



Dengue virus stimulation of dendritic cells induces phosphorylation- and proteolysis-dependent signalling

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ABSTRACT

Aims: Dengue virus (DENV) infection is commonly observed in countries with tropical climates and remains a significant health hazard. No real cure has been established for the infection to date.

Methodology and results: To better understand the very early molecular events during the initial infection process, we exposed primary dendritic cells with Dengue virus and analysed proteins with increased phosphorylation signatures in the first 10 min using phospho-protein enrichment and tandem mass spectrometry analysis. Upon initial viral interaction, strong phosphorylation was observed for Endoplasmic reticulum chaperones, BiP, BID, Dok-2, GEF-H1 and Calpain-2. Reduced phosphorylation was noted for Importin-5, ERp72 and Rho-GDI. Knockdown of Calpain-2, a protease activated by calcium flux, reduced DENV infection rates of primary dendritic cells as measured by focus-forming units (FFUs).

Conclusion, significance and impact of study: We conclude that Calpain-2, BID, Importin 5 and ATP/GTPases are all active along the apoptosis pathway axis, indicating that dendritic cells commit to early signalling steps of cell death upon initial viral contact.

Keywords: Dengue Virus, signalling, phosphorylation, proteomics, mass spectrometry

INTRODUCTION

Dengue virus (DENV) is a mosquito-borne pathogen with the genus *Aedes*, principally *A. aegypti*, which affects an expanding geographical range, resulting in an increased number of incidences. According to WHO, half of the world's population is now at risk of exposure to this virus. Disease manifestation upon DENV infection can be classified into grades I-IV. Grade I is fever with the presence only of easy bruising or a positive tourniquet test. Grade II is the presence of spontaneous bleeding into the skin and elsewhere. Grade III is the clinical evidence of shock, and grade IV is shock so severe that blood pressure and pulse cannot be detected, also referred to as the "dengue shock syndrome (DSS)" (Rodenhuis-Zybert *et al.*, 2010; Costa *et al.*, 2013). At present, the treatment of various forms of DENV infection is largely supportive due to the lack of specific therapeutics, placing an enormous burden on health systems in low-income countries. Studying the pathologic aspects of Dengue disease suffer from a technical barrier due to the absence of suitable animal models that adequately mimic disease progression. Initially, Langerhans cells, dermal cells and interstitial dendritic cells (DCs) are thought to represent the initial targets of

DENV infection at the site of the mosquito bite (Costa *et al.*, 2013). The ability of these cells to support DENV replication *in vitro* is not yet well established, but DCs are suspected to support the initial antiviral responses at the first stages of DENV infection (Chen *et al.*, 1997). Pattern recognition receptors such as TLR3 and TLR7 as well as Heparan Sulphate, RIG-I/MDA5 and C-type lectins including DC-SIGN (CD209) and CLEC5A have been demonstrated to be involved in DENV sensing (Chen *et al.*, 1997; Tassaneetrithep *et al.*, 2003; Dalrymple *et al.*, 2014). Despite these studies, it has become clear that diverse innate and inflammatory pathways are activated during Dengue disease, but with protective as well as pathological consequences for the host. Little is known about initial molecular events after initial binding that lead to internalisation, fusion and subsequently host cell infection (Kaufmann and Rossmann, 2011), and understanding the mechanisms involved in very early stages of infection is critical in order to develop effective antiviral treatment strategies and to prevent severe outcome of disease. To this end, we have developed an *in vitro* DENV infection model using primary peripheral blood mononuclear cells (PBMCs) derived dendritic cells as well as the THP-1 cell line to study the earliest signalling events upon exposure to DENV. A phospho-

proteomics approach was used to identify and characterise proteins and enzymes involved in calcium-dependent proteolysis during early stages of host-pathogen interactions.

MATERIALS AND METHODS

Cells, antibodies and reagents

Chemicals and solvents were purchased from Sigma unless indicated otherwise. Monocyte derived dendritic cells (MDDCs) 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 transferred into tubes, and these were centrifuged for 20 min at 2000 rpm. PBMC were isolated from the gradient and washed with 10 mL RPMI-1640 three times. PBMC were subsequently re-suspended in 3 mL cold MACS buffer (PBS, 2% FCS, 2 mM EDTA) and 250 μ L CD14+ microbeads (Miltenyi Biotech) were added. Cells were incubated at 40 °C for 20 min, 30 mL MACS buffer was added to wash and the pellet was re-suspended in 3 mL 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 40 ng/mL each of IL-4 and GM-CSF (Peprotech). Antibodies used for western blotting: anti-calpain-2, anti-BID, anti-Bcl-2, anti-Dok-2, anti-RhoGDI, anti-Erp72 and anti-importin-5 (Cell Signalling, all used at 1:1000 dilution), anti- β actin (Sigma) and secondary antibody (goat anti-rabbit/mouse, LiCor).

Dengue Virus (DENV) and Influenza infection assays

The previously reported Dengue virus strain 16681 (DENV-2) (Kinney *et al.*, 1997) was used in all experiments described in this study. Virus was propagated in C6/36 mosquito *Aedes Albopictus* cells (Igarashi, 1979) by culturing cells to approximately 80% confluency, removing media and infecting in a 4 mL volume (MOI – 0.1, R2.5 diluent) for 2 h at 28 °C (flasks were tilted every 20 min). Subsequently, 25 mL R15 culture media was added and infected cells were incubated for up to 11 days at 28 °C, culture supernatant was harvested, centrifuged at 1500 rpm for 5 min, aliquoted and stored at –80 °C until use. Viral stock titres were measured by DENV FFU assay (described below). A time-course experiment investigating optimal time-point for viral harvest was conducted, taking aliquots from Day 1 to Day 15. It was seen that 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). 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). Virus containing supernatant was plaqued to determine

the concentration of infectious virus particles and stored at –80 °C.

Focus-forming unit (FFU) assay

African green monkey kidney (Vero) cells (Naoki *et al.*, 2014) were seeded in 100 μ L M5 at a confluency of 20,000 cells per well in flat-bottom 96-well plates. Cells were grown overnight at 37 °C with 5% CO₂ in Eagle's Minimum Essential Medium (EMEM) supplemented with 10% FBS, 1% Penicillin/Streptomycin and 1% Glutamine. The following day samples were serially diluted 1:10 from 10⁻¹ to 10⁻⁶ in M2.5. Media was removed from wells and 50 μ L of diluted sample was added to each, after which plates were incubated for 2 h at 37 °C (5% CO₂). 100 μ L of overlay medium was then added to each well and plates were again incubated at 37 °C (5% CO₂) 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/avicol solution. This ensures that each virus particle that successfully infected a cell is capable of generating one focus only. To detect the number of focus forming units, cells were washed three times with 100 μ L PBS and once with fixing solution (20% paraformaldehyde diluted to 1% in PBS). A hundred microlitre 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 100 μ L of permeabilisation solution (0.1% Saponin (Sigma), 0.1% bovine serum albumin in PBS) for 10 min at room temperature. Wells were washed three times with 100 μ L PBS, and 100 μ L of anti-DENV envelope antibody (Sigma) solution (~200 ng/mL) was added to each well. Plates were incubated at 4 °C overnight. The next day plates were washed with 100 μ L PBS three times and 50 μ L HRP-conjugated secondary antibody solution (~500 ng/mL) was added for 1 h during which plates were incubated at 37 °C. Subsequently, plates were again washed three times with 100 μ L PBS and 50 μ L of 3,3'-diaminobenzidine (DAB) substrate solution (Sigma) was added to each well for 10-15 min or until visible dark spots begin to appear. The reaction was stopped by washing plates with 100 μ L/well distilled water. Staining was carried out after washing away Avicol/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.

Silencing of Calpain-2 using an siRNA pool

siRNAs were re-suspended as per manufacturer instructions (Dharmacon, Thermo Scientific). In brief, 1× siRNA re-suspension buffer was prepared by diluting Dharmacon 5× siRNA buffer into sterile RNase-free water. Tubes containing lyophilised siRNA were pulse centrifuged and 50 µL of 1× siRNA buffer was added to obtain a 100 µM 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 °C. The required volume of cell suspension was centrifuged at 1500 rpm for 5 min. Supernatant was discarded and the pellet re-suspended in Accell delivery media (Dharmacon, Thermo Fisher) and this was supplemented with 2% HI FCS and subsequently split into two 15 mL 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 1 µL/10⁶ 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 °C for 72 h. To check the level of silencing, whole cell lysate was obtained from dedicated wells and western blotting was carried out with a Calpain-2 specific antibody (Cell Signalling, 1:1000 dilution) and a secondary antibody (goat anti-rabbit/mouse, 1:1000 dilution, LiCor) used for detection. Band intensity was quantified using the LiCor imaging system (LiCor Biosciences).

Phosphoprotein enrichment experiments

Phosphoproteomics analysis using the phosphoprotein enrichment strategy was performed essentially as described (Lochmatter *et al.*, 2016). In brief, MOI used was 1 unless stated otherwise. Cells were harvested and washed in 5 mL RPMI-1640 at 4 °C before stimulation with either Mock (supernatant from non-infected C6/36 cells), DENV, IAV or LPS (10 ng/mL). For 10 min stimulation: cells were incubated in a 37 °C water bath for 2 min and then transferred to a 37 °C incubator for 8 min, for longer stimulation: cells were incubated at 37 °C in an incubator for the relevant time. All subsequent steps were carried out on ice. Cells were washed in 5 mL HEPES buffered saline (HBS) – 500mL distilled water, 4.4 g NaCl, 10 mL 1 M HEPES pH 7.4, 500 µL 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 13000 rpm for 30 min at 4 °C to remove cellular debris and supernatant was collected as whole-cell lysate. Purification was carried out as per manufacturer instruction (PhosphoProtein Purification Kit, Qiagen). In brief, whole-cell lysate protein concentration was ascertained by Bradford assay (Bio-Rad). Samples were diluted to 0.1 mg/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. Iso-electric focussing was carried out as per manufacturer instructions (3100 OFF-GEL High Resolution kit, Agilent). In brief, eluates from the phospho-purification columns were de-salted using ZEBRA spin de-salting columns and concentrated to 500 µL volumes using 7K ultra-centrifugal concentrator tubes (Pierce). All samples were adjusted to a concentration of ~100 µg. Protein OFFGEL Stock Solution was prepared to 1.25× concentration. This was used to formulate IPG Strip Rehydration Solution and to dilute protein samples to the appropriate concentration. IPG strip (pH 3-10), frames and electrodes were assembled as described and 40 µL 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, 150 µL of pre-prepared OFF GEL 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 (OFFGEL, Agilent) at a maximum current of 50 µA and maximum power of 200 mill watts. Samples collected from each of the 12 chambers and desalted again using ZEBRA columns (Pierce) prior to subsequent SDS-PAGE analysis to ensure the removal of urea, thiourea and glycerol. 20% of each sample was analysed by SDS-PAGE followed by western blotting using anti-phosphotyrosine antibody-HRP conjugate (Zymed, 1:000 dilution), followed by enhanced chemiluminescence (ECL, Amersham) and exposure to film (Amersham) using Xograph. 80% of each sample was separated by SDS-PAGE, and bands were visualised by silver staining (Thermo Fisher Scientific).

Analysis by mass spectrometry

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 liquid chromatography tandem mass spectrometry (LC-MS/MS) using an 3000 Ultimate (LC-Packings, Dionex, Amsterdam, NL) HPLC system coupled on-line to a 3D high-capacity ion trap (HCT plus, Bruker Daltonics, Bremen, Germany) mass spectrometer via a pneumatically assisted nano-electrospray source as described previously (Batycka *et al.*, 2006). MS/MS spectra (peak lists) were searched against the SwissProt database using Mascot version 2.4 (Matrix science, London, UK) and the following parameters: peptide tolerance 2.5 Da, ¹³C=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 (Abbatiello

et al., 2017). Protein ID was 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 (Lochmatter *et al.*, 2016).

RESULTS AND DISCUSSION

In order to establish an *in vitro* DENV infection model, DENV was propagated in C6/36 mosquito cells to create viral stocks. Monocyte-derived dendritic cells (moDCs) isolated from Leukocyte Cones were infected with DENV using different titres for two to five days. We observed efficient infection at the expected virus concentrations determined by a limited dilution focus forming unit (FFU) assay as described previously (Luhn *et al.*, 2007). To explore early host cell signalling events, PBMC derived DCs were either left untreated or exposed to DENV. After 10 min, cells were collected, lysed and phosphorylated proteins isolated through column-based enrichment (Figure 1), separated by SDS-PAGE and displayed by anti-phosphotyrosine immunoblotting as a representation of global changes in phosphorylation profiles. A complex pattern of tyrosine phosphorylated proteins was observed after 10 min (Figure 2A). To increase the resolution of protein phosphorylation (Lochmatter *et al.*, 2016), the phosphoproteins were, after enrichment, separated by preparative isoelectric focussing (OFFGEL) prior to SDS-PAGE analysis (Figure 2B). Interestingly, the acidic fractions (Chen *et al.*, 1997; Rodenhuis-Zybert *et al.*, 2010;) contained most proteins that were found to be differentially phosphorylated after DENV infection. This experimental approach allowed the visualisation of differentially phosphorylated proteins even by silver staining. Therefore, gel bands representing differentially abundant proteins were excised, digested and subjected to analysis by liquid chromatography tandem mass spectrometry (LC-MS/MS, Figures 1 and 2). Alternatively, isolated OFF GEL fractions were concentrated and digested in-solution prior to LC-MS/MS analysis. We identified proteins that showed differential abundance between DENV infected and control dendritic cells, indicating differential phosphorylation (Figure 2 and Table S1). LC-MS/MS analysis revealed 22 proteins from gel bands (Table 1). To test whether these changes are specific to DENV, we compared the phosphorylation status of eight proteins that showed a marked difference in abundance between DENV exposure and control. We tested their differential abundance on the total protein level as well as after phosphoprotein enrichment induced either by DENV, Influenza virus (IAV), a broader stimulus such as lipopolysaccharide (LPS) or no stimulation (Figure 3). The most striking selectivity for DENV in phosphorylation was observed for Calpain-2 and BID, a Bcl-2 protein family member. The phosphorylated fractions of Dok-2, RhoGDI, Erp72 and importin-5 were all reduced upon DENV exposure and not specific to this stimulus, and phosphorylated endoplasmic reticulum protein was found to be induced by all three stimuli (Figure 3). As Calpain-2 is

generally involved in calcium-dependent early signalling pathways and could therefore mediate an immediate host cell response to DENV exposure (Lu *et al.*, 2013), we examined the effect of Calpain-2 knockdown on the DENV infection process. Calpain-2 was knocked down to ~60% without any noticeable effect on the viability and integrity of primary DCs (Figure 4A-C). In contrast, when control and DCs with reduced levels of Calpain-2 were exposed to DENV, the infectivity and subsequent colony formation (FFU) was greatly impaired after 48 h and also up to five days (Figure 4 D, E and F). Our data indicates that DENV binding to DCs within the first few min can trigger and modulate apoptotic pathways through a calpain-2 dependent process, possibly by binding to a G-protein coupled receptor and Calcium flux (Figure 5), in a similar fashion that has been observed for HCV (Simonin *et al.*, 2009) and enterovirus 71 (Lu *et al.*, 2013).

Our study describes early signalling events upon binding of DENV to the host cell leading to fusion and internalisation. Clues for this are provided by characterising the initial receptors involved in recognising DENV particles. Putative primary protein receptors for flaviviruses in general include Fc receptors, C-type lectins including DC-SIGN (Dendritic Cell-Specific Intercellular adhesion molecule-3 (ICAM3)-Grabbing Non-integrin) (or CD209), mannose receptor, heparin sulfate or proteoglycans on monocyte-derived dendritic cells and DC-SIGNR on microvascular endothelial cells (Dalrymple *et al.*, 2014).

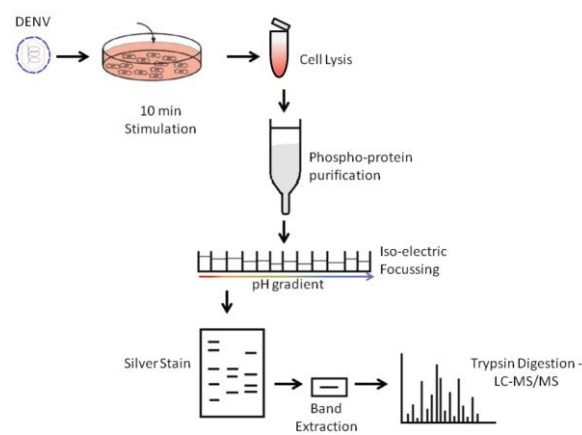


Figure 1: Experimental workflow for the discovery of phosphorylated proteins after early encounter of DCs with DENV. Dendritic cells (DCs) exposed to DENV for 10 min were lysed and phosphoproteins enriched by ion metal solid phase extraction. Enriched phosphoproteins were separated by SDS-PAGE, visualised by immunoblotting or silver staining, and differentially abundant gel bands were processed, digested, and proteins were identified by liquid chromatography tandem mass spectrometry (LC-MS/MS). Alternatively, individual OFFGEL (preparative iso-electric focussing) fractions were digested in-solution and analysed by LC-MS/MS.

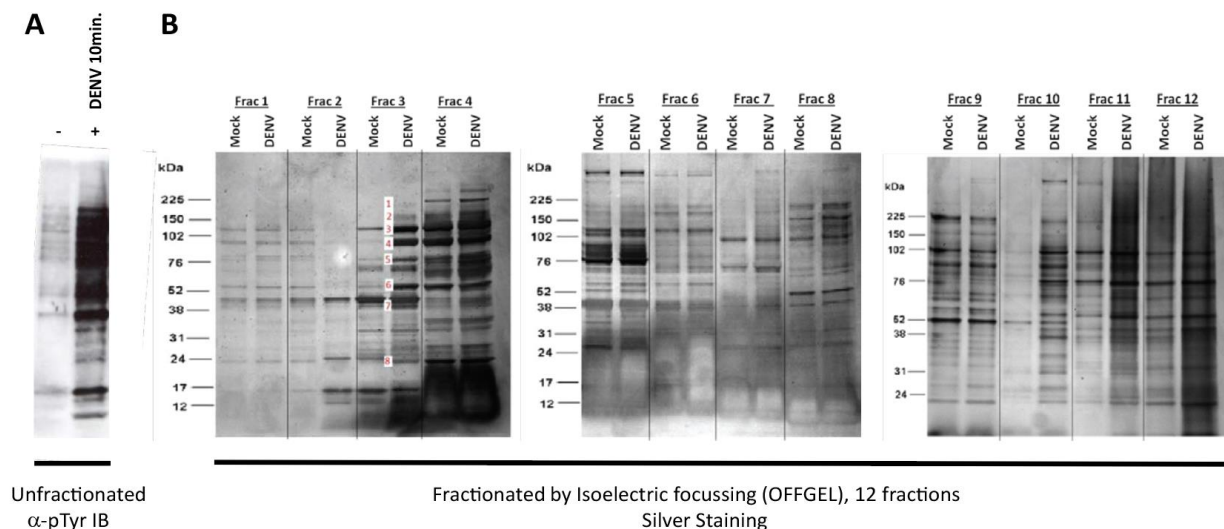


Figure 2: Altered protein phosphorylation profiles after DC exposure to DENV. (A) DENV binding to DCs induced phosphorylation that was resolved by phosphoprotein enrichment, separation by SDS-PAGE and anti-pTyr immunoblotting; (B) Alternatively, protein extracts were enriched for phosphoproteins followed by separation using isoelectric focussing, SDS-PAGE and visualised by silver staining. Bands indicated with numbers were excised and processed for analysis by tandem mass spectrometry (Table 1).

Table 1: Candidate proteins that are differentially phosphorylated upon Dengue virus (DENV) infection identified by tandem mass spectrometry using a gel-based approach. Gel band number: corresponding numbers on the gel (Figure 2) for bands 1-8; molecular weights observed on gel and expected (actual) are indicated for each protein identified; accession number (UniProtKB); protein score: Mascot Mowse probability score representing confidence of identification.

Gel band number	Molecular weight (kDa)		Protein name	Accession number	Protein score
	Gel	Actual			
1	200	104	Protein Niban	Q9BZQ8	600
2	150	125	Importin-5	O00410	558
3	120	93	Endoplasmic	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

Initial signalling after receptor attachment triggers phosphorylation events that include Raf1, CNK, KSR1, MK2 and LSP1 (Gringhuis *et al.*, 2014). Our results indicate that within the first min after binding, Calpain-2 appears to be phosphorylated to a greater extent (Figures 2 and 3) consistent with its activation through cleavage (Figure 4). Calcium-dependent activation of calpain has been reported to be associated with immune activation, and several phosphorylation sites are known, such as Ser50/Ser369/Thr370 that are altered during cell adhesion and motility changes, and Ser369/Ser370 that appear to have an inhibitory effect (Hornbeck *et al.*, 2015). Our phosphoprotein enrichment approach is not optimal for the mapping of phosphosites as there is no enrichment at the phosphopeptide level, but it increases

confidence in identifying proteins by the detection of multiple peptides (Simonin *et al.*, 2009). Despite this caveat, we detected evidence for phosphorylation at Ser67/68 after DENV exposure that is not found in the unstimulated counterpart (Figure 6). Calpain-2 is a calcium-dependent protease, thereby suggesting that an increased calcium flux may lead to phosphorylation/activation of this enzyme. In the context of DENV recognition, calcium-dependent signals are triggered possibly through interaction with a G-protein coupled receptors (GPCRs), thereby leading to calpain activation/phosphorylation. However, despite that many receptors on DCs have been associated with DENV binding, subsequently leading to entry; GPCRs have not yet been directly linked to this event.

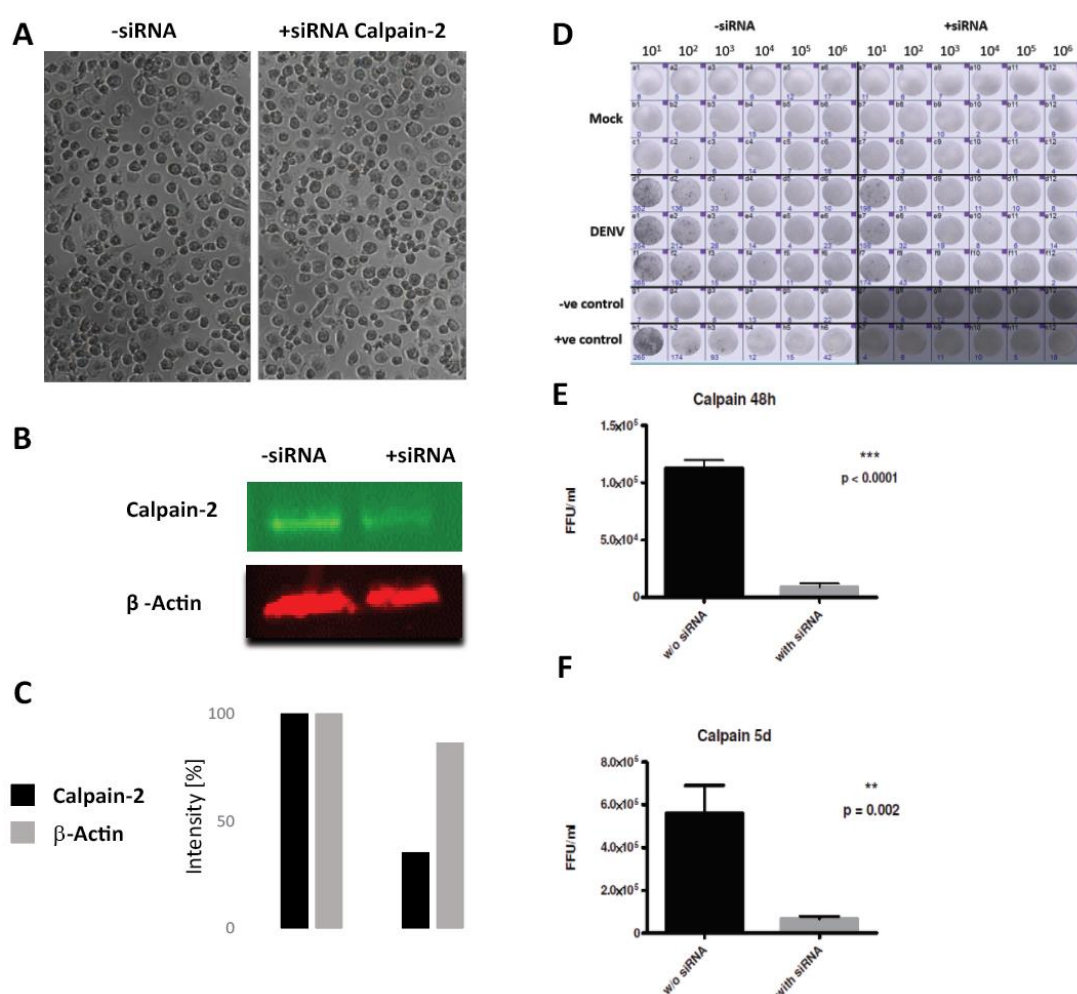


Figure 4: Altered dengue virus infection in Calpain-2 knockdown DCs. (A) Phase contrast microscope picture of DCs treated with either control (left panel) or siRNA against calpain-2 (right panel); (B) Immunoblotting of DC whole cell extracts from cells treated with control or siRNA against calpain-2 using antibodies against calpain-2 (upper panel) and β -actin (lower panel); (C) Quantitation of proteins shown in panel (B); (D) Representative FFU assay for DENV infection after 48 hours. Control and Calpain-2 knockdown cells were infected with DENV or left untreated (Mock) using different dilutions of virus; (E) Quantitation of FFU after 48 h of DENV infection of DCs previously treated with either control (black bars) or calpain-2 siRNA (grey bars); (F) Same as (E) with an infection of 5 days. The data illustrated in B-E represents one out of six independent experiments.

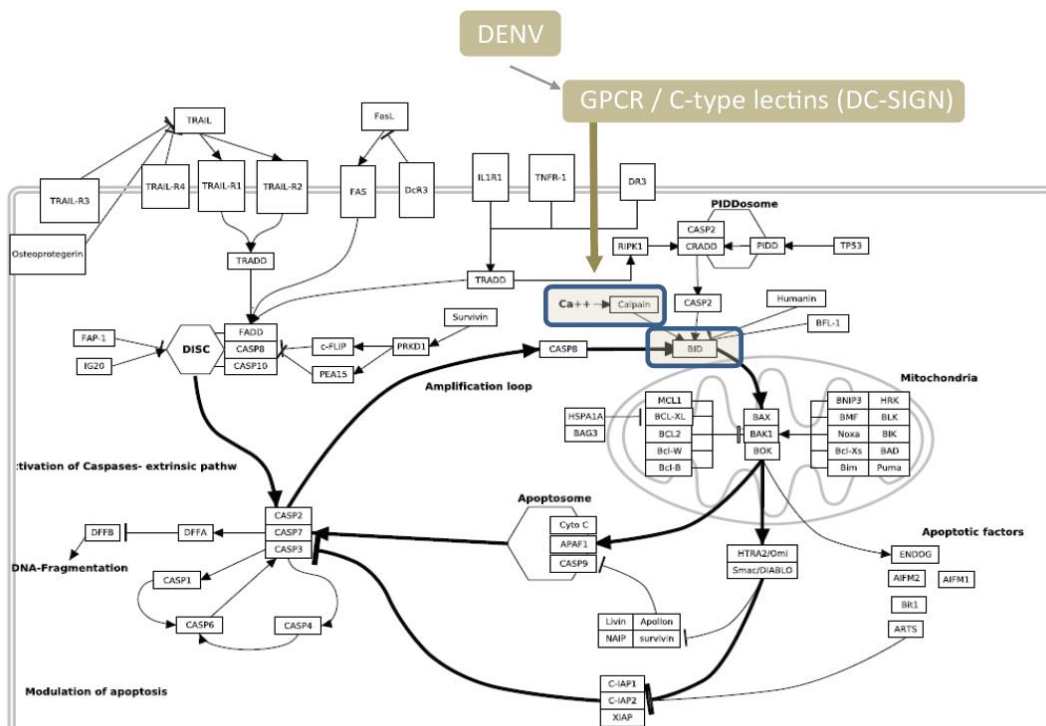


Figure 5: Pathway Analysis revealing activated apoptotic signalling networks during Dengue virus infection. Wikipedia pathway analysis of the apoptosis pathway. Relevant molecules detected in this study are highlighted in grey areas.

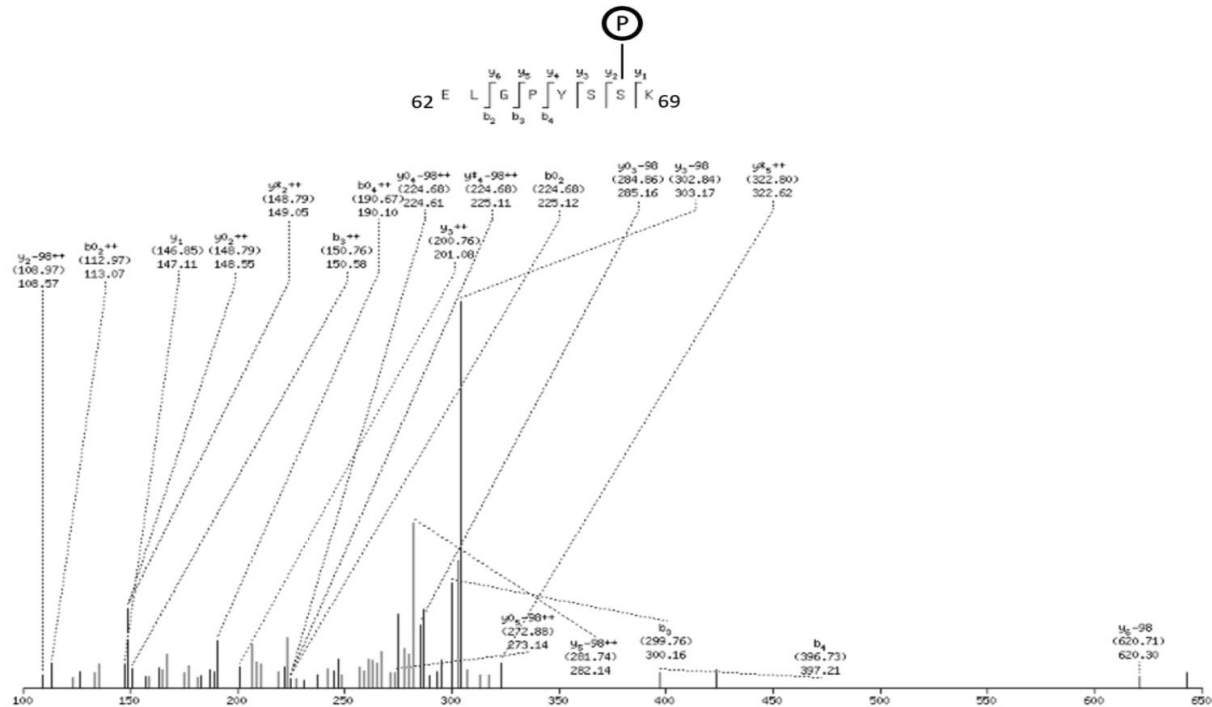


Figure 6: Phosphorylation of Calpain-2 Ser67/68 upon DENV exposure. MS/MS spectrum of the human calpain-2 (UniProt Nr P17622) derived peptide 62-69 ELGPYSSpK containing a phosphorylation at Ser68. Ions assigned as b and y fragments, observed masses (in brackets) and theoretical masses are indicated. Evidence for phosphorylation is also provided by a loss of -98Da, and the assignment is equally probable for Ser67 (data not shown).

Calpains have been previously found to be modulated during viral infection. For instance, calpain activation by hepatitis C virus proteins inhibits the extrinsic apoptotic signalling pathway (Simonin *et al.*, 2009). In addition, calcium flux and calpain-mediated activation of the apoptosis-inducing factor contribute to enterovirus 71-induced apoptosis (Lu *et al.*, 2013). In contrast, calpain 1 and 2 were found not to be required for echovirus 1 (EV1) entry, but for later stages of infection such as replication (Upla *et al.*, 2008). Also, other pathogens such as group B Streptococcus (GBS) disrupts the actin and microtubule cytoskeleton of macrophages by calpain activation (Fettucciari *et al.*, 2011). Pathway analysis reveals that the proteins calpain / bid / importin are all acting along the apoptosis pathway axis (Figure 5). 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.

CONCLUSION

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. Our results, although preliminary, describe early phosphorylation signalling events triggered upon initial host cell contact with DENV. Our data support the notion that targeting calpain proteases maybe a predicted pharmaceutical approach to interfere with infectious agents such as DENV.

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