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1 **Dengue virus infection of primary endothelial cells (EC) induces innate immune responses,**
2 **changes in EC function and is restricted by interferon stimulated responses.**

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1 **Abstract**

2 Although endothelial cell (EC) infection is not widespread during dengue virus (DENV) infection *in*
3 *vivo*, the endothelium is the site of the pathogenic effects seen in severe DENV disease. In this study
4 we investigated DENV infection of primary EC and defined factors that influence infection in this cell
5 type.

6 Consistent with *in vivo* findings where EC infection is infrequent, only 3-15% of EC became
7 productively DENV-2-infected *in vitro*. This low level infection could not be attributed to inhibition
8 by heparin, EC donor variation, heterogeneity, or biological source. DENV-infection of EC was
9 associated with induction of innate immune responses, including increased STAT1 protein, STAT1-
10 phosphorylation, and IFN- β , OAS-1, IFIT-1/ISG56 and viperin mRNA. Antibody blocking of IFN- β
11 inhibited the induction of OAS1, IFIT1/ISG56 and viperin while shRNA knockdown of viperin
12 enhanced DENV-infection in EC. DENV-infection of EC resulted in increased activity of
13 sphingosine kinase 1, a factor important in maintaining vascular integrity, and altered basal and
14 stimulated changes in barrier integrity of DENV-infected EC monolayers.

15 Thus, DENV productively infects only a small percentage of primary EC but this has a major
16 influence on induction of IFN- β driven innate immune responses that can restrict infection while the
17 EC themselves are functionally altered. These changes may have important consequences for the
18 endothelium and are reflective of pathogenic changes associated with vascular leakage, as seen in
19 DENV disease.

20

1 **Introduction**

2 Dengue virus (DENV) can infect endothelial cell lines (Avirutnan and others 1998; Basu and others
3 2011; da Conceicao and others 2013) and primary endothelial cells (EC) derived from a number of
4 different sources *in vitro* (Arevalo and others 2009; Bunyaratvej and others 1997; Dalrymple and
5 Mackow 2011; Huang and others 2000), but the significance of DENV infection of the endothelium *in*
6 *vivo* and the roles of this in DENV pathogenesis has been debated (Basu and Chaturvedi 2008;
7 Dalrymple and Mackow 2012b; 2014; Martina and others 2009; Spiropoulou and Srikiatkachorn
8 2013; Trung and Wills 2010). Mouse models have shown that DENV infection of the endothelium is
9 linked to vascular leakage (Chen and others 2007; Yen and others 2008; Zellweger and others 2010)
10 and human studies have detected the presence of DENV-infected EC in post-mortem samples from
11 infected patients (Balsitis and others 2009; Jessie and others 2004; Pova and others 2014). The low
12 number of DENV-infected EC along with the lack of overall damage to the EC observed in these
13 post-mortem samples and rapid and full recovery of many DENV patients with vascular leakage has
14 favoured a model where the vascular changes associated with severe DENV are primarily due to
15 indirect effects following infection, mediated by changes in circulating cytokines and chemokines.
16 Many studies have described low level DENV-infection of primary EC (Anderson and others 1997;
17 Arevalo and others 2009; Bunyaratvej and others 1997; Diamond and others 2000a; Huang and others
18 2000; Liu and others 2009; Warke and others 2003) or EC lines *in vitro* (Peyrefitte et al., 2006).
19 More recently, up to 90% of human umbilical vein endothelial cells (HUVEC) were DENV infected
20 when heparin, a common inclusion in culture medium following isolation of primary HUVEC, was
21 removed from culture (Dalrymple and Mackow 2011), suggesting most HUVEC are susceptible to
22 DENV but infection is inhibited by heparin. Microarray analysis has demonstrated up-regulation of a
23 number of cytokines, chemokines and interferon stimulated genes (ISGs) in DENV-infected EC
24 (Dalrymple and Mackow 2012a; Warke and others 2003). Antibody blocking studies have suggested
25 that it is the action of IFN- β that induces these ISGs and limits DENV infection (Dalrymple and
26 Mackow 2012a). Thus, the literature is conflicting on the degree of susceptibility of ECs to DENV
27 infection, but suggests that EC can be infected by DENV that induces an inflammatory response in
28 these cells with a central role for IFN- β .

1

2 In the current study we have extended this understanding of DENV infection of EC. Using primary
3 ECs derived from two different sources and multiple EC donors we demonstrate a productive DENV-
4 2 infection in only a small percentage of cells. DENV-infection of EC was accompanied by early
5 innate immune responses, including the induction of IFN- β mRNA and the ISG viperin, which acts to
6 restrict viral infection. Importantly, even in the face of only a small percentage of productively
7 DENV-infected EC, we observe a clear change in cellular protein and mRNA profiles related to the
8 host innate immune response and altered EC function. Our data suggest that EC may not be a major
9 target for DENV infection but the responses to this low level DENV infection in EC are reflective of
10 pathogenic changes in the vasculature and may have important consequences for vascular leakage and
11 DENV disease *in vivo*.

12

13 **Materials and Methods**

14 **HUVEC and ECFC.** Human umbilical cords and cord blood were collected with informed consent
15 and in line with Central Northern Adelaide Health Service human ethics approval and in accordance
16 with the 'Declaration of Helsinki'. Primary HUVEC were isolated by collagenase digestion of human
17 umbilical veins, cultured on gelatin (Sigma) coated flasks in M199 media (Sigma) supplemented with
18 20% (v/v) fetal calf serum (FCS) (Hyclone, Utah, USA), penicillin, streptomycin, 20mM HEPES,
19 1.5% (w/v) sodium pyruvate (all Gibco, MD, USA), non-essential amino acids (Sigma) and
20 endothelial cell growth supplement (BD Bioscience) (HUVEC medium) as previously described
21 (Litwin and others 1997). HUVEC were utilised in infection studies at passage 1-4.

22

23 Endothelial colony forming cells (ECFC), formerly known as late outgrowth endothelial progenitor
24 cells were isolated by CD133 magnetic bead isolation of cells from human umbilical cord blood
25 collected and processed as previously described (Appleby and others 2012). CD133- mononuclear
26 cells were then cultured in EGM-2 endothelial growth media (Lonza, Switzerland) supplemented with
27 an EGM2 bullet kit (Lonza) without heparin and 20% (v/v) FCS. Further culture, isolation of

1 colonies and passaging occurred as previously described (Ingram and others 2004). ECFC were
2 utilised at passages 4-8.

3

4 **DENV infection and plaque assay.** Cells were infected at a multiplicity (MOI) of 1 for 90 mins
5 using Mon601, a laboratory clone of the DENV type-2 (DENV-2) strain New Guinea C (Gualano and
6 others 1998). Virus stocks were produced from *in vitro* transcribed RNA that was transfected into
7 baby hamster kidney (BHK-21) cells and supernatant amplified in C6/36 insect cells. Titre of virus
8 stocks and cell culture supernatant samples was determined by plaque assay on Vero, African Green
9 monkey kidney cells. Plaques were detected by neutral red overlay and quantitated as pfu per ml. For
10 IFN- β blocking studies HUVEC were infected at an MOI of 1 then cultured *in* media supplemented
11 with control or neutralising IFN- β antibody (Pbl interferon source) at 1000 U/ml.

12

13 **RNA extraction and RT-PCR.** Total RNA was extracted from cells using Trizol (Gibco) and
14 subjected to reverse transcription using random hexamers (NEB). cDNA was subjected to real time
15 PCR as follows: DENV primers, 5.1 GCAGATCTCTGATGAATAACCAAC and 3.2:
16 TTGTCAGCTGTTGTACAGTCG; IFN- β primers, TGTC AACATGACCAACAAGTGTCT and
17 GCAAGTTGTAGCTCATGGAAAGAG; OAS-1 primers, TCCACCTGCTTCACAGAACTACA and
18 GGCGGATGAGGCTCTTGAG; IFIT-1 primers, AACTTAATGCAGGAAGAACATGACAA and
19 CTGCCAGTCTGCCCATGTG; viperin primers, GTGAGCAATGGAAGCCTGATC and
20 GCTGTCACAGGAGATAGCGAGAA. Semi-quantitative RT-PCR was performed by amplification
21 of a standard curve in parallel and results were normalised against cyclophilin quantitated using
22 primers GGCAAATGCTGGACCCAACACAAA and CTAGGCATGGGAGGGGAACAAGGAA.
23 Reactions were performed in a Corbett Rotorgene 6000 using sybergreen reaction mix (iTaq,
24 BioRad).

25

26 **Immunostaining and confocal microscopy.** Cells were grown on gelatin coated coverslips, fixed in
27 4% (w/v) paraformaldehyde and stored at 4°C. Fixed cells were permeabilised, blocked and
28 immunostained for dsRNA (goat anti-mouse antibody at 1/200 dilution, English and Scientific

1 Consulting, J2), DENV antigen (DENV patient sera at 1/5000 dilution), as described previously (Carr
2 and others 2013). Bound antibody was detected with goat-anti mouse-555 (red) or goat-anti human-
3 488 (green), nuclei were stained with Hoechst (5ug/ml, blue) and images captured by confocal
4 microscopy (Leica Sp5 Spectral Confocal Microscope).

5

6 **Crystal violet staining.** Cell were fixed and stained in 50% methanol containing 0.2% (w/v) crystal
7 violet. Unbound stain was removed and fixed cells washed extensively with water. Bound stain was
8 solubilised with 2% (w/v) SDS and absorbance at 540 nm was quantitated (Titertek Twinreader).

9

10 **SDS-PAGE and Western blotting.** Cells were lysed in passive lysis buffer (Promega) with protease
11 inhibitors (Roche CompleteMini) and protein levels quantitated (Biorad). 15ug of total protein was
12 subjected to SDS-PAGE, proteins transferred to nitrocellulose membranes and membranes serially
13 probed for STAT1-Y701 (Cell Signalling), STAT1-S727 (Cell Signalling), total STAT1 (Cell
14 Signalling) and actin (Millipore). Bound antibody complexes were detected by chemiluminescence
15 (Biorad), images captured (LAS4000, Fuji Film) and quantitated using Image J (Schneider and others
16 2012).

17

18 **shRNA production and infection of HUVEC.** Viperin and control shRNA lentivirus was made as
19 previously described (Helbig and others 2011). Following filtration of cultured media from lentivirus
20 transduced cells through a 0.45 um filter, the viral supernatants were diluted 1:3 in HUVEC media
21 (M199 media containing the supplements described above) and incubated with HUVEC for 8h.
22 Lentivirus containing supernatant was removed and replaced with fresh HUVEC media and cells
23 incubated for approximately a further 64h, prior to DENV infection, as above.

24

25 **SK1 activity assay.** Cells were lysed and SK1 activity quantitated by transfer of ³²P to sphingosine *in*
26 *vitro*, as described previously (Carr and others 2013; Pitman and others 2012). Total protein was
27 quantitated (Biorad protein assay) and results expressed as SK1 activity/ug of protein.

28

1 **EC permeability assay.** Cells were DENV or mock infected (heat inactivated virus, 80°C, 20 mins)
2 at an MOI=1, as described above, trypsinised and replated in complete HUVEC medium at 15,000
3 cells/well in 16 well E-view plates (ACEA Biosciences Inc). Cells were allowed to recover in
4 complete HUVEC media and media changed at 2-3h post plating. Plates were incubated and the cell
5 index measured by an impedance based real time cell analysis using the x-celligence system (ACEA
6 Biosciences Inc). Prior to plating, E-plates were normalised with medium alone and measurements
7 were taken every hour. In relevant experiments, at 3h pi, media was changed to M199 + 0.1% (v/v)
8 FCS, without growth factors and cells incubated overnight. At 24h pi media was changed to test
9 stimulus (i) complete HUVEC media or (ii) M199 + 0.1% (v/v) FCS + 0.5 ng/ml recombinant human
10 TNF- α (ProSpec). Cells were incubated for a further 12h and cell index measured hourly.

11

12 **Statistical analysis.** Normally distributed data sets were compared using an unpaired Student's t-
13 tests. Experiments were replicated as indicated in each figure legend. Statistical analysis was
14 performed using Microsoft Excel.

15

16 **Results**

17 **Dengue virus productively infects only a small percentage of EC including ECFC.** Recent
18 studies have shown that heparin inhibits DENV infection of HUVEC and that by 24h pi, the majority
19 of HUVEC can become DENV-antigen positive (Dalrymple and Mackow 2011). To assess this in
20 freshly isolated primary HUVEC, we cultured these cells in the presence or absence of heparin,
21 DENV-infected and measured infectious virus release by plaque assay. Results confirm increased
22 DENV-infection in the absence of heparin (Figure 1A), but still only a low percentage of the total cell
23 population was observed to be DENV-antigen positive (Table 1). DENV-infection at a higher MOI of
24 5 produced a modest increase in infectious virus release (data not shown) and HUVEC showed
25 slightly higher infected cell numbers when using an alternative DENV-2 strain, POU-312 (Table 1).
26 Overall, however the percentage of DENV-antigen positive cells was still low and in the order of 3-
27 15% (Table 1).

28

1 We considered that this low susceptibility of EC to DENV-2 infection noted above could be due to
2 donor variation, heterogeneity in the primary HUVEC population or variation in EC susceptibility due
3 to the location in the body they are isolated from. To address this we next DENV-infected primary
4 HUVEC, endothelial colony forming cells (ECFC) and Vero cells, a control DENV-susceptible cell
5 type and quantitated DENV-infection by plaque assay. ECFC represent a second novel source of
6 primary EC and are derived clonally from a single circulating EC progenitor. Both HUVEC and
7 ECFC demonstrated productive DENV-infection, comparable to Vero cells (Figure 1B). DENV-
8 infection was highest early (days 24-72h post infection, pi) and persisted but declined with time. No
9 cytopathic effect (CPE) was associated with DENV-infection of primary HUVEC or ECFC cultures,
10 determined visually or as quantitated by crystal violet vital dye staining (Figure 1C). In contrast,
11 DENV-infection of Vero cells produced infectious DENV (Figure 1B) but by day 3 pi, a clear visual
12 DENV-induced CPE was observed with a reduction in vital dye staining (Figure 1C). The level of
13 DENV-2 infectious virus release was comparable in cell preparations from different EC donors,
14 although some donor variation was observed (Figure 1B, D). DENV-infected cultures from multiple
15 donors supported active DENV-2 replication, as measured by high DENV titres and the presence of
16 DENV-2 RNA by RT-PCR at day 2 pi (Figure 1D). Additionally, dual DENV-2 antigen and dsRNA
17 positive EC were observed by immunostaining and fluorescence microscopy (Figure 1E). The
18 DENV-infection was restricted to a small number of cells and did not spread throughout the entire
19 culture (Figure 1E). The number of DENV-infected cells was quantitated by cell counting from
20 multiple EC donors and observed to be in the range of 3-15% (Table 1), much lower than the routine
21 infection of the majority of Vero cells under the same conditions (data not shown). Thus, primary EC
22 from 2 different sources and multiple donors are susceptible to a low level productive DENV-2
23 infection *in vitro*.

24

25 **DENV infection of EC induces innate immune responses that restrict DENV-2 infection.** To
26 assess the ability of DENV-2 to initiate an innate immune response in EC we investigated the
27 phosphorylation status of STAT1, a key molecule in IFN signalling. Results demonstrate a clear time
28 and MOI dependent induction of STAT1-Y701 and S727 phosphorylation in DENV-infected EC from

1 24h pi and still elevated at 72h pi (Figure 2). Interestingly, we also observed an increase in total
2 STAT1 protein (Figure 2). We next assessed the up-regulation of IFN- β mRNA and a number of
3 known anti-viral effector ISGs (IFIT-1/ISG56, OAS-1, and viperin) in response to DENV-2 infection
4 of EC by RT-PCR. DENV infection was confirmed by the detection of DENV RNA, which increased
5 from 24-72h pi (Figure 3A) and was detected in all cases and in multiple EC donors at 48h pi (Figure
6 3B). The levels of IFN- β , IFIT-1/ISG56, OAS-1, and viperin mRNAs were undetectable or negligible
7 in mock-infected cells (Figure 3). IFN- β was induced in DENV-infected cells at 24h pi and still
8 present at 72h pi (Figure 3A). Levels of IFN- β mRNA, were elevated at 48h pi in all cases and in
9 multiple EC donors (Figure 3B). In contrast, mRNA for IFIT1/ISG56, OAS-1 and viperin were all
10 induced in DENV-infected cells at 24h pi, further elevated at 48h pi but declining by 72h pi (Figure
11 3A). Again elevated mRNA levels at 48h pi were observed in all cases and in multiple EC donors
12 (Figure 3B). These results indicate that a robust innate antiviral response is initiated in EC cells
13 following DENV infection.

14
15 Cells were next infected with DENV-2 and incubated with a neutralising antibody against IFN- β . At
16 48h pi cells were fixed for immunostaining and RNA extracted for RT-PCR. At 48h pi DENV-2
17 infection was enhanced in HUVEC incubated with antibody against IFN- β , as measured by
18 production of DENV-2 RNA by RT-PCR (Fig 4A) although production of infectious virus was not
19 significantly different (Fig 4B). Consistent with this, staining for dsRNA was visualised in increased
20 numbers of HUVEC in cells treated with the IFN- β antibody but the majority of cells still remained
21 uninfected (Fig 4C). Treatment of cells with a neutralising antibody against IFN- β also reduced the
22 level of mRNA for OAS-1, IFIT1/ISG56 and viperin but not IFN- β itself (Fig 4D). Immunostaining
23 of fixed cells for viperin and STAT1-S727 unfortunately was not successful and thus we could not
24 formally demonstrate if the induction of this response was in DENV-2-infected or uninfected
25 bystander cells.

26
27 We have previously reported that viperin has anti-viral actions against DENV (Helbig and others
28 2013) and thus we next investigated the specific role of viperin in restricting DENV infection by

1 shRNA-mediated knockdown of viperin in EC. HUVEC were transduced with a viperin shRNA
2 lentivirus that has been previously demonstrated to reduce the induction of viperin via IFN- α
3 stimulation by 90-95% (Helbig and others 2011), a control lentivirus or mock transduced. Cells were
4 allowed to recover and 3 days later challenged with DENV-2. A reduction in viperin mRNA levels
5 was achieved in viperin shRNA compared with control lentivirus transduced cells at day 2 and 3 post
6 lentivirus transduction (supplementary Fig 1). Following DENV-2 infection there was a modest
7 although significant increase in production of infectious virus from viperin shRNA cells at 48h pi
8 (Figure 5A). Immunostaining for dsRNA and quantitation by visual cell counting demonstrated an
9 increased number of infected cells in viperin shRNA cultures, although the majority of EC still
10 remained uninfected (Figure 5B, Table 2). This suggests that viperin can inhibit, but does not
11 completely restrict DENV-2 infection of EC.

12

13 **DENV infection of EC induces sphingosine kinase-1 activity and alters EC barrier integrity.**

14 Our prior studies have identified sphingosine kinase-1 (SK-1) as a vital molecule for EC function such
15 as survival and differentiation (Bonder and others 2009; Gamble and others 2009) that we know is
16 also altered during DENV infection (Carr and others 2013; Wati and others 2011). We thus
17 quantified SK1 activity in DENV-infected EC lysates. At 24h pi, SK1 activity was significantly
18 increased in DENV-2 infected EC, however, by 48h pi, SK1 activity had normalised and showed no
19 difference between mock and DENV-2 infected EC (Figure 6).

20

21 The induction of components of the innate immune response (Figure 3) and changes in SK1 activity
22 (Figure 6) suggest the potential for functional changes in DENV-2 infected EC and we therefore next
23 assessed the barrier integrity of DENV-2 infected EC monolayers. HUVEC were mock or DENV-2
24 infected, then plated in a chamber slide at a density we previously defined to form a monolayer. The
25 cell index, which reflects changes in cell number or function was quantified by real time measurement
26 of electrical impedance using the x-celligence system. Cell monolayers were viewed by microscopy
27 using the E-view plate system to confirm cell viability and confluence. Results were normalised in
28 each experiment against mock infected controls and demonstrate changes in cell index or barrier

1 integrity with time (Figure 7). The initial barrier integrity of DENV-infected EC appears lower at 1h
2 post infection, although this is not significant. The spike at 2h pi reflects a transient change in barrier
3 integrity due to removal of the chamber slide from the incubator to change the media. A significant
4 reduction in cell index, reflecting a decrease in the integrity of the EC barrier, was observed in
5 DENV-infected EC that subsequently recovered to normal within the first 24h of infection. This
6 effect was observed with either EC cultured in complete HUVEC media (Figure 7A) or in low serum
7 media (0.1% [v/v] FCS; Figure 7B). Consistent with our prior observations (Figure 1C), in multiple
8 infection experiments from different EC donors there was no evidence of a visual DENV-associated
9 CPE.

10

11 EC responses to vasoactive stimuli were also assessed at 24h pi. EC were DENV-2 infected and
12 plated in x-celligence chambers, allowed to recover for 3h in complete HUVEC media, then serum
13 starved overnight (0.1% FCS, no growth factors). Cultures were then treated with TNF- α or re-fed
14 complete HUVEC media. Notably, at 24h pi (time 0 for addition of TNF- α or complete media) the
15 barrier integrity of DENV-EC appears reduced, although this is not significant. At 6-7h post addition
16 of TNF- α , a greater reduction in EC barrier integrity was observed in DENV-2 compared to mock
17 infected EC (Figure 8A). In contrast, re-stimulation of EC with 20% FCS/growth factors following
18 serum starvation, maintained the barrier integrity of the mock-infected EC monolayer for up to 8h, but
19 that of DENV-infected EC declined (Figure 8B). Together these results suggest time-dependent
20 changes in SK-1 activity and functional changes in both basal and growth factor stimulated EC barrier
21 integrity.

22

23 **Discussion**

24 Emerging evidence suggests an important role of the endothelium for innate immune responses during
25 viral infections that could also be a potential therapeutic target to prevent tissue and vascular damage
26 (Baccala and others 2014; Oldstone 2013; Safronetz and others 2014; Teijaro and others 2011). The
27 role of the endothelium during DENV-infection is not well defined, however the endothelium is a

1 clear target for the pathogenesis of disease. While the degree to which the endothelium contributes to
2 DENV replication is controversial there are well described functional changes in the endothelium that
3 are linked to severe DENV disease, (Basu and Chaturvedi 2008; Dalrymple and Mackow 2012b;
4 2014). Most studies in primary EC and EC lines demonstrate a low level of EC susceptibility to
5 productive DENV-infection with anywhere from 2-30% of EC becoming infected within 1-7 days of
6 infection (Andrews and others 1978; Arevalo and others 2009; Bunyaratvej and others 1997;
7 Diamond and others 2000a; Huang and others 2000; Liu and others 2009; Peyrefitte and others 2006;
8 Vervaeke and others 2013; Warke and others 2003; Zamudio-Meza and others 2009). DENV is
9 reported to utilise heparin sulphate proteoglycans (HSPG) for entry into a variety of cell types
10 (Artpradit and others 2013; Chen and others 1997; Germi and others 2002; Hilgard and Stockert 2000;
11 Lin and others 2002) including EC (Dalrymple and Mackow 2011; Vervaeke and others 2013) and in
12 the study by Dalrymple and Mackow, DENV-4 infection of EC at MOI of 6 in the absence of heparin
13 resulted in up to 90% of HUVEC DENV-antigen positive at day 1 pi, as assessed by
14 immunohistochemistry (Dalrymple and Mackow 2011). Similarly, in our study, exclusion of heparin
15 from the cell culture medium increased by 0.5-1.5 log production of infectious virus from DENV-2
16 infected HUVEC. However, in our experiments, at best only around 15% of the EC population
17 became DENV antigen and dsRNA positive. The study of Vervaeke *et al.*, 2013 utilised primary
18 dermal microvascular EC or microvascular EC lines infected with DENV-2 (NGC) and achieved
19 infection of 10-50% of cells in the absence of heparin that was dependent on MOI (Vervaeke and
20 others 2013), while approximately 40% of commercially acquired HUVEC were reported to become
21 DENV-2 (16681) infected at day 3-7 pi in the absence of heparin (Raekiansyah and others 2014).
22 Clearly, heparin inhibits DENV-infection of EC and the discrepancies between studies in the actual
23 level of EC susceptibility to DENV-infection may be due to technical factors such as EC senescence
24 (AbuBakar and others 2014), the DENV strain, including potential adaptation to utilise heparin
25 (Artpradit and others 2013; Celis and others 2014; Lee and others 2006) or the presence of DENV
26 NS1 in the inoculum which can bind to glycosaminoglycans (GAGs) and heparin (Avirutnan and
27 others 2007) and may competitively inhibit DENV infection of EC.

28

1 Regardless, EC are susceptible to DENV infection and in our infection system DENV-2 does not
2 spread to infect the majority of cells in culture when using EC from multiple donors, representing two
3 different biological sources of EC and including the novel ECFC type, derived from EC progenitors.
4
5 We confirmed the induction of IFN- β mRNA in DENV-infected EC as previously reported (da
6 Conceicao and others 2013; Dalrymple and Mackow 2012a; Warke and others 2003). Additionally,
7 we report the induction of OAS-1, IFIT1/ISG56, and viperin mRNA as well as an increased level of
8 both total STAT1 protein and STAT1-S727 and Y701 phosphorylation in DENV-2 infected EC. The
9 observation that total STAT1 protein is increased at 72h pi in DENV-2 infected EC is interesting and
10 may in part account for the increase seen in STAT1 phosphoprotein. Recent work has demonstrated
11 that a second wave of induction of ISGs is generated through a prolonged production of IFN- β , which
12 could drive an increase in unphosphorylated forms of STAT1, STAT2 and IRF9 (Cheon and others
13 2013). Similarly, the production of IFN- β , for which we observed mRNA out to at least 72h pi, may
14 be responsible for the increased total STAT1 in our study. As expected, IFN- β blocking experiments
15 reduced the RNA levels of OAS-1, IFIT1/ISG56 and viperin demonstrating that induction of these
16 genes is dependent on the action of IFN- β . Blocking of IFN- β enhanced DENV RNA production and
17 number of dsRNA containing cells, but not the amount of infectious virus in cell culture supernatants.
18 This suggests that when IFN- β actions are blocked after infection, production of virus from existing
19 infected cells is unaltered, as expected (Diamond and others 2000b) but additional cells become
20 DENV susceptible and new rounds of infection are increased. These results strongly imply an IFN- β
21 dependent induction of responses and restriction of infection in bystander cells. Unfortunately,
22 immunostaining for endogenous viperin or STAT-1-S727 in HUVEC could not be achieved to
23 conclusively demonstrate this. Additionally, lentivirus transduction of EC with shRNA against the
24 ISG viperin also increased DENV infection.. This result is similar to our described anti-viral role for
25 viperin against DENV-2 infection in macrophages, another important target cell type for DENV
26 infection and pathogenesis, and where similarly a low percentage of cells are DENV-infected which is
27 associated with induction of viperin, particularly in uninfected bystander cells (Helbig and others
28 2013). Notably, transduction of viperin shRNA into EC in our study, or blocking with a neutralising

1 IFN- β antibody did not result in a complete take-over of the EC culture by the DENV. We
2 demonstrated lower levels of viperin mRNA in viperin shRNA cells prior to and at the time of
3 DENV-infection, have previously validated our viperin shRNA to yield 95% knockdown of IFN-
4 stimulated viperin in Huh7 cells (Helbig and others 2011) and our prior work has shown a high
5 lentivirus transduction rate of EC (Barrett and others 2011). Our results herein, however, suggest that
6 the viperin shRNA knockdown and blocking of IFN- β activity may be incomplete or perhaps
7 overwhelmed by the strong induction of innate responses by DENV infection of EC. Additionally,
8 there are likely to be other anti-viral factors that are IFN- β -independent or unaffected by viperin
9 shRNA that can still act to restrict infection in these cells, as suggested by the large number of ISGs
10 that are known to target positive strand RNA viruses in other cell types (Schoggins and others 2014).
11 Although DENV-infection of EC does not occur in a major fraction of the cell population, the
12 infection clearly still has a significant effect on EC function. We demonstrate an increase in SK1
13 activity at 24h pi, a factor that we have previously shown to be an essential regulator of EC survival,
14 growth and differentiation (Bonder and others 2009; Gamble and others 2009). The increase in SK1
15 activity in DENV-infected EC may reflect a viral-induced response to prevent pre-mature death of
16 newly infected cells, as has been suggested for the increased SK1 seen early following RSV and
17 HCMV infection *in vitro* (Machesky and others 2008; Monick and others 2004). Additionally, SK1
18 has been proposed to play a role in influencing the JAK/STAT pathway following IFN signalling to
19 activate ISGs (Seo and others 2011). The detected increase in SK1 activity early in DENV infection
20 differs to our prior work showing reduced SK1 activity late in DENV-infected cells which is mediated
21 via high levels of the DENV 3'UTR of the genome (Carr and others 2013; Wati and others 2011).
22 Thus, the increase in SK1 seen early in DENV-infected HUVEC is mechanistically different to the
23 later decrease in SK1 activity and likely to be driven by anti-viral host responses rather than viral
24 replication per se.

25

26 Based on our prior work with SK1 and EC, we would expect that elevated levels of SK1 activity
27 would promote EC barrier integrity (Li and others 2008) and sensitise EC to stimulation with
28 inflammatory cytokines (Limaye and others 2009). We assessed this by quantifying real time changes

1 in EC barrier integrity *in vitro*. Our data demonstrate significantly different changes in EC barrier
2 integrity in mock and DENV-2 infected EC. Barrier integrity of DENV-2-infected EC was decreased
3 in the early hours following infection, was more pronounced in EC grown in the absence of growth
4 factor supplementation but normalised again by 24h pi. This suggests an acute response following the
5 insult of a viral infection where the integrity of the EC barrier is compromised. The increased SK1
6 activity we detected at 24h pi may subsequently restore normal vascular barrier function.
7 Additionally, we observed that DENV-infected EC have altered responsiveness to TNF- α and growth
8 factor stimuli, as previously reported (Cardier and others 2005; Carr and others 2003; Dewi and others
9 2004; 2008) and consistent with elevated SK1 activity at 24h pi sensitising cells to the actions of
10 TNF- α (Limaye and others 2009). Some prior studies have suggested that DENV-infected EC do not
11 show altered permeability or responses to stimuli such as TNF- α *in vitro* (Liu and others 2009;
12 Raekiansyah and others 2014; Talavera and others 2004). These studies, however, utilised single time
13 points at later times pi for analysis of barrier integrity (eg. 7 days pi), and thus may have missed the
14 transient early changes in EC function identified in our study. The changes in DENV-EC barrier
15 function we have identified *in vitro* are likely to have functional implications for the endothelium
16 during DENV infection *in vivo*, in particular for relevant biological stimuli, such as TNF- α , that are
17 known to be elevated in the circulation of DENV infected patients.

18

19 In conclusion, we have presented data demonstrating that DENV-2 can productively infect EC, but
20 infection is restricted and associated with a strong induction of the innate immune response and
21 functional changes in EC. These results support the growing evidence for an important role of
22 DENV-infected EC, not as a major site of viral replication, but as a contributor to the inflammatory
23 response and functional changes in the endothelium that may be associated with severe DENV-
24 disease *in vivo*.

25

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8

9 **Author Disclosure Statement.**

10 No competing financial interests exist.

11

12 **Figure Legends**

13 **Figure 1. DENV-2 productively infects EC.** HUVEC or ECFC were isolated and mock or DENV-2
14 infected at an MOI=1. **A.** At 24h prior to infection cells from 3 independent cord donors were plated
15 in media with or without heparin, DENV-2 infected and cultured \pm heparin. Culture supernatant was
16 collected, clarified and infectious virus release quantitated by plaque assay. Results represent mean \pm
17 SD from duplicate infections from each donor. * = $p < 0.05$, Students unpaired t-test. **B.** At the
18 indicated time point pi, culture supernatant was harvested and infectious virus release determined, as
19 in A. Results represent mean \pm SD from duplicate infections from each donor. * = $p < 0.05$, Students
20 unpaired t-test. **C.** At 72h pi, cells were fixed and stained with crystal violet and A540nm
21 quantitated. Results represent mean \pm SEM (n=5). * = $p < 0.05$, Students unpaired t-test. **D.** At 48h
22 pi, supernatant was collected and infectious virus release determined as in A (left panel). Cells were
23 lysed and total DENV RNA quantitated by RT-PCR (right panel). Results represent single
24 measurements from individual donors (n=15, pfu/ml) and (n=10, DENV RNA). **E.** At 48h pi cells
25 were fixed and immunostained for dsRNA (red) and DENV antigen (Ag, green). Nuclei were stained
26 with Hoechst (blue) and images captured by confocal microscopy. Images are representative,
27 showing very few DENV positive cells in each field and co-staining for dsRNA and DENV Ag.

28

1 **Figure 2. DENV-2 infection of EC induces STAT1 phosphorylation and total STAT1 protein.**

2 **A.** EC were mock or DENV-2 infected at an MOI = 1 or 5 and at 24 and 72h pi, cells were lysed and
3 subjected to SDS-PAGE and western blot. Filters were serially probed for STAT1-Y701, S727, total
4 STAT1 and actin. Bound proteins were detected by chemiluminescence and images captured. Images
5 show results from two independent EC donors and infections. **B.** Images from EC1 were quantitated
6 using ImageJ and normalised against actin.

7

8 **Figure 3. DENV-2 infection of EC induces innate immune responses.** EC were mock or DENV-2

9 infected, RNA extracted and subjected to RT-PCR. Results were normalised against cyclophilin. **A.**
10 Time course for production of DENV RNA and induction of IFN- β , viperin, IFIT1/ISG56 and OAS-
11 1. Results represent duplicate infections from a single donor. **B.** RT-PCR results from duplicate
12 infections in multiple donors (n=4-5).

13

14 **Figure 4. Blocking of IFN- β actions increases DENV-2 infection and reduces mRNAs for ISGs.**

15 HUVEC were DENV-2 infected and incubated with control or 1000 U/ml of a neutralising antibody
16 against IFN- β . At 48h pi cells were lysed and RNA extracted or cells fixed and immunostained. **A.**
17 DENV-2 RNA by RT-PCR; **B.** infectious virus release by plaque assay; **C.** dsRNA (green), nuclei
18 (blue) were stained with Hoechst and images captured by confocal microscopy (i) mock infected, (ii)
19 control and (iii) IFN- β antibody; **D.** IFN- β , OAS-1, viperin and IFIT1/ISG56 mRNA detected by RT-
20 PCR. PCR results were normalised against cyclophilin and are expressed relative to no control
21 antibody treatment and are representative of duplicate infections from n=2 different HUVEC donors.

22

23 **Figure 5. Viperin shRNA transduction enhances DENV-2 infection of EC.** HUVEC were mock

24 transduced or transduced with a control or viperin shRNA lentivirus. At 72h post transduction, EC
25 were DENV-2 infected and at 48h pi **A.** supernatant collected and analysed for infectious virus
26 release by plaque assay. Results represent mean \pm SD from duplicate infections from 3 different
27 donors and lentivirus transduction experiments. *= p<0.05, Student's unpaired t-test. **B.** cells were

1 fixed and immunostained for dsRNA (green). Nuclei were stained with Hoechst (blue) and images
2 captured by confocal microscopy.

3

4 **Figure 6. SK1 activity is increased early following DENV-2 infection of EC.** EC were mock or
5 DENV-2 infected and at the indicated time point pi, cells were lysed and SK1 activity quantitated.
6 Results were normalised against total protein and represent mean \pm SEM from duplicate results from
7 3 independent donor and infection experiments. *= $p < 0.05$, Student's unpaired t-test.

8

9 **Figure 7. DENV-2 infection of EC decreases basal barrier integrity in the early hours following**
10 **infection.** EC were mock or DENV-2 infected, replated in E-view plates and incubated in the x-
11 celligence with hourly measurements of barrier integrity. Cells were allowed to recover for 2h, media
12 changed and incubation continued for a further 22h in either **A.** complete HUVEC media or **B.** 0.1%
13 (v/v) serum containing media. Results were normalised against mock-infected control cells and
14 represent mean \pm SEM of n=2-4 measurements at each time point from n=4 independent donors and
15 infection experiments. *= $p < 0.05$, Student's unpaired t-test.

16

17 **Figure 8. DENV-2 infection of EC sensitises EC to stimulation.** EC were mock or DENV-2
18 infected, plated in E-view plates and allowed to recover for 3h. Media was changed to 0.1% (v/v)
19 serum media and cells incubated overnight. Media was then replaced with **A.** M199 + 0.1% (v/v)
20 FCS + 0.5 ng/ml recombinant human TNF- α or **B.** complete HUVEC media (20% [v/v] serum +
21 endothelial cell growth supplements) and incubated for a further 12h. Results were normalised
22 against mock at the time of addition of TNF- α or serum (t=0) and represent mean \pm SEM of n=6
23 measurements at each time point from two independent donor and infection experiments. *= $p < 0.05$,
24 Student's unpaired t-test.

25

26 **References**

27 AbuBakar S, Shu MH, Johari J, Wong PF. 2014. Senescence affects endothelial cells susceptibility to
28 dengue virus infection. Int J Med Sci 11(6):538-44.

- 1 Anderson R, Wang S, Osiowy C, Issekutz AC. 1997. Activation of endothelial cells via antibody-
2 enhanced dengue virus infection of peripheral blood monocytes. *Journal of Virology*
3 71(6):4226-32.
- 4 Andrews BS, Theofilopoulos AN, Peters CJ, Loskutoff DJ, Brandt WE, Dixon FJ. 1978. Replication of
5 dengue and junin viruses in cultured rabbit and human endothelial cells. *Infect Immun*
6 20(3):776-81.
- 7 Appleby SL, Cockshell MP, Pippal JB, Thompson EJ, Barrett JM, Tooley K, Sen S, Sun WY, Grose R,
8 Nicholson I, Levina V, Cooke I, Talbo G, Lopez AF, Bonder CS. 2012. Characterization of a
9 distinct population of circulating human non-adherent endothelial forming cells and their
10 recruitment via intercellular adhesion molecule-3. *PLoS One* 7(11):e46996.
- 11 Arevalo MT, Simpson-Haidaris PJ, Kou Z, Schlesinger JJ, Jin X. 2009. Primary human endothelial cells
12 support direct but not antibody-dependent enhancement of dengue viral infection. *J Med*
13 *Virol* 81(3):519-28.
- 14 Artpradit C, Robinson LN, Gavrillov BK, Rurak TT, Ruchirawat M, Sasisekharan R. 2013. Recognition of
15 heparan sulfate by clinical strains of dengue virus serotype 1 using recombinant subviral
16 particles. *Virus Res* 176(1-2):69-77.
- 17 Avirutnan P, Malasit P, Seliger B, Bhakdi S, Husmann M. 1998. Dengue virus infection of human
18 endothelial cells leads to chemokine production, complement activation, and apoptosis.
19 *Journal of Immunology* 161(11):6338-46.
- 20 Avirutnan P, Zhang L, Punyadee N, Manuyakorn A, Puttikhunt C, Kasinrerak W, Malasit P, Atkinson JP,
21 Diamond MS. 2007. Secreted NS1 of dengue virus attaches to the surface of cells via
22 interactions with heparan sulfate and chondroitin sulfate E. *PLoS Pathog* 3(11):e183.
- 23 Baccala R, Welch MJ, Gonzalez-Quintal R, Walsh KB, Teijaro JR, Nguyen A, Ng CT, Sullivan BM,
24 Zarpellon A, Ruggeri ZM, de la Torre JC, Theofilopoulos AN, Oldstone MB. 2014. Type I
25 interferon is a therapeutic target for virus-induced lethal vascular damage. *Proc Natl Acad*
26 *Sci U S A* 111(24):8925-30.
- 27 Balsitis SJ, Coloma J, Castro G, Alava A, Flores D, McKerrow JH, Beatty PR, Harris E. 2009. Tropism of
28 dengue virus in mice and humans defined by viral nonstructural protein 3-specific
29 immunostaining. *Am J Trop Med Hyg* 80(3):416-24.
- 30 Barrett JM, Parham KA, Pippal JB, Cockshell MP, Moretti PA, Brice SL, Pitson SM, Bonder CS. 2011.
31 Over-expression of sphingosine kinase-1 enhances a progenitor phenotype in human
32 endothelial cells. *Microcirculation* 18(7):583-97.
- 33 Basu A, Chaturvedi UC. 2008. Vascular endothelium: the battlefield of dengue viruses. *FEMS*
34 *Immunol Med Microbiol* 53(3):287-99.
- 35 Basu A, Jain P, Sarkar P, Gangodkar S, Deshpande D, Ganti K, Shetty S, Ghosh K. 2011. Dengue virus
36 infection of SK Hep1 cells: inhibition of in vitro angiogenesis and altered cytomorphology by
37 expressed viral envelope glycoprotein. *FEMS Immunol Med Microbiol* 62(2):140-7.
- 38 Bonder CS, Sun WY, Matthews T, Cassano C, Li X, Ramshaw HS, Pitson SM, Lopez AF, Coates PT, Proia
39 RL, Vadas MA, Gamble JR. 2009. Sphingosine kinase regulates the rate of endothelial
40 progenitor cell differentiation. *Blood* 113(9):2108-17.
- 41 Bunyaratvej A, Butthep P, Yoksan S, Bhamarapravati N. 1997. Dengue viruses induce cell
42 proliferation and morphological changes of endothelial cells. *Southeast Asian Journal of*
43 *Tropical Medicine & Public Health* 28 Suppl 3:32-7.
- 44 Cardier JE, Marino E, Romano E, Taylor P, Liprandi F, Bosch N, Rothman AL. 2005. Proinflammatory
45 factors present in sera from patients with acute dengue infection induce activation and
46 apoptosis of human microvascular endothelial cells: possible role of TNF-alpha in endothelial
47 cell damage in dengue. *Cytokine* 30(6):359-65.
- 48 Carr JM, Hocking H, Bunting K, Wright PJ, Davidson A, Gamble J, Burrell CJ, Li P. 2003. Supernatants
49 from dengue virus type-2 infected macrophages induce permeability changes in endothelial
50 cell monolayers. *J Med Virol* 69(4):521-8.

- 1 Carr JM, Kua T, Clarke JN, Calvert JK, Zebol JR, Beard MR, Pitson SM. 2013. Reduced sphingosine
2 kinase 1 activity in dengue virus type-2 infected cells can be mediated by the 3' untranslated
3 region of dengue virus type-2 RNA. *J Gen Virol* 94(Pt 11):2437-48.
- 4 Celis A, Moros Z, Gerder M, Pagano F, Vizzi E, Liprandi F. 2014. [Selection of heparin-sensitive
5 dengue virus variants in BHK-21 cells]. *Invest Clin* 55(2):155-67.
- 6 Chen HC, Hofman FM, Kung JT, Lin YD, Wu-Hsieh BA. 2007. Both virus and tumor necrosis factor
7 alpha are critical for endothelium damage in a mouse model of dengue virus-induced
8 hemorrhage. *J Virol* 81(11):5518-26.
- 9 Chen Y, Maguire T, Hileman RE, Fromm JR, Esko JD, Linhardt RJ, Marks RM. 1997. Dengue virus
10 infectivity depends on envelope protein binding to target cell heparan sulfate. *Nat Med*
11 3(8):866-71.
- 12 Cheon H, Holvey-Bates EG, Schoggins JW, Forster S, Hertzog P, Imanaka N, Rice CM, Jackson MW,
13 Junk DJ, Stark GR. 2013. IFNbeta-dependent increases in STAT1, STAT2, and IRF9 mediate
14 resistance to viruses and DNA damage. *EMBO J* 32(20):2751-63.
- 15 da Conceicao TM, Rust NM, Berbel AC, Martins NB, do Nascimento Santos CA, Da Poian AT, de
16 Arruda LB. 2013. Essential role of RIG-I in the activation of endothelial cells by dengue virus.
17 *Virology* 435(2):281-92.
- 18 Dalrymple N, Mackow ER. 2011. Productive dengue virus infection of human endothelial cells is
19 directed by heparan sulfate-containing proteoglycan receptors. *J Virol* 85(18):9478-85.
- 20 Dalrymple NA, Mackow ER. 2012a. Endothelial cells elicit immune-enhancing responses to dengue
21 virus infection. *J Virol* 86(12):6408-15.
- 22 Dalrymple NA, Mackow ER. 2012b. Roles for endothelial cells in dengue virus infection. *Adv Virol*
23 2012:840654.
- 24 Dalrymple NA, Mackow ER. 2014. Virus interactions with endothelial cell receptors: implications for
25 viral pathogenesis. *Curr Opin Virol* 7C:134-140.
- 26 Dewi BE, Takasaki T, Kurane I. 2004. In vitro assessment of human endothelial cell permeability:
27 effects of inflammatory cytokines and dengue virus infection. *J Virol Methods* 121(2):171-80.
- 28 Dewi BE, Takasaki T, Kurane I. 2008. Peripheral blood mononuclear cells increase the permeability of
29 dengue virus-infected endothelial cells in association with downregulation of vascular
30 endothelial cadherin. *J Gen Virol* 89(Pt 3):642-52.
- 31 Diamond MS, Edgil D, Roberts TG, Lu B, Harris E. 2000a. Infection of human cells by dengue virus is
32 modulated by different cell types and viral strains. *Journal of Virology*. 74(17):7814-23.
- 33 Diamond MS, Roberts TG, Edgil D, Lu B, Ernst J, Harris E. 2000b. Modulation of Dengue virus
34 infection in human cells by alpha, beta, and gamma interferons. *Journal of Virology*
35 74(11):4957-66.
- 36 Gamble JR, Sun WY, Li X, Hahn CN, Pitson SM, Vadas MA, Bonder CS. 2009. Sphingosine kinase-1
37 associates with integrin $\alpha\beta_3$ to mediate endothelial cell survival. *Am J Pathol*
38 175(5):2217-25.
- 39 Germi R, Crance JM, Garin D, Guimet J, Lortat-Jacob H, Ruigrok RW, Zarski JP, Drouet E. 2002.
40 Heparan sulfate-mediated binding of infectious dengue virus type 2 and yellow fever virus.
41 *Virology* 292(1):162-8.
- 42 Gualano RC, Pryor MJ, Cauchi MR, Wright PJ, Davidson AD. 1998. Identification of a major
43 determinant of mouse neurovirulence of dengue virus type 2 using stably cloned genomic-
44 length cDNA. *Journal of General Virology* 79(Pt 3):437-46.
- 45 Helbig KJ, Carr JM, Calvert JK, Wati S, Clarke JN, Eyre NS, Narayana SK, Fiches GN, McCartney EM,
46 Beard MR. 2013. Viperin is induced following dengue virus type-2 (DENV-2) infection and has
47 anti-viral actions requiring the C-terminal end of viperin. *PLoS Negl Trop Dis* 7(4):e2178.
- 48 Helbig KJ, Eyre NS, Yip E, Narayana S, Li K, Fiches G, McCartney EM, Jangra RK, Lemon SM, Beard MR.
49 2011. The antiviral protein viperin inhibits hepatitis C virus replication via interaction with
50 nonstructural protein 5A. *Hepatology* 54(5):1506-17.

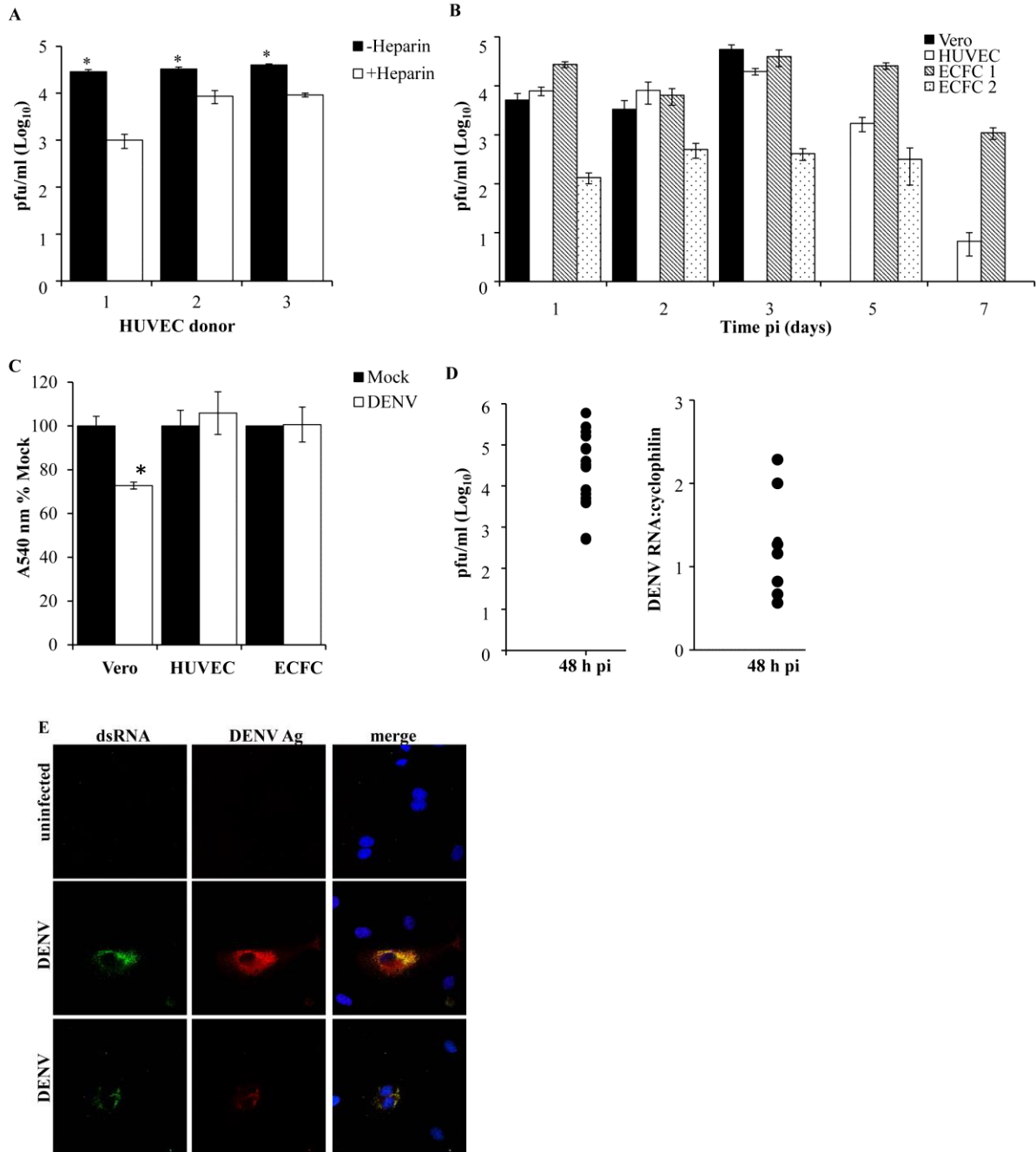
- 1 Hilgard P, Stockert R. 2000. Heparan sulfate proteoglycans initiate dengue virus infection of
2 hepatocytes. *Hepatology* 32(5):1069-77.
- 3 Huang YH, Lei HY, Liu HS, Lin YS, Liu CC, Yeh TM. 2000. Dengue virus infects human endothelial cells
4 and induces IL-6 and IL-8 production. *American Journal of Tropical Medicine & Hygiene* 63(1-
5 2):71-5.
- 6 Ingram DA, Mead LE, Tanaka H, Meade V, Fenoglio A, Mortell K, Pollok K, Ferkowicz MJ, Gilley D,
7 Yoder MC. 2004. Identification of a novel hierarchy of endothelial progenitor cells using
8 human peripheral and umbilical cord blood. *Blood* 104(9):2752-60.
- 9 Jessie K, Fong MY, Devi S, Lam SK, Wong KT. 2004. Localization of dengue virus in naturally infected
10 human tissues, by immunohistochemistry and in situ hybridization. *J Infect Dis* 189(8):1411-
11 8.
- 12 Lee E, Wright PJ, Davidson A, Lobigs M. 2006. Virulence attenuation of Dengue virus due to
13 augmented glycosaminoglycan-binding affinity and restriction in extraneural dissemination. *J*
14 *Gen Virol* 87(Pt 10):2791-801.
- 15 Li X, Stankovic M, Bonder CS, Hahn CN, Parsons M, Pitson SM, Xia P, Proia RL, Vadas MA, Gamble JR.
16 2008. Basal and angiopoietin-1-mediated endothelial permeability is regulated by
17 sphingosine kinase-1. *Blood* 111(7):3489-97.
- 18 Limaye V, Xia P, Hahn C, Smith M, Vadas MA, Pitson SM, Gamble JR. 2009. Chronic increases in
19 sphingosine kinase-1 activity induce a pro-inflammatory, pro-angiogenic phenotype in
20 endothelial cells. *Cell Mol Biol Lett* 14(3):424-41.
- 21 Lin JC, Lin SC, Chen WY, Yen YT, Lai CW, Tao MH, Lin YL, Miaw SC, Wu-Hsieh BA. 2014. Dengue viral
22 protease interaction with NF-kappaB inhibitor alpha/beta results in endothelial cell
23 apoptosis and hemorrhage development. *J Immunol* 193(3):1258-67.
- 24 Lin YL, Lei HY, Lin YS, Yeh TM, Chen SH, Liu HS. 2002. Heparin inhibits dengue-2 virus infection of five
25 human liver cell lines. *Antiviral Res* 56(1):93-6.
- 26 Litwin M, Clark K, Noack L, Furze J, Berndt M, Albelda S, Vadas M, Gamble J. 1997. Novel cytokine-
27 independent induction of endothelial adhesion molecules regulated by platelet/endothelial
28 cell adhesion molecule (CD31). *J Cell Biol* 139(1):219-28.
- 29 Liu P, Woda M, Ennis FA, Libraty DH. 2009. Dengue virus infection differentially regulates endothelial
30 barrier function over time through type I interferon effects. *J Infect Dis* 200(2):191-201.
- 31 Machesky NJ, Zhang G, Raghavan B, Zimmerman P, Kelly SL, Merrill AH, Jr., Waldman WJ, Van
32 Brocklyn JR, Trgovcich J. 2008. Human cytomegalovirus regulates bioactive sphingolipids. *J*
33 *Biol Chem* 283(38):26148-60.
- 34 Martina BE, Koraka P, Osterhaus AD. 2009. Dengue virus pathogenesis: an integrated view. *Clin*
35 *Microbiol Rev* 22(4):564-81.
- 36 Monick MM, Cameron K, Powers LS, Butler NS, McCoy D, Mallampalli RK, Hunninghake GW. 2004.
37 Sphingosine kinase mediates activation of extracellular signal-related kinase and Akt by
38 respiratory syncytial virus. *American Journal of Respiratory Cell and Molecular Biology*
39 30(6):844-852.
- 40 Oldstone MB. 2013. Lessons learned and concepts formed from study of the pathogenesis of the two
41 negative-strand viruses lymphocytic choriomeningitis and influenza. *Proc Natl Acad Sci U S A*
42 110(11):4180-3.
- 43 Peyrefitte CN, Pastorino B, Grau GE, Lou J, Tolou H, Couissinier-Paris P. 2006. Dengue virus infection
44 of human microvascular endothelial cells from different vascular beds promotes both
45 common and specific functional changes. *J Med Virol* 78(2):229-42.
- 46 Pitman MR, Pham DH, Pitson SM. 2012. Isoform-selective assays for sphingosine kinase activity.
47 *Methods Mol Biol* 874:21-31.
- 48 Pova TF, Alves AM, Oliveira CA, Nuovo GJ, Chagas VL, Paes MV. 2014. The pathology of severe
49 dengue in multiple organs of human fatal cases: histopathology, ultrastructure and virus
50 replication. *PLoS One* 9(4):e83386.

- 1 Raekiansyah M, Espada-Murao LA, Okamoto K, Kubo T, Morita K. 2014. Dengue virus neither directly
2 mediates hyperpermeability nor enhances tumor necrosis factor-alpha-induced permeability
3 in vitro. *Jpn J Infect Dis* 67(2):86-94.
- 4 Safronetz D, Prescott J, Feldmann F, Haddock E, Rosenke R, Okumura A, Brining D, Dahlstrom E,
5 Porcella SF, Ebihara H, Scott DP, Hjelle B, Feldmann H. 2014. Pathophysiology of hantavirus
6 pulmonary syndrome in rhesus macaques. *Proc Natl Acad Sci U S A* 111(19):7114-9.
- 7 Schneider CA, Rasband WS, Eliceiri KW. 2012. NIH Image to ImageJ: 25 years of image analysis. *Nat*
8 *Methods* 9(7):671-5.
- 9 Schoggins JW, MacDuff DA, Imanaka N, Gainey MD, Shrestha B, Eitson JL, Mar KB, Richardson RB,
10 Ratushny AV, Litvak V, Dabelic R, Manicassamy B, Aitchison JD, Aderem A, Elliott RM, Garcia-
11 Sastre A, Racaniello V, Snijder EJ, Yokoyama WM, Diamond MS, Virgin HW, Rice CM. 2014.
12 Pan-viral specificity of IFN-induced genes reveals new roles for cGAS in innate immunity.
13 *Nature* 505(7485):691-5.
- 14 Seo YJ, Alexander S, Hahm B. 2011. Does cytokine signaling link sphingolipid metabolism to host
15 defense and immunity against virus infections? *Cytokine Growth Factor Rev* 22(1):55-61.
- 16 Spiropoulou CF, Srikiatkachorn A. 2013. The role of endothelial activation in dengue hemorrhagic
17 fever and hantavirus pulmonary syndrome. *Virulence* 4(6):525-36.
- 18 Talavera D, Castillo AM, Dominguez MC, Gutierrez AE, Meza I. 2004. IL8 release, tight junction and
19 cytoskeleton dynamic reorganization conducive to permeability increase are induced by
20 dengue virus infection of microvascular endothelial monolayers. *J Gen Virol* 85(Pt 7):1801-
21 13.
- 22 Teijaro JR, Walsh KB, Cahalan S, Fremgen DM, Roberts E, Scott F, Martinborough E, Peach R,
23 Oldstone MB, Rosen H. 2011. Endothelial Cells Are Central Orchestrators of Cytokine
24 Amplification during Influenza Virus Infection. *Cell* 146(6):980-91.
- 25 Trung DT, Wills B. 2010. Systemic vascular leakage associated with dengue infections - the clinical
26 perspective. *Curr Top Microbiol Immunol* 338:57-66.
- 27 Vervaeke P, Alen M, Noppen S, Schols D, Oreste P, Liekens S. 2013. Sulfated Escherichia coli K5
28 polysaccharide derivatives inhibit dengue virus infection of human microvascular endothelial
29 cells by interacting with the viral envelope protein E domain III. *PLoS One* 8(8):e74035.
- 30 Warke RV, Xhaja K, Martin KJ, Fournier MF, Shaw SK, Brizuela N, de Bosch N, Lapointe D, Ennis FA,
31 Rothman AL, Bosch I. 2003. Dengue virus induces novel changes in gene expression of
32 human umbilical vein endothelial cells. *J Virol* 77(21):11822-32.
- 33 Wati S, Rawlinson SM, Ivanov RA, Dorstyn L, Beard MR, Jans DA, Pitson SM, Burrell CJ, Li P, Carr JM.
34 2011. Tumour necrosis factor alpha (TNF-alpha) stimulation of cells with established dengue
35 virus type 2 infection induces cell death that is accompanied by a reduced ability of TNF-
36 alpha to activate nuclear factor kappaB and reduced sphingosine kinase-1 activity. *J Gen*
37 *Virol* 92(Pt 4):807-18.
- 38 Yen YT, Chen HC, Lin YD, Shieh CC, Wu-Hsieh BA. 2008. Enhancement by tumor necrosis factor alpha
39 of dengue virus-induced endothelial cell production of reactive nitrogen and oxygen species
40 is key to hemorrhage development. *J Virol* 82(24):12312-24.
- 41 Zamudio-Meza H, Castillo-Alvarez A, Gonzalez-Bonilla C, Meza I. 2009. Cross-talk between Rac1 and
42 Cdc42 GTPases regulates formation of filopodia required for dengue virus type-2 entry into
43 HMEC-1 cells. *J Gen Virol* 90(Pt 12):2902-11.
- 44 Zellweger RM, Prestwood TR, Shresta S. 2010. Enhanced infection of liver sinusoidal endothelial cells
45 in a mouse model of antibody-induced severe dengue disease. *Cell Host Microbe* 7(2):128-
46 39.

47

48

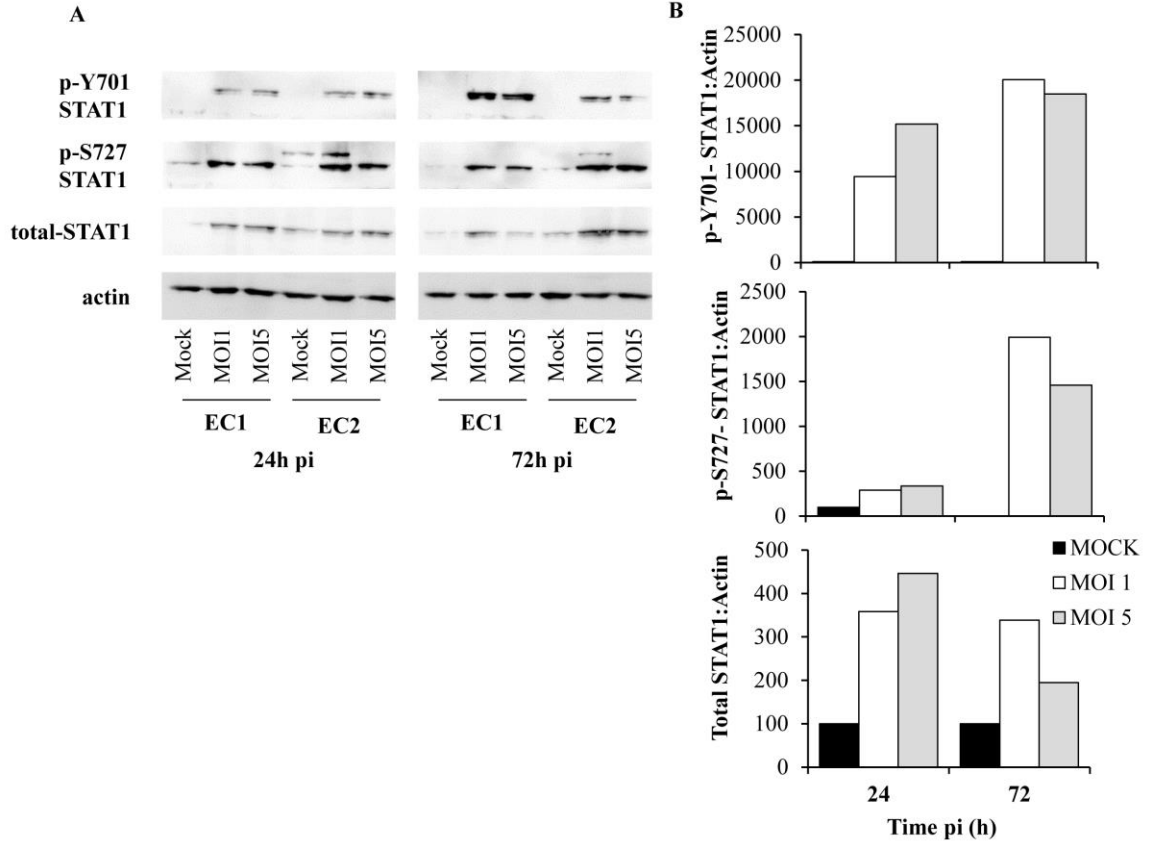
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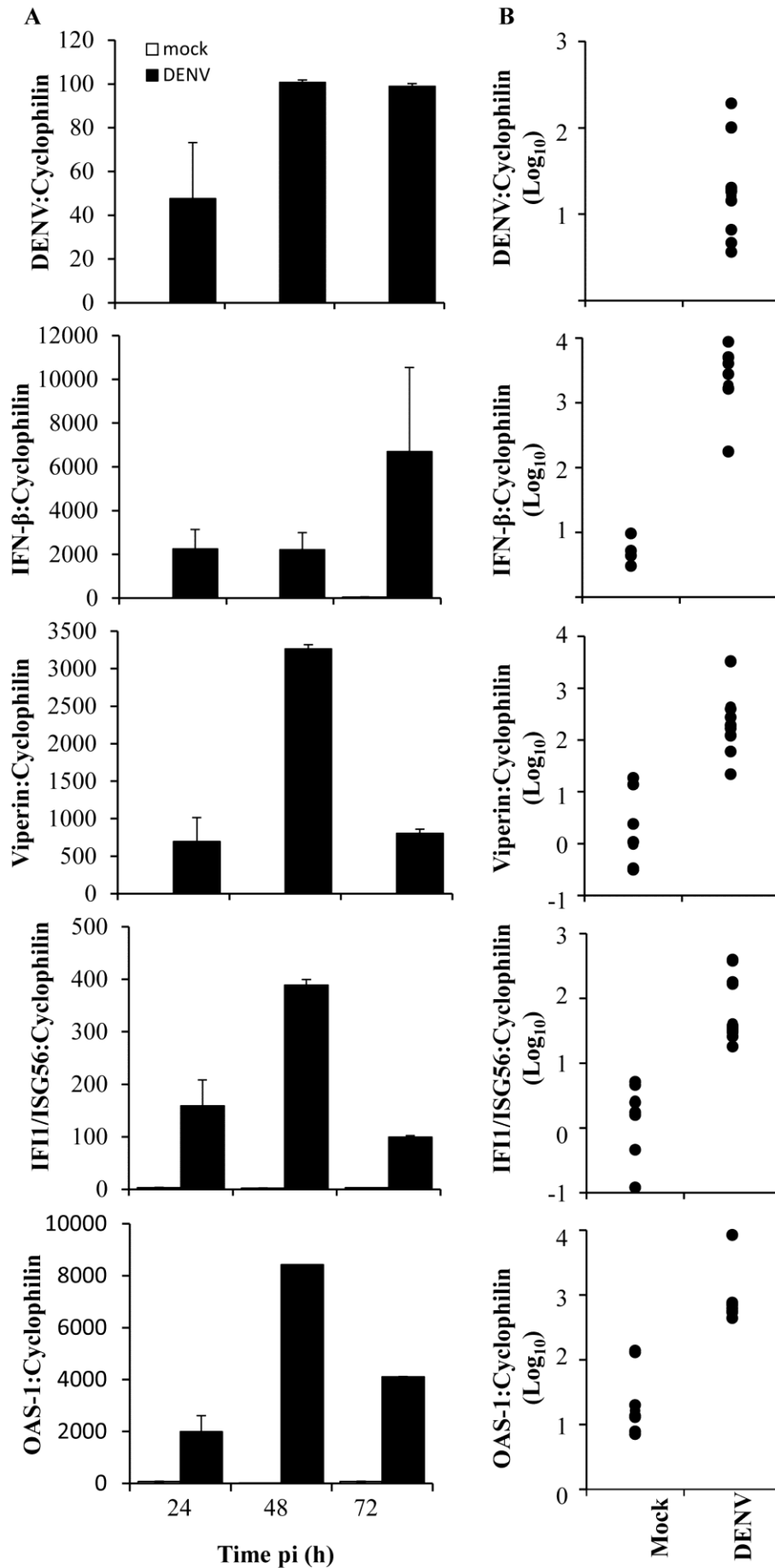
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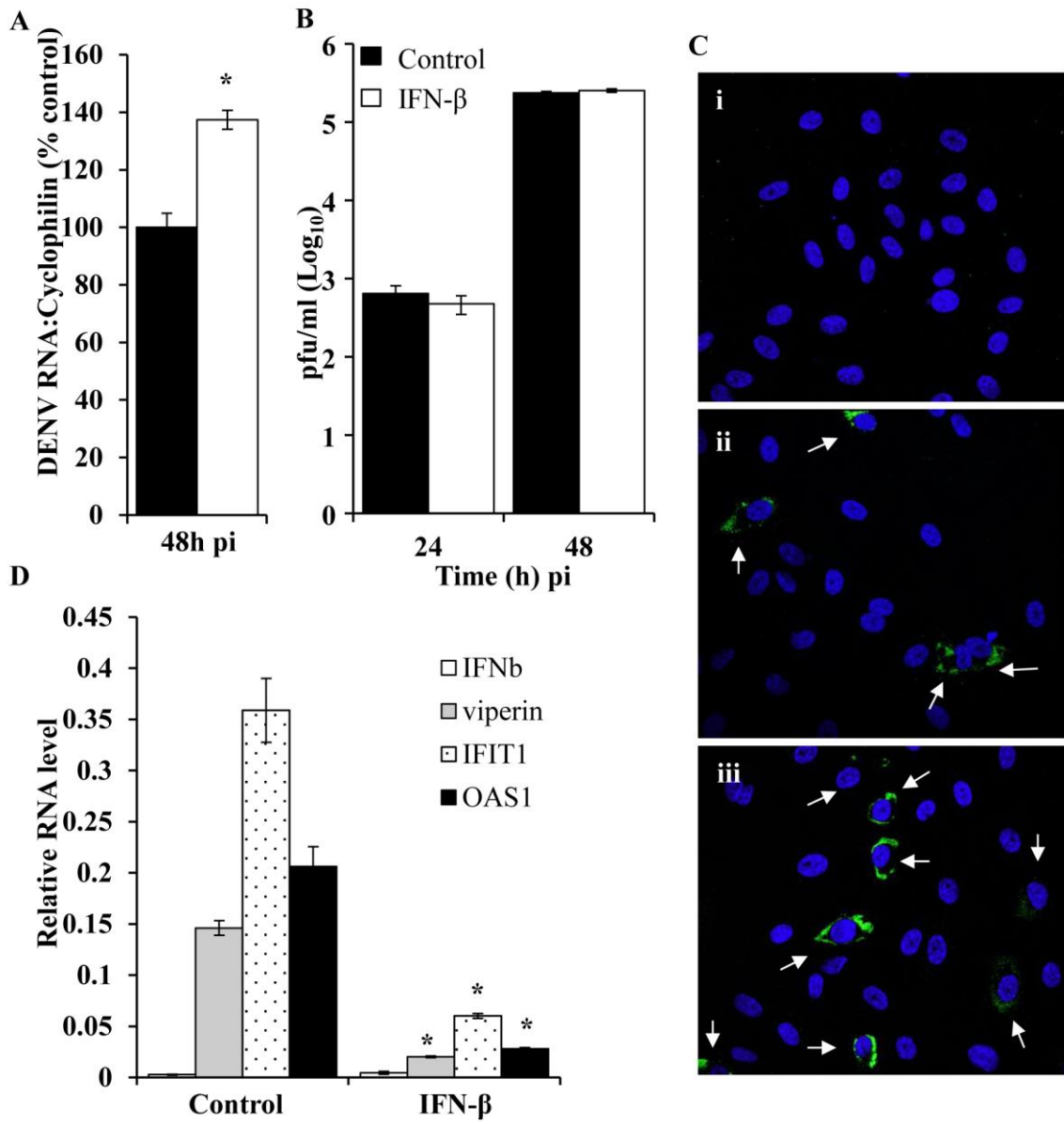
Figure 1



1 **Figure 2**

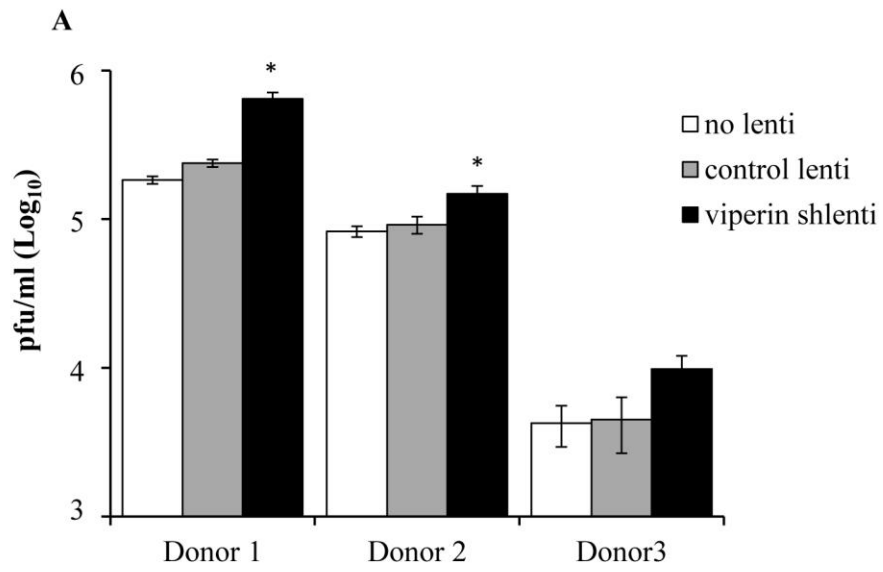


1 **Figure 3**

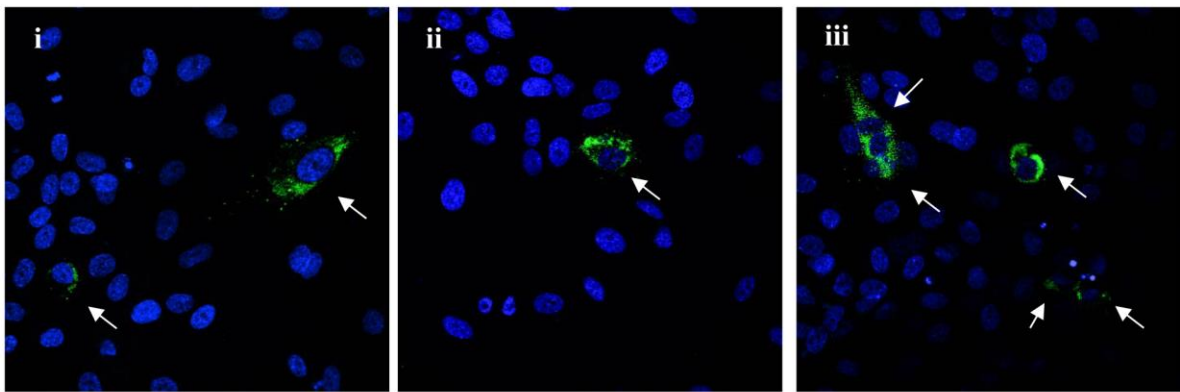


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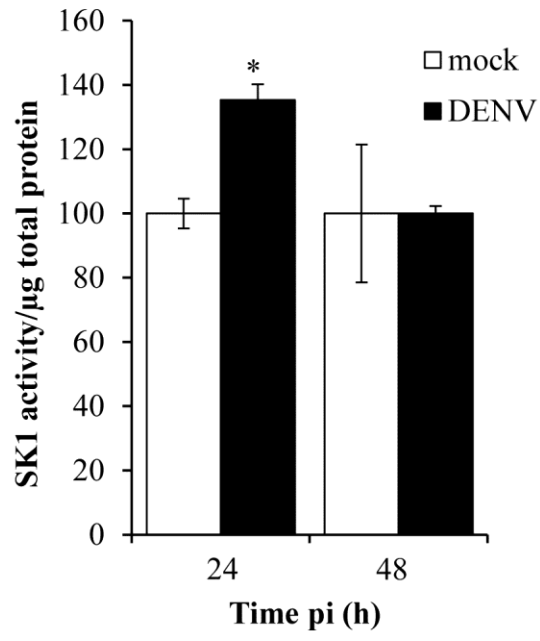
2 **Figure 4**



B

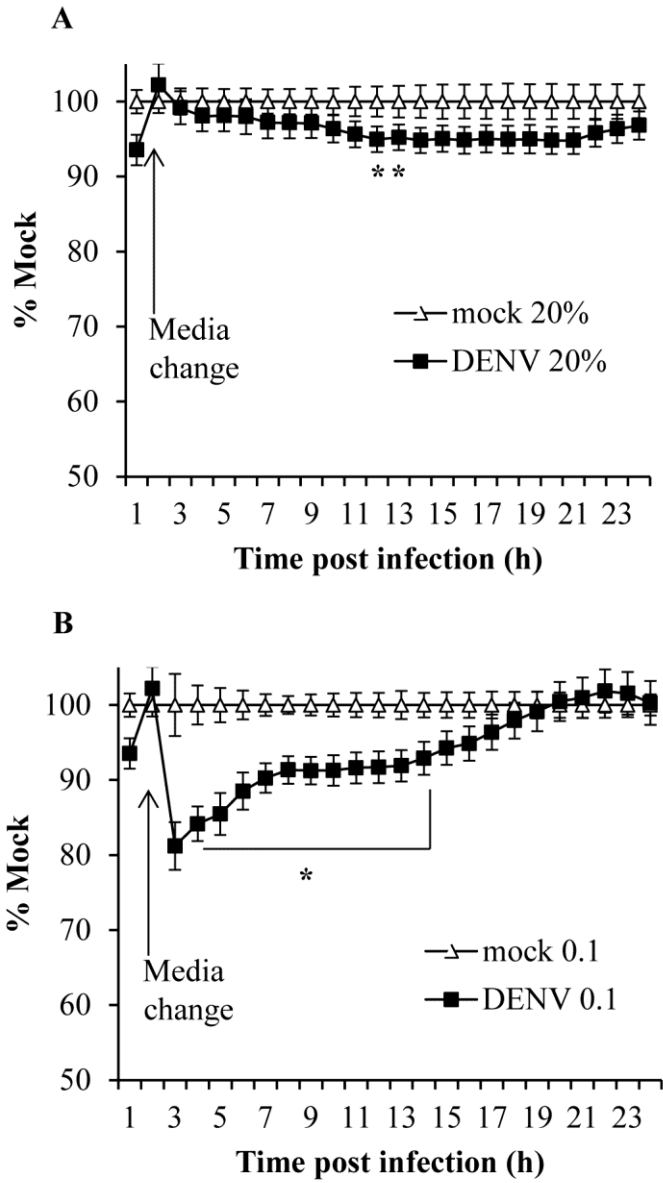


1 **Figure 5**



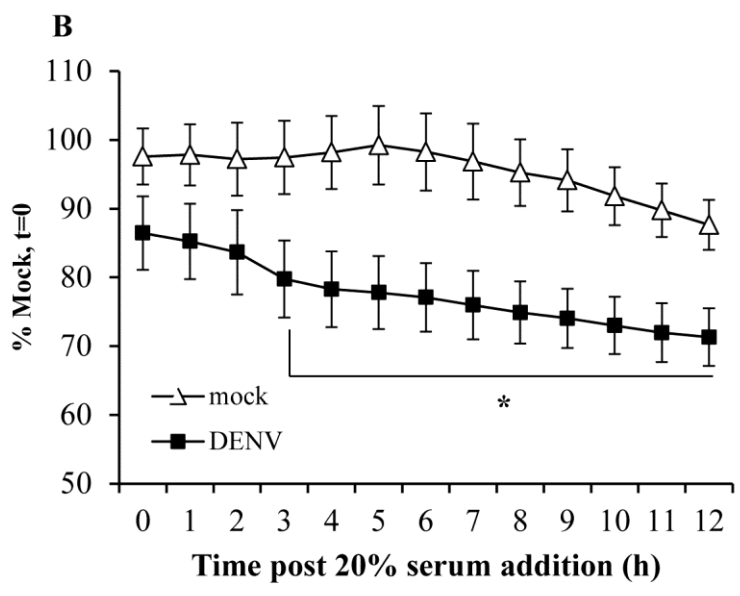
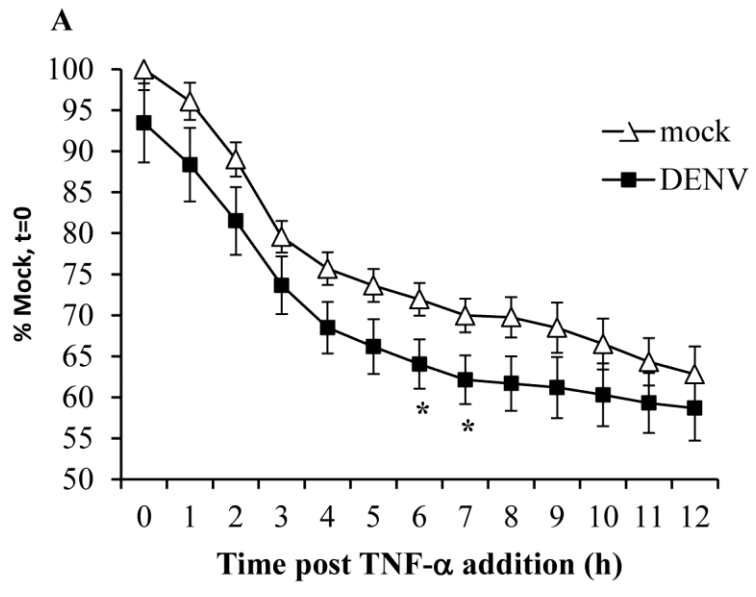
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2 **Figure 6**



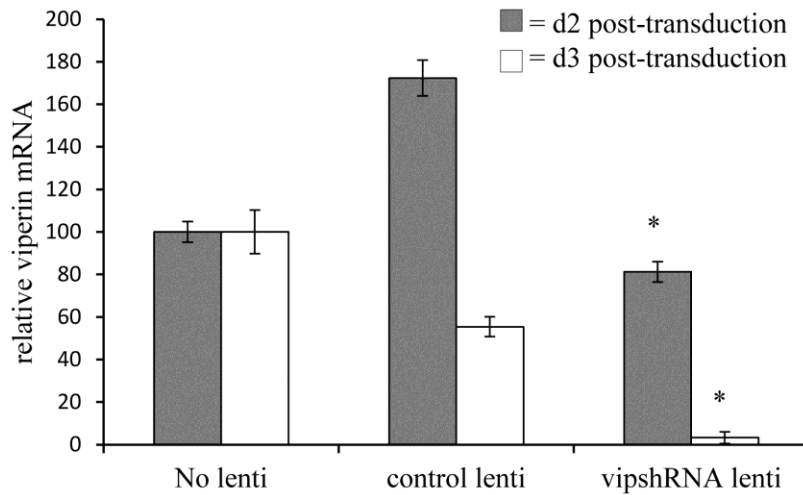
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2 **Figure 7**



1

2 **Figure 8**



Sup Fig 1. viperin shRNA transduction reduces viperin mRNA levels. HUVEC were isolated and transduced under mock conditions (no lentivirus), a control lentivirus or viperin shRNA lentivirus. RNA was extracted from cells at day 2 post transduction (n=1, HUVEC donor) or day 3 post transduction (ie day of DENV infection) (n=1 HUVEC donor) and subjected to RT-PCR for viperin. Results were normalised against cyclophilin and expressed as a % of the no lenti control. * = $p < 0.05$, Students t-test compared to control lentivirus transduced cells. Results show that control lentivirus transduction induces viperin at day 2 post-transduction which declines by day 3 post-transduction. At both time points, viperin RNA levels are significantly lower in the viperin shRNA transduced cells.

1