Rac exchanger 2; DEP domain-containing protein 2	28
Complement component 1 Q subcomponent-binding protein, mitochondrial; GC1q-R protein; Glycoprotein gC1qBP; C1qBP; Hyaluronan-binding protein 1; Mitochondrial matrix protein	25
Serine/threonine-protein kinase MARK1; EC=2.7.11.1; MAP/microtubule affinity-regulating kinase 1	24

Less in DENV than Flu	Mock	DENV	IAV	LPS
Plasminogen activator inhibitor 1 RNA-binding protein; PAI1 RNA-binding protein 1; PAI-RBP1SERPINE1 mRNA-binding protein 1;	256	87	222	120
Ena/VASP-like protein; Ena/vasodilator-stimulated phosphoprotein-like;	96	47	142	253
Protein disulfide-isomerase A4; EC=5.3.4.1; Endoplasmic reticulum resident protein 70; ER protein 70; ERp70; Endoplasmic reticulum resident protein 72; ER protein 72; ERp-72; ER	30		360	41
Heat shock protein HSP 90-alpha; Heat shock 86 kDa; HSP 86; HSP86; Renal carcinoma antigen NY-REN-38;	343	422	494	436
Heat shock 70 kDa protein 1-like; Heat shock 70 kDa protein 1L; Heat shock 70 kDa protein 1-Hom; HSP70-Hom;	119	121	189	129
ATP synthase subunit alpha, mitochondrial;		31	82	
Nucleobindin-1; CALNUC;		63	103	47
POTE ankyrin domain family member E; ANKRD26-like family C member 1A; Prostate, ovary, testis-expressed protein on chromosome 2; POTE-2;	73	47	81	
Putative heat shock protein HSP 90-alpha A5; Heat shock protein 90-alpha E; Heat shock protein 90Ae;		25	48	
Actin, cytoplasmic 1; Beta-actin;	218	154	175	231
Heat shock-related 70 kDa protein 2; Heat shock 70 kDa protein 2;	84	60	81	

Less in DENV than Mock	Mock	DENV	IAV	LPS
High mobility group protein B1; High mobility group protein 1; HMG-1;	367	142	91	428
Plasminogen activator inhibitor 1 RNA-binding protein; PAI1 RNA-binding protein 1; PAI-RBP1; SERPINE1 mRNA-binding protein 1;	256	87	222	120
Glucosidase 2 subunit beta; 80K-H protein; Glucosidase II subunit beta; Protein kinase C substrate 60.1 kDa protein heavy chain; PKCSH;	296	127		
Rho GDP-dissociation inhibitor 1; Rho GDI 1; Rho-GDI alpha;	21	161		
Actin, aortic smooth muscle; Alpha-actin-2; Cell growth-inhibiting gene 46 protein; ;	214	136		198
Cofilin-118 kDa phosphoprotein; p18; Cofilin, non-muscle isoform;	101	29		
Calumenin; Crocalbin; IEF SSP 9302;	125	54		
Actin, cytoplasmic 1; Beta-actin;	218	154	175	231
Ena/VASP-like protein; Ena/vasodilator-stimulated phosphoprotein-like;	96	47	142	253
Splicing factor 1; Mammalian branch point-binding protein; BBP; mBBP; Transcription factor ZFM1; Zinc finger gene in MEN1 locusZinc finger protein 162;	63	26		52
Protein canopy homolog 3; CTG repeat protein 4a; Expanded repeat-domain protein CAG/CTG 5; Protein associated with TLR4; Trinucleotide repeat-containing gene 5 protein;	67	33	39	23
POTE ankyrin domain family member E; ANKRD26-like family C member 1A; Prostate, ovary, testis-expressed protein on chromosome 2POTE-2;	73	47	81	
POTE ankyrin domain family member I;	73		81	
Heat shock-related 70 kDa protein 2; Heat shock 70 kDa protein 2;	84	60	81	88
[3-methyl-2-oxobutanoate dehydrogenase [lipoamide]] kinase, mitochondrial; EC=2.7.11.4; Branched-chain alpha-ketoacid dehydrogenase kinase; BCKD-kinase; BCKDHKIN;	85	61	70	100
Beta-actin-like protein 2; AltName: Full=Kappa-actin;	117	94		124
Endoplasmin; 94 kDa glucose-regulated protein; GRP-94; Heat shock protein 90 kDa beta member 1; Tumor rejection antigen 1; gp96 homolog;	199	177	122	200
Rho GDP-dissociation inhibitor 2; Rho GDI 2; Ly-GDI; Rho-GDI beta;	344	323	277	321
Protein S100-A9; Calgranulin-B; Calprotectin L1H subunit; Leukocyte L1 complex heavy chain; Migration inhibitory factor-related protein 14; MRP-14; Short=p14;	45	25	39	67

Less in DENV than either other	Mock	DENV	IAV	LPS
Glucosidase 2 subunit beta; 80K-H protein; Glucosidase II subunit beta; Protein kinase C substrate 60.1 kDa protein heavy chain; PKCSH;	296	127		
Plasminogen activator inhibitor 1 RNA-binding protein; PAI1 RNA-binding protein 1; PAI-RBP1; SERPINE1 mRNA-binding protein 1;	256	87	222	120
Ena/VASP-like protein; Ena/vasodilator-stimulated phosphoprotein-like;	96	47	142	253
Calumenin; Crocalbin; IEF SSP 9302; ;	125	54		
Actin, cytoplasmic 1; Beta-actin;	218	154	175	231
POTE ankyrin domain family member E; ANKRD26-like family C member 1A; Prostate, ovary, testis-expressed protein on chromosome 2; POTE-2;	73	47	81	
Heat shock-related 70 kDa protein 2; Heat shock 70 kDa protein 2;	84	60	81	88
[3-methyl-2-oxobutanoate dehydrogenase [lipoamide]] kinase, mitochondrial; EC=2.7.11.4; Branched-chain alpha-ketoacid dehydrogenase kinase; BCKD-kinase; BCKDHKIN;	85	61	70	100
Protein S100-A9; Calgranulin-B; Calprotectin L1H subunit; Leukocyte L1 complex heavy chainMigration inhibitory factor-related protein 14; MRP-14; p14;	45	25	39	67

Higher phosphorylation level in DENV:

Only in DENV	DENV
Heat shock protein HSP 90-beta; HSP 90; Heat shock 84 kDa; HSP 84; HSP84;	523
Heat shock protein HSP 90-alpha; Heat shock 86 kDa; HSP 86; HSP86; Renal carcinoma antigen NY-REN-38;	261
14-3-3 protein eta; Protein AS1;	216
Putative heat shock protein HSP 90-beta-3; Heat shock protein 90-beta c; Heat shock protein 90Bc;	170
Tubulin beta chainTubulin beta-5 chain;	166
Tubulin beta-3 chain; Tubulin beta-4 chain; Tubulin beta-III;	166
Antileukoproteinase; ALP; BLPI; HUSI-1; Mucus proteinase inhibitor; MPI; Protease inhibitor WAP4; Secretory leukocyte protease inhibitor; S	153
Lactotransferrin; Lactoferrin; EC=3.4.21.-; Talalactoferrin;	122
Immunoglobulin J chain;	121
Heat shock 70 kDa protein 1-like; Heat shock 70 kDa protein 1L; Heat shock 70 kDa protein 1-HomHSP70-Hom;	116
Proteasome subunit alpha type-5; EC=3.4.25.1; Macropain zeta chain; Multicatalytic endopeptidase complex zeta chain; Proteasome zeta chain;	102
Charged multivesicular body protein 4b; Chromatin-modifying protein 4b; CHMP4bSNF7 homolog associated with Alix 1; SNF7-2; Short=hSnf7-2; Vacuolar protein sorting-associat	102
Tubulin beta-2C chain; Tubulin beta-2 chain;	101
Stathmin-2; Superior cervical ganglion-10 protein; Protein SCG10;	79
Putative heat shock protein HSP 90-beta 2; Heat shock protein 90-beta b; Heat shock protein 90Bb;	77
Heat shock 70 kDa protein 6; Heat shock 70 kDa protein B~;	77
Heat shock cognate 71 kDa protein; Heat shock 70 kDa protein 8;	77
Putative heat shock protein HSP 90-beta 4;	72
Deoxyribonucleoside 5~-monophosphate N-glycosidase; EC=3.2.2.-; c-Myc-responsive protein Rcl;	70
Protein disulfide-isomerase A6; EC=5.3.4.1; Endoplasmic reticulum protein 5; ER protein 5; ERp5; Protein disulfide isomerase P5; Thioredoxin domain-containing protein 7;	64
CapZ-interacting protein; Protein kinase substrate CapZIP; RCSD domain-containing protein 1;	61
Drebrin-like protein; Cervical SH3P7; Cervical mucin-associated protein; Drebrin-F; HPK1-interacting protein of 55 kDa; HIP-55; SH3 domain-containing protein 7	59

Calpastatin; Calpain inhibitor; Sperm BS-17 component;	57
Outer dense fiber protein 2; Cenexin; Outer dense fiber of sperm tails protein 2;	57
Peroxiredoxin-4; EC=1.11.1.15; Antioxidant enzyme AOE372; AOE37-2; Peroxiredoxin IV; Short=Prx-IV; Thioredoxin peroxidase AO372; Thioredoxin-dependent peroxide reductase A03	52
Receptor-type tyrosine-protein phosphatase C; EC=3.1.3.48; Leukocyte common antigen; L-CA; T200; CD_antigen=CD45	49
Nucleobindin-1; CALNUC;	45
60 kDa heat shock protein, mitochondrial; 60 kDa chaperonin; Chaperonin 60; CPN60; Heat shock protein 60; HSP-60; Short=Hsp60; HuCHA60; Mitochondrial mat	45
Cofilin-2; Cofilin, muscle isoform;	45
Golgi-specific brefeldin A-resistance guanine nucleotide exchange factor 1; BFA-resistant GEF 1;	44
Docking protein 2; Downstream of tyrosine kinase 2; p56(dok-2);	44
X-ray repair cross-complementing protein 5; EC=3.6.4.-; 86 kDa subunit of Ku antigen; ATP-dependent DNA helicase 2 subunit 2; ATP-dependent DNA helicase II 80 kDa subunit; CTC box	44
Putative endoplasmin-like protein; Putative heat shock protein 90 kDa beta member 2;	42
Leucine-rich repeat and guanylate kinase domain-containing protein;	42
Myosin-9; Cellular myosin heavy chain, type A; Myosin heavy chain 9; Myosin heavy chain, non-muscle IIa; Non-muscle myosin heavy chain A; NMMHC-A; Non-muscle m	42
DNA-binding protein RFXANK; Ankyrin repeat family A protein 1; Regulatory factor X subunit B; RFX-B; Regulatory factor X-associated ankyrin-containing protein;	42
Zinc finger protein 280D; Suppressor of hairy wing homolog 4; Zinc finger protein 634;	41
GRIP and coiled-coil domain-containing protein 1; Golgi coiled-coil protein 1;	41
Ig lambda-2 chain C regions;	41
Fc receptor-like protein 2; FcR-like protein 2; FcRL2; Fc receptor homolog 2; FcRH2; IFGP family protein 4; Immunoglobulin receptor translocation-associated protein 4; SH2 domain-containing phosphatase anchor protein 1; CD_antigen=CD307b	40
Protein CDV3 homolog;	40
Leucine-rich repeat and guanylate kinase domain-containing protein;	39
Golgi-specific brefeldin A-resistance guanine nucleotide exchange factor 1; BFA-resistant GEF 1;	39
BH3-interacting domain death agonist; p22 BID; BID;	39
Pleckstrin homology domain-containing family O member 2; PH domain-containing family O member 2; Pleckstrin homology domain containing family Q member 1; PH domain-containing family Q member 1;	38
Pyridoxal kinase; EC=2.7.1.35; Pyridoxine kinase;	36
Splicing factor 3B subunit 5; SF3b5Pre-mRNA-splicing factor SF3b 10 kDa subunit;	34
EF-hand domain-containing protein D2; Swiprosin-1;	34
Reticulocalbin-1;	33

DDB1- and CUL4-associated factor 10; WD repeat-containing protein 32;	33
Deleted in malignant brain tumors 1 protein; Glycoprotein 340Gp-340; Hensin; Salivary agglutinin; SAG; Surfactant pulmonary-associated D-binding protein;	33
Interferon alpha-inducible protein 27-like protein 2; Interferon-stimulated gene 12b protein; Short=ISG12(b); Protein TLH29; pIFI27-like protein;	31
LIM domain and actin-binding protein 1; Epithelial protein lost in neoplasm;	29
Proteasome subunit beta type-1; EC=3.4.25.1; Macropain subunit C5; Multicatalytic endopeptidase complex subunit C5; Proteasome component C5; Proteasome gamma chain	29
UPF0552 protein C15orf38;	28
Calcineurin subunit B type 2; Calcineurin B-like protein; CBLP; Calcineurin BII; Short=CNBII; Protein phosphatase 2B regulatory subunit 2; Protein phosphatase 3 regulatory s	27
Calcium-regulated heat stable protein 1; Calcium-regulated heat-stable protein of 24 kDa; CRHSP-24;	27
Guanine nucleotide-binding protein-like 3; E2-induced gene 3 protein; Novel nucleolar protein 47; NNP47; Nucleolar GTP-binding protein 3; Nucleostemin;	27
Mucin-5B; MUC-5B; Cervical mucin; High molecular weight salivary mucin MG1; Mucin-5 subtype B, tracheobronchial; Sublingual gland mucin;	27
Lanosterol 14-alpha demethylase; LDM; EC=1.14.13.70; CYPLI; Cytochrome P450 51A1; Cytochrome P450-14DM; Cytochrome P45014DM; Cytochrome P450LI; Sterol 14	27
Glycogenin-1; GN-1; GN1; EC=2.4.1.186;	26
Macrophage-capping protein; Actin regulatory protein CAP-G;	26
Lysophospholipid acyltransferase LPCAT4; EC=2.3.1.-; 1-acylglycerol-3-phosphate O-acyltransferase 7; 1-AGP acyltransferase 7; 1-AGPAT 7; Acyltransferase-like 3; Lysophosphatidylchol	26
Dystrophin;	25
Pyrin and HIN domain-containing protein 1; Interferon-inducible protein X;	25
Elongation factor 1-delta; EF-1-delta; Antigen NY-CO-4;	20
Uncharacterized protein C11orf66;	20

More in DENV than Flu	Mock	DENV	IAV	LPS
Tubulin beta chain; Tubulin beta-5 chain;	635	1214	938	1070
Tubulin alpha-1B chain; Alpha-tubulin ubiquitous; Tubulin K-alpha-1; Tubulin alpha-ubiquitous chain;	319	513	301	
Protein disulfide-isomerase A6; EC=5.3.4.1; Endoplasmic reticulum protein 5; ER protein 5; ERp5; Protein disulfide isomerase P5; Thioredoxin domain-containing protein 7;	419	770	571	736
Coagulation factor XIII A chain; Coagulation factor XIIIa; EC=2.3.2.13; Protein-glutamine gamma-glutamyltransferase A chain; Transglutaminase A chain;	144	312	143	253
Tubulin beta-3 chain; Tubulin beta-4 chain; Tubulin beta-III;	365	816	655	669
Tubulin beta-6 chain;	118	278	168	229
Tubulin beta-2C chain; Tubulin beta-2 chain;	420	951	849	870
Serine/threonine-protein kinase PAK 2; EC=2.7.11.1Gamma-PAK; PAK65; S6/H4 kinase; p21-activated kinase 2; PAK-2; p58;	137	216	121	111
EF-hand domain-containing protein D2; Swiprosin-1;	65	129	37	46
Rho GDP-dissociation inhibitor 1; Rho GDI 1; Rho-GDI alpha;	195	251	162	161
78 kDa glucose-regulated protein; GRP-78; Endoplasmic reticulum lumenal Ca(2+)-binding protein grp78; Heat shock 70 kDa protein 5; Immunoglobulin heavy chain-binding protein; BiP; F	1143	1788	1708	1416
Plastin-2; L-plastin; LC64P; Lymphocyte cytosolic protein 1; LCP-1;	345	457	381	360
Tubulin beta-8 chain;		324	252	298
High mobility group protein B1; High mobility group protein 1; HMG-1;	367	142	91	428
Tubulin beta-1 chain;	92	193	138	169
Endoplasmin94 kDa glucose-regulated protein; GRP-94; Heat shock protein 90 kDa beta member 1; Tumor rejection antigen 1; gp96 homolog;	199	177	122	200
Rho GDP-dissociation inhibitor 2; Rho GDI 2Ly-GDI; Rho-GDI beta;	344	323	277	321
Stathmin; Leukemia-associated phosphoprotein p18; Metablastin; Oncoprotein 18; Short=Op18; Phosphoprotein p19; pp19; Prosolin; Protein Pr22; AltN		89	52	149
Putative heat shock protein HSP 90-beta 2; Heat shock protein 90-beta b; Heat shock protein 90Bb;		131	96	
Protein disulfide-isomerase A3; EC=5.3.4.1; 58 kDa glucose-regulated protein; 58 kDa microsomal protein; p58; Disulfide isomerase ER-60; Endoplasmic reticulum resident prote	261	310	280	354
Nucleophosmin; Short=NPM; Nucleolar phosphoprotein B23; Nucleolar protein NO38; Numatrin;	65	55		58
Cellular nucleic acid-binding protein; CNBP; Zinc finger protein 9;	36	80	53	85

More in DENV than Mock	Mock	DENV	IAV	LPS
78 kDa glucose-regulated protein; GRP-78; Endoplasmic reticulum lumenal Ca(2+)-binding protein grp78; Heat shock 70 kDa protein 5; Immunoglobulin heavy chain-binding protein; BiP; F	1143	1788	1708	1416
Protein disulfide-isomerase; PDI; EC=5.3.4.1; Cellular thyroid hormone-binding protein; Prolyl 4-hydroxylase subunit beta; p55;			574	242
Tubulin beta chain; Tubulin beta-5 chain;	635	1214	938	1070
Tubulin beta-2C chainTubulin beta-2 chain;	420	951	849	870
Tubulin beta-3 chain; Tubulin beta-4 chain; Tubulin beta-III;	365	816	655	669
Endoplasmin; 94 kDa glucose-regulated protein; Short=GRP-94; Heat shock protein 90 kDa beta member 1; Tumor rejection antigen 1; gp96 homolog;	199	177	122	200
Protein disulfide-isomerase A6; EC=5.3.4.1; Endoplasmic reticulum protein 5; ER protein 5; ERp5Protein disulfide isomerase P5; Thioredoxin domain-containing protein 7;	419	770	571	736
Tubulin alpha-1B chain; Alpha-tubulin ubiquitous; Tubulin K-alpha-1; Tubulin alpha-ubiquitous chain;	319	513	301	
Tropomyosin beta chain; Beta-tropomyosin; Tropomyosin-2;	322	500		
40S ribosomal protein SA; 37 kDa laminin receptor precursor; 37LRP; 37/67 kDa laminin receptor; LRP/LR; 67 kDa laminin receptor; 67LR; Colon carcinoma laminin-bi	72	250		
Tropomyosin alpha-4 chain; TM30p1; Tropomyosin-4;	540	708		
Coagulation factor XIII A chain; Coagulation factor XIIIa; EC=2.3.2.13; Protein-glutamine gamma-glutamyltransferase A chain; Transglutaminase A chain;	144	312	143	253
Tropomyosin alpha-3 chain; Gamma-tropomyosin; Tropomyosin-3; Tropomyosin-5; hTM5;	604	769		
Tubulin beta-6 chain;	118	278	168	229
Rho GDP-dissociation inhibitor 1; Rho GDI 1; Rho-GDI alpha;	195	251	162	161
Putative tubulin beta chain-like protein ENSP00000290377;	92	225		
14-3-3 protein zeta/delta; Protein kinase C inhibitor protein 1; KCIP-1;	244	377		
Calreticulin; CRP55; Calregulin; Endoplasmic reticulum resident protein 60; ERp60; HACBP; grp60;	364	481		
Heat shock protein HSP 90-beta; HSP 90; Heat shock 84 kDa; HSP 84; HSP84;	476	592	596	677
Plastin-2; L-plastin; LC64P; Lymphocyte cytosolic protein 1; LCP-1;	345	457	381	360
Sorting nexin-2; Transformation-related gene 9 protein; TRG-9;	52	161		
Tropomyosin alpha-1 chain; Alpha-tropomyosin; Tropomyosin-1;	257	362		
Tubulin beta-1 chain;	92	193	138	169
Drebrin-like protein; Cervical SH3P7; Cervical mucin-associated protein; Drebrin-F; HPK1-interacting protein of 55 kDa; HIP-55; SH3 domain-containing protein 7	122	218	235	147
Heat shock protein HSP 90-alpha; Heat shock 86 kDa; HSP 86; HSP86; Renal	343	422	494	436

carcinoma antigen NY-REN-38;				
Serine/threonine-protein kinase PAK 2; EC=2.7.11.1; Gamma-PAK; PAK65; S6/H4 kinase; p21-activated kinase 2; PAK-2; p58;	137	216	121	111
Elongation factor 1-delta; EF-1-delta; Antigen NY-CO-4;	96	164	169	108
14-3-3 protein beta/alpha; Protein 1054; Protein kinase C inhibitor protein 1; KCIP-1;	368	434		
EF-hand domain-containing protein D2; Swiprosin-1;	65	129	37	46
Adenylyl cyclase-associated protein 1; CAP 1;	40	103	103	56
Vimentin;	95	152		
Rho GDP-dissociation inhibitor 1; Rho GDI 1; Rho-GDI alpha;	195	251	162	161
Protein disulfide-isomerase A3; EC=5.3.4.1; 58 kDa glucose-regulated protein; 58 kDa microsomal protein; p58; Disulfide isomerase ER-60; Endoplasmic reticulum resident prote	261	310	280	354
CapZ-interacting protein; Protein kinase substrate CapZIP; RCSD domain-containing protein 1;	30	75		27
Cellular nucleic acid-binding protein; CNBP; Zinc finger protein 9;	36	80	53	85
Coronin-1B; Coronin-2;	184	226	218	85
Proteasome activator complex subunit 1; 11S regulator complex subunit alpha; Short=REG-alpha; Activator of multicatalytic protease subunit 1; Interferon gamma up-regulated I-5111 protein; S	36	75	73	82
Switch-associated protein 70; SWAP-70;	91	119	134	116
Eukaryotic translation initiation factor 2 subunit 1; Eukaryotic translation initiation factor 2 subunit alpha; eIF-2-alpha; eIF-2A; eIF-2alpha;	107	135	146	148
Transaldolase; EC=2.2.1.2;	26	48		
Hematopoietic lineage cell-specific protein; Hematopoietic cell-specific LYN substrate 1; LckBP1; p75;	109	130	211	202
Serine/threonine-protein kinase PAK 1; EC=2.7.11.1; Alpha-PAK; p21-activated kinase 1; PAK-1; p65-PAK;	26	46		

More in DENV than either other	Mock	DENV	IAV	LPS
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Tropomyosin alpha-4 chainTM30p1; Tropomyosin-4;	540	708		
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Serine/threonine-protein kinase PAK 2; EC=2.7.11.1; Gamma-PAK; PAK65; S6/H4 kinase; p21-activated kinase 2PAK-2; p58;	137	216	121	111

Tubulin beta-8 chain;		324	252	298
14-3-3 protein beta/alpha; Protein 1054; Protein kinase C inhibitor protein 1; KCIP-1;	368	434		
Adenylyl cyclase-associated protein 1; CAP 1;	40	103	103	56
Hypoxia up-regulated protein 1; 150 kDa oxygen-regulated protein; ORP-150; 170 kDa glucose-regulated protein; GRP-170;		92	34	59
Vimentin;	95	152		
Tubulin alpha-8 chain; Alpha-tubulin 8; Tubulin alpha chain-like 2;		179		124
EF-hand domain-containing protein D2; Swiprosin-1;	65	129	37	46
Protein disulfide-isomerase A3; EC=5.3.4.1; 58 kDa glucose-regulated protein; 58 kDa microsomal protein; p58; Disulfide isomerase ER-60; Endoplasmic reticulum resident prote	261	310	280	354
CapZ-interacting protein; Protein kinase substrate CapZIP; RCSD domain-containing protein 1;	30	75		27
Cellular nucleic acid-binding protein; CNBP; Zinc finger protein 9;	36	80	53	85
Coronin-1B; Coronin-2;	184	226	218	85
Proteasome activator complex subunit 1; 11S regulator complex subunit alpha; REG-alpha; Activator of multicatalytic protease subunit 1; Interferon gamma up-regulated I-5111 protein;	36	75	73	82
Stathmin; Leukemia-associated phosphoprotein p18; Metablastin; Oncoprotein 18; Op18; Phosphoprotein p19; pp19; Prosolin; Protein Pr22; AltN		89	52	149
Transaldolase; EC=2.2.1.2;	26	48		

Table of interesting hits

Protein	Condition
Calmodulin; CaM	Not in DENV
14-3-3 protein gamma; Protein kinase C inhibitor protein 1; KCIP-1	
Vascular endothelial growth factor C; VEGF-C; Flt4 ligand; Flt4-L; Vascular endothelial growth factor-related protein; VRP	
SAM and SH3 domain-containing protein 1Proline-glutamate repeat-containing protein	
Ribonuclease inhibitor; Placental ribonuclease inhibitor; Placental RNase inhibitor; Ribonuclease/angiogenin inhibitor 1; RAI	
Serine/threonine-protein kinase MARK1; EC=2.7.11.1; MAP/microtubule affinity-regulating kinase 1	
Ena/VASP-like protein; Ena/vasodilator-stimulated phosphoprotein-like;	Less in DENV than Flu
Protein disulfide-isomerase A4; EC=5.3.4.1; Endoplasmic reticulum resident protein 70; ER protein 70; ERp70; Endoplasmic reticulum resident protein 72; ER protein 72; ERp-72; ER	
High mobility group protein B1; High mobility group protein 1; HMG-1;	Less in DENV than Mock
Rho GDP-dissociation inhibitor 1; Rho GDI 1; Rho-GDI alpha;	
Cofilin-118 kDa phosphoprotein; p18; Cofilin, non-muscle isoform;	
Calumenin; Crocalbin; IEF SSP 9302;	
Ena/VASP-like protein; Ena/vasodilator-stimulated phosphoprotein-like;	
Endoplasmin; 94 kDa glucose-regulated protein; GRP-94; Heat shock protein 90 kDa beta member 1; Tumor rejection antigen 1; gp96 homolog;	
Rho GDP-dissociation inhibitor 2; Rho GDI 2; Ly-GDI; Rho-GDI beta;	
Protein S100-A9; Calgranulin-B; Calprotectin L1H subunit; Leukocyte L1 complex heavy chain; Migration inhibitory factor-related protein 14; MRP-14; Short=p14;	
Ena/VASP-like protein; Ena/vasodilator-stimulated phosphoprotein-like;	Less in DENV than either other
Calumenin; Crocalbin; IEF SSP 9302; ;	
POTE ankyrin domain family member E; ANKRD26-like family C member 1A; Prostate, ovary, testis-expressed protein on chromosome 2; POTE-2;	
Protein S100-A9; Calgranulin-B; Calprotectin L1H subunit; Leukocyte L1 complex heavy chainMigration inhibitory factor-related protein 14; MRP-14; p14;	
Antileukoproteinase; ALP; BLPI; HUSI-1; Mucus proteinase inhibitor; MPI; Protease inhibitor WAP4; Secretory leukocyte protease inhibitor; S	Only in DENV
Lactotransferrin; Lactoferrin; EC=3.4.21.-; Talalactoferrin;	
Charged multivesicular body protein 4b; Chromatin-modifying protein 4b; CHMP4bSNF7 homolog associated with Alix 1; SNF7-2; Short=hSnf7-2; Vacuolar protein sorting-associat	
Stathmin-2; Superior cervical ganglion-10 protein; Protein SCG10;	
Protein disulfide-isomerase A6; EC=5.3.4.1; Endoplasmic reticulum protein 5; ER protein 5; ERp5; Protein disulfide isomerase P5; Thioredoxin domain-containing protein 7;	
Calpastatin; Calpain inhibitor; Sperm BS-17 component;	
Golgi-specific brefeldin A-resistance guanine nucleotide exchange factor 1; BFA-resistant GEF 1;	
Docking protein 2; Downstream of tyrosine kinase 2; p56(dok-2);	

GRIP and coiled-coil domain-containing protein 1; Golgi coiled-coil protein 1;	
BH3-interacting domain death agonist; p22 BID; BID;	
EF-hand domain-containing protein D2; Swiprosin-1;	
Interferon alpha-inducible protein 27-like protein 2; Interferon-stimulated gene 12b protein; Short=ISG12(b); Protein TLH29; pIFI27-like protein;	
Calcineurin subunit B type 2; Calcineurin B-like protein; CBLP; Calcineurin BII; Short=CNBII; Protein phosphatase 2B regulatory subunit 2; Protein phosphatase 3 regulatory s	
Calcium-regulated heat stable protein 1; Calcium-regulated heat-stable protein of 24 kDa; CRHSP-24;	
Guanine nucleotide-binding protein-like 3; E2-induced gene 3 protein; Novel nucleolar protein 47; NNP47; Nucleolar GTP-binding protein 3; Nucleostemin;	
Macrophage-capping protein; Actin regulatory protein CAP-G;	
Lysophospholipid acyltransferase LPCAT4; EC=2.3.1.-; 1-acylglycerol-3-phosphate O-acyltransferase 7; 1-AGP acyltransferase 7; 1-AGPAT 7; Acyltransferase-like 3; Lysophosphatidylchol	
Pyrin and HIN domain-containing protein 1; Interferon-inducible protein X;	
Serine/threonine-protein kinase PAK 2; EC=2.7.11.1Gamma-PAK; PAK65; S6/H4 kinase; p21-activated kinase 2; PAK-2; p58;	**More in DENV than Flu**
Rho GDP-dissociation inhibitor 1; Rho GDI 1; Rho-GDI alpha;	
78 kDa glucose-regulated protein; GRP-78; Endoplasmic reticulum lumenal Ca(2+)-binding protein grp78; Heat shock 70 kDa protein 5; Immunoglobulin heavy chain-binding protein; BiP; F	
Plastin-2; L-plastin; LC64P; Lymphocyte cytosolic protein 1; LCP-1;	
High mobility group protein B1; High mobility group protein 1; HMG-1;	
Endoplasmin94 kDa glucose-regulated protein; GRP-94; Heat shock protein 90 kDa beta member 1; Tumor rejection antigen 1; gp96 homolog;	
Rho GDP-dissociation inhibitor 2; Rho GDI 2Ly-GDI; Rho-GDI beta;	
Stathmin; Leukemia-associated phosphoprotein p18; Metablastin; Oncoprotein 18; Short=Op18; Phosphoprotein p19; pp19; Prosolin; Protein Pr22; AltN	
78 kDa glucose-regulated protein; GRP-78; Endoplasmic reticulum lumenal Ca(2+)-binding protein grp78; Heat shock 70 kDa protein 5; Immunoglobulin heavy chain-binding protein; BiP; F	**More in DENV than Mock**
Protein disulfide-isomerase; PDI; EC=5.3.4.1; Cellular thyroid hormone-binding protein; Prolyl 4-hydroxylase subunit beta; p55;	
78 kDa glucose-regulated protein; GRP-78; Endoplasmic reticulum lumenal Ca(2+)-binding protein grp78; Heatshock 70 kDa protein 5; Immunoglobulin heavy chain-binding protein; BiP; F	
Endoplasmin; 94 kDa glucose-regulated protein; Short=GRP-94; Heat shock protein 90 kDa beta member 1; Tumor rejection antigen 1; gp96 homolog;	
Protein disulfide-isomerase A6; EC=5.3.4.1; Endoplasmic reticulum protein 5; ER protein 5; ERp5Protein disulfide isomerase P5; Thioredoxin domain-containing protein 7;	
Rho GDP-dissociation inhibitor 1; Rho GDI 1; Rho-GDI alpha;	
14-3-3 protein zeta/delta; Protein kinase C inhibitor protein 1; KCIP-1;	
Calreticulin; CRP55; Calregulin; Endoplasmic reticulum resident protein 60; ERp60; HACBP; grp60;	
Plastin-2; L-plastin; LC64P; Lymphocyte cytosolic protein 1; LCP-1;	
Serine/threonine-protein kinase PAK 2; EC=2.7.11.1; Gamma-PAK; PAK65; S6/H4 kinase; p21-activated kinase 2; PAK-2; p58;	

Elongation factor 1-delta; EF-1-delta; Antigen NY-CO-4;	
14-3-3 protein beta/alpha; Protein 1054; Protein kinase C inhibitor protein 1; KCIP-1;	
EF-hand domain-containing protein D2; Swiprosin-1;	
Vimentin;	
Rho GDP-dissociation inhibitor 1; Rho GDI 1; Rho-GDI alpha;	
Protein disulfide-isomerase A3; EC=5.3.4.1; 58 kDa glucose-regulated protein; 58 kDa microsomal protein; p58; Disulfide isomerase ER-60; Endoplasmic reticulum resident prote	
CapZ-interacting protein; Protein kinase substrate CapZIP; RCSD domain-containing protein 1;	
Coronin-1B; Coronin-2;	
Proteasome activator complex subunit 1; 11S regulator complex subunit alpha; Short=REG-alpha; Activator of multicatalytic protease subunit 1; Interferon gamma up-regulated I-5111 protein; S	
Switch-associated protein 70; SWAP-70;	
Serine/threonine-protein kinase PAK 1; EC=2.7.11.1; Alpha-PAK; p21-activated kinase 1; PAK-1; p65-PAK;	
78 kDa glucose-regulated protein; GRP-78; Endoplasmic reticulum lumenal Ca(2+)-binding protein grp78; Heat shock 70 kDa protein 5; Immunoglobulin heavy chain-binding protein; BiP; F	**More in DENV than either other**
Protein disulfide-isomerase; PDI; EC=5.3.4.1; Cellular thyroid hormone-binding protein; Prolyl 4-hydroxylase subunit beta; p55;	
78 kDa glucose-regulated protein; GRP-78; Endoplasmic reticulum lumenal Ca(2+)-binding protein grp78; Heat shock 70 kDa protein 5; Immunoglobulin heavy chain-binding protein; BiP; F	
Endoplasmin; 94 kDa glucose-regulated protein; GRP-94; Heat shock protein 90 kDa beta member 1; Tumor rejection antigen 1; gp96 homolog; ;	
Protein disulfide-isomerase A6; EC=5.3.4.1; Endoplasmic reticulum protein 5; ER protein 5; ERp5Protein disulfide isomerase P5; Thioredoxin domain-containing protein 7;	
Rho GDP-dissociation inhibitor 1; Rho GDI 1; Rho-GDI alpha;	
14-3-3 protein zeta/delta; Protein kinase C inhibitor protein 1; KCIP-1;	
Calreticulin; CRP55; Calregulin; Endoplasmic reticulum resident protein 60; ERp60; HACBP; grp60;	
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Serine/threonine-protein kinase PAK 2; EC=2.7.11.1; Gamma-PAK; PAK65; S6/H4 kinase; p21-activated kinase 2PAK-2; p58;	
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CapZ-interacting protein; Protein kinase substrate CapZIP; RCSD domain-containing protein 1;	
Coronin-1B; Coronin-2;	
Proteasome activator complex subunit 1; 11S regulator complex subunit alpha; REG-alpha; Activator of multicatalytic protease subunit 1; Interferon gamma up-regulated I-5111 protein;	
Stathmin; Leukemia-associated phosphoprotein p18; Metablastin; Oncoprotein 18; Op18;	

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