Reduction in sphingosine kinase 1 influences the susceptibility to dengue virus infection by altering antiviral responses

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Sphingosine kinase (SK) 1 is a host kinase that enhances some viral infections. Here we investigated the ability of SK1 to modulate dengue virus (DENV) infection in vitro. Overexpression of SK1 did not alter DENV infection; however, targeting SK1 through chemical inhibition resulted in reduced DENV RNA and infectious virus release. DENV infection of SK1−/− murine embryonic fibroblasts (MEFs) resulted in inhibition of infection in an immortalized line (iMEF) but enhanced infection in primary MEFs (18 MEFs). Global cellular gene expression profiles showed expected innate immune mRNA changes in DENV-infected WT but no induction of these responses in SK1−/−iMEFs. Reverse transcription PCR demonstrated a low-level induction of IFN-α and poor induction of mRNA for the interferon-stimulated genes (ISGs) viperin, IFIT1 and CXCL10 in DENV-infected SK1−/− compared with WT iMEFs. Similarly, reduced induction of ISGs was observed in SK1−/− 18 MEFs, even in the face of high-level DENV replication. In both iMEFs and 18 MEFs, DENV infection induced production of IFN-α protein. Additionally, higher basal levels of antiviral factors (IRF7, CXCL10 and OAS1) were observed in uninfected SK1−/− iMEFs but not 18 MEFs. This suggests that, in this single iMEF line, lack of SK1 upregulates the basal levels of factors that may protect cells against DENV infection. More importantly, regardless of the levels of DENV replication, all cells that lacked SK1 produced IFN-α but were refractory to induction of ISGs such as viperin, IFIT1 and CXCL10. Based on these findings, we propose new roles for SK1 in affecting innate responses that regulate susceptibility to DENV infection.

INTRODUCTION

A growing literature suggests that sphingosine kinase (SK) 1 is a host cell enzyme that phosphorylates the lipid sphingosine to form sphingosine 1-phosphate (S1P), can promote infections by some viruses (Carr et al., 2013b). For human cytomegalovirus (HCMV) (Machesky et al., 2008), respiratory syncytial virus (RSV) (Monick et al., 2004), influenza A virus (IAV) (Seo et al., 2010, 2013) and measles virus (Vijayan et al., 2014), high levels of SK1 in cells prior to infection assist viral replication. The ability of SK1 to promote infection has been proposed to occur via a number of different mechanisms, such as preventing viral-induced cell death (Machesky et al., 2008; Monick et al., 2004), increasing production of viral RNA and export of viral proteins (Seo et al., 2013), NFκB-mediated effects on viral protein synthesis (Vijayan et al., 2014) and phosphorylation of STAT1 (Seo et al., 2011). There are two forms of SK, SK1 and SK2, and these may have similar or contrasting roles in different systems (Neubauer & Pitson, 2013; Oskeritzian et al., 2008; Pitson, 2011). Recently, SK2 has been shown to increase the replication of some hepatitis C virus strains by interfering with...
oxidative stress and lipid peroxidation in infected cells (Yamane et al., 2014). The above studies linking high levels of SK with enhancement of viral infection are in contrast to our own observations of a reduction in SK1 activity in response to dengue virus (DENV) infection (Wati et al., 2011), as also reported for the related flavivirus bovine viral diarrhea virus (Yamane et al., 2009). Close analysis of the time-course of the reduction in SK1 in DENV-infected cells demonstrates a correlation with high levels of the DENV 3’ UTR, which can compete for the SK1 activator eEF1A (Carr et al., 2013a; Leclercq et al., 2008, 2011), which is utilized by flaviviruses to assist replication (Blackwell & Brinton, 1997; Davis et al., 2007). Additionally, our previous data (Carr et al., 2013a; Calvert et al., 2015), as well as that of Yamane et al. (2009), suggest an increase in SK1 activity in the early hours following infection. Thus the roles of SK1 in viral infection may vary in relation to the time post-infection (p.i.).

Therefore, we further investigated the role of SK1 during the early stages of DENV infection. DENV is a significant mosquito-borne human viral infection for which there are currently only supportive treatments (Guzman et al., 2010; Simmons et al., 2012; Whitehorn et al., 2014). DENV disease can vary from mild febrile illness (‘dengue’) to ‘dengue with warning signs’ and even potentially life threatening ‘severe dengue’ (WHO, 2009). The more serious forms of the disease are associated with thrombocytopenia, haemoconcentration, bleeding tendency and vascular leak syndrome with the potential for hypovolaemic shock. The severe symptoms of DENV infection are believed to be largely driven by the host cell immune/inflammatory response to infection (Halstead, 1989, 2010; Nascimento et al., 2014) and thus we have investigated the role of host cell factors such as SK1 in this process.

SK1 has well-described functions in cell signalling, such as TNF-α-stimulated NFκB activation (Alvarez et al., 2010; Leclercq & Pitson, 2006; Maceyka et al., 2009; Xia et al., 2002), and in cell death/survival decisions (Pitson, 2011). The product of SK activity, S1P, can have intracellular signalling functions or be secreted from cells to act on cell surface S1P receptors (Leclercq & Pitson, 2006; Rosen et al., 2014; Spiegel & Milstien, 2011; Xia & Wadham, 2011). A number of studies have shown that modulating the S1P/receptor can reduce virus-induced disease and tissue injury associated with IAV (Marsolais et al., 2009; Oldstone & Rosen, 2014; Walsh et al., 2011) and in an animal model of RSV infection (Walsh et al., 2014). Thus, studies of the SK–virus interaction are of significant interest to potential therapeutic interventions for viral diseases. Herein, we show that endogenous SK1 is not altered early on in DENV infection, but a reduction in SK1 is associated with modulation of innate immune responses that influence cellular susceptibility to DENV infection. Specifically, without SK1 there is a reduction in the induction of interferon-stimulated genes (ISGs) such as those for viperin, IFIT1 and CXCL10 following DENV infection. These new roles for SK1 during DENV infection will have important implications for our understanding of innate immunity to RNA viruses, the cross-talk between pathways controlling growth/survival and antiviral responses, and the future potential for modulation of the SK/S1P axis for treatment of viral infections.

RESULTS

Elevated SK1 has no effect on productive DENV infection

Previous data have suggested that exogenous expression of SK1 in cells promotes HCMV, IAV and measles virus infection (Machesky et al., 2008; Seo et al., 2010; Vijayan et al., 2014). We quantified the level of endogenous SK1 in five different human cell lines and assessed their ability to support DENV infection. The level of endogenous SK1 activity negatively correlated with the level of infectious DENV release at 48 h p.i. (r = −0.89) (Fig. 1a), suggesting that high levels of endogenous SK1 prior to infection may be detrimental to DENV infection. We next used stable cell lines engineered to induce expression of WT-SK1, LCK-SK1 (a form of SK1 constitutively localized to the plasma membrane; Pitson et al., 2005) and G82D-SK1 (a catalytically inactive form of SK1; Pitson et al., 2000) following treatment with doxycycline (DOX). We validated DOX induction of SK1 protein and activity (Fig. 1b, c), where treatment with as little as 1 ng ml⁻¹ DOX for 18 h before infection induced approximately a 500-fold increase in SK1 activity (Fig. 1b). As expected, no SK1 activity was detected for the inactive G82D-SK1 mutant (Fig. 1b). Higher levels of DOX were used (10 ng ml⁻¹) to induce SK1 protein, with 1 μg ml⁻¹ DOX used to induce comparable protein levels of the G82D-SK1 mutant (Fig. 1c). DENV infection of cells pre-treated with DOX and thus expressing elevated levels of SK1 did not produce any significant difference in the level of infectious virus release in comparison with untreated DENV-infected cells (Fig. 1d). This suggests that in our infection system and similar to our prior observation, where increasing SK1 after an infection was established had no effect on DENV replication (Carr et al., 2013a), increased levels of exogenous SK1 prior to DENV challenge also has no significant impact on DENV infection.

We next assessed if DENV infection induced changes in endogenous SK1 mRNA, protein and activity in DENV-infected human embryonic kidney (HEK) 293 cells at early time points p.i. RNA extraction and reverse transcription PCR (RT-PCR) analysis of mock- and DENV-infected HEK293 cells up to 24 h p.i. did not show any significant difference in SK1 mRNA levels (Fig. 2a). We next analysed SK1 protein and phospho-SK1 (p-SK1) levels (a modification that increases SK1 activity; Pitson et al., 2003) by Western blotting (Fig. 2b). While both SK1 and p-SK1 levels were very low in HEK293 cells, no change in p-SK1 or SK1 protein was observed upon DENV infection.
Fig. 1. Overexpression of SK1 does not alter DENV viral production in HEK293 cells. (a) Endogenous SK1 activity was quantified in five different uninfected human cell lines grown at subconfluence. Cells were DENV-infected at an m.o.i. of 0.1, supernatant was harvested at 48 h p.i., and infectious virus release was measured by plaque assay. Results represent mean ± SD from n=3 (SK1 activity assay) and n=2 (plaque assay). HEK293 cells engineered to overexpress WT SK1, LCK-SK1 and G82D-SK1 upon DOX induction were treated or not treated with DOX for 18 h. (b) Cells were pre-treated with 1–5 ng ml⁻¹ DOX and lysed, and SK1 activity was quantified. SK1 activity was normalized against total protein content and expressed as fold change compared with HEK293 cells. Data represent mean ± SD. (c) Cells were pre-treated with 10 ng ml⁻¹ or 1 µg ml⁻¹ DOX (for G82D-SK1) and lysed, and induction of SK1 protein was confirmed by Western blot analysis. (d) Cells were pre-treated with 10 ng ml⁻¹ or 1 µg ml⁻¹ DOX (for G82D-SK1) and DENV-infected at an m.o.i. of 1, and supernatants were harvested and analysed for infectious virus at 6, 24 and 48 h p.i. by plaque assay. Data are expressed as the mean ± SE of three replicates and are representative of three independent experiments for WT-SK1 and two independent experiments for LCK-SK1 and G82D-SK1.
SK1 mRNA, protein or activity is not altered early in DENV-infected cells. HEK293 cells were left uninfected (U), mock-infected with heat-inactivated virus (M) or DENV-infected at an m.o.i. of 1 (D). At the indicated time point p.i., RNA and protein lysates were collected. (a) Total SK1 mRNA was quantified by RT-PCR. Results represent mean ± SE of four replicates from two independent experiments. Relative gene expression was determined by the ΔΔCt method and data are normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and expressed as a percentage of the mock-infected value. (b) Cells were lysed and analysed by Western blotting by serially re-probing the membrane for p-SK1, total SK1 and tubulin. Results are representative of duplicate samples from replicate experiments. Lysates from cells expressing high levels of WT SK1 and S225A-SK1, a mutant of SK1 that is not phosphorylated, were used as controls. (c) Cells were lysed and SK1 activity was quantified using an SK1-specific in vitro assay. Results were normalized against total protein concentration and are expressed relative to the SK1 activity in mock-infected control lysates. Results represent normalized data from duplicate samples from n=4 independent experiments.

**Fig. 2.** SK1 mRNA, protein or activity is not altered early in DENV-infected cells. HEK293 cells were left uninfected (U), mock-infected with heat-inactivated virus (M) or DENV-infected at an m.o.i. of 1 (D). At the indicated time point p.i., RNA and protein lysates were collected. (a) Total SK1 mRNA was quantified by RT-PCR. Results represent mean ± SE of four replicates from two independent experiments. Relative gene expression was determined by the ΔΔCt method and data are normalized against glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and expressed as a percentage of the mock-infected value. (b) Cells were lysed and analysed by Western blotting by serially re-probing the membrane for p-SK1, total SK1 and tubulin. Results are representative of duplicate samples from replicate experiments. Lysates from cells expressing high levels of WT SK1 and S225A-SK1, a mutant of SK1 that is not phosphorylated, were used as controls. (c) Cells were lysed and SK1 activity was quantified using an SK1-specific in vitro assay. Results were normalized against total protein concentration and are expressed relative to the SK1 activity in mock-infected control lysates. Results represent normalized data from duplicate samples from n=4 independent experiments.

Reduced viral replication when SK1 is absent in iMEFs is not due to an over-exuberant response to DENV infection

We next aimed to define the cellular changes associated with this altered DENV infection. We performed global

**Lower SK1 reduces DENV infection**

Previous data suggested that targeting SK using broad-spectrum SK1/SK2 inhibitors reduced viral infection (Machesky et al., 2008; Seo et al., 2010; Vijayan et al., 2014). Thus, we pre-treated HEK293 cells with SK1-specific inhibitors, SK1-I [1, 2, 4-trideoxy-4-(methylamino)-1-(4-pentylphenyl)-d-erythro-pent-1-enitol hydrochloride; Paugh et al., 2008], also reduced infectious virus release (Fig. 3c), with approximately a 1–2 log reduction in DENV infectious virus release at 24 h p.i. in multiple experiments. This effect of SKi was also reproducible in a number of different cell types (Fig. 3b). Similarly, pre-treatment of HEK293 cells with an SK1-specific inhibitor, SK1-1 [1, 2, 4-trideoxy-4-(methylamino)-1-(4-pentylphenyl)-d-erythro-pent-1-enitol hydrochloride; Paugh et al., 2008], also reduced infectious virus release (Fig. 3c), with approximately a 1–2 log reduction in DENV infectious virus release at 24 h p.i. in multiple experiments. These results suggest that a reduction in endogenous SK1 prior to infection reduces DENV infection and that this effect is mediated by SK1.

To further assess the effect of lack of SK1 on DENV infection, we challenged both immortalized and primary murine embryonic fibroblasts (MEFs) isolated from WT and SK1−/− embryos (iMEFs and 1 uMEFs, respectively) with DENV. Results demonstrated a significant 2–3 log decrease in infectious DENV release from SK1−/− compared with WT iMEFs (Fig. 4a). This result was observed using a single SK1−/− iMEF line in comparison with two independently isolated WT iMEF isolates. In contrast, DENV infection of 1 uMEF resulted in a significant increase in infectious DENV release at 48 h p.i. in SK1−/− compared with WT 1 uMEFs (Fig. 4b). This result was reproduced in four independent 1 uMEF isolates. RT-PCR analysis of DENV RNA at 24 and 48 h p.i. confirmed a significant reduction in RNA in SK1−/− iMEFs (Fig. 4c) but enhanced DENV RNA in SK1−/− 1 uMEFs (Fig. 4d). Thus, in the absence of SK1, DENV infection is altered: reduced in iMEFs but enhanced in 1 uMEFs.
Fig. 3. Chemical reduction in SK1 reduces DENV infection. HEK293 cells were pre-treated for 18 h with drug or with DMSO control and DENV-infected at an m.o.i. of 0.1, and infectious virus release was measured in supernatants harvested at the indicated time p.i. by plaque assay. (a) Time-course with 5 μM SK1 pre-treatment. (b) Pre-treatment of different cell types with 5 μM SK1. Results are expressed as a percentage of mock-infected control at 24 h p.i. (c) HEK293 cells pre-treated with 5 μM SK1-I, 90 min. Results represent mean ± SD of duplicate infections. Results were replicated in n=3 independent biological experiments (a, c). *Significantly different from vehicle control, P<0.05, Student’s unpaired t-test.

transcriptome analysis at 24 h p.i., when DENV infection in WT iMEFs is clearly different from that in SK1−/− iMEFs, but both cell types are still producing infectious virus and viral RNA. DENV infection of WT iMEFs caused a significant change in levels of 126 mRNAs compared with uninfected iMEFs, the majority of which RNAs are associated with known cellular responses to viral infection. Heat-map profiles of the normalized intensity array data for 45 selected genes of interest from uninfected WT compared with WT DENV-infected iMEFs demonstrate a clear induction of a host cell response to infection (Fig. 5a). Data were further analysed statistically and a summary of the significant (more than twofold) RNA changes in uninfected compared with DENV-infected WT iMEFs is shown in Table S1, available in the online Supplementary Material. The induction of RNA for viperin, IFIT1, IRF7 and CXCL10 in DENV-infected WT iMEFs was validated by RT-PCR (Fig. 5b). Table S2 compares the calculated fold change for viperin, IFIT1, IRF7 and CXCL10 mRNA levels indicated from the normalized intensity array data (Fig. 5a), the array data after further statistical correction (Table S1) and the quantitative RT-PCR. Although the absolute fold change differs, there is consistency in the pattern of induction of these genes in uninfected WT compared with DENV-infected iMEFs.

In contrast, no RNAs were significantly altered by DENV infection of SK1−/− iMEFs as determined by ANOVA. This is illustrated visually by heat-map representation of the normalized intensity array data for the same 45 selected genes shown for DENV infection of WT iMEFs (Fig. 5a). Levels of RNA for viperin, IFIT1, IRF7 and CXCL10 were assessed by RT-PCR, which similarly confirmed no significant induction of these RNAs in comparison with uninfected SK1−/− iMEFs (Fig. 5b). The clear difference in levels of some mRNAs in uninfected SK1−/− compared with WT iMEFs is further illustrated in Fig. 7.

Without SK1, both iMEFs and 1′MEFs are refractory to DENV induction of ISGs

We next analysed the time-course for induction of genes selected from the transcriptome array above as well as IFN-β, to assess if the refractory nature of SK1−/− MEFS to induction of ISGs was due to the kinetics of the response, the level of DENV replication in these cells or the type 1 interferon response. As expected, results showed increasing levels of RNA with time for IFN-β and ISGs for vaperin, IFIT1 and CXCL10 in DENV-infected WT iMEFs (Fig. 6a) and 1′MEFs (Fig. 6b). Of note is the much lower relative gene expression seen for IFN-β and ISGs in the 1′MEF than in the iMEF system. This is despite comparable levels of DENV RNA and infectious virus release in both cells, and thus the rationale for this difference is unclear.

An induction of IFN-β was observed at 48 h p.i. in DENV-infected SK1−/− iMEFs, but at significantly lower levels than in WT iMEFs, and a similar induction of IFN-β protein was confirmed by ELISA (Fig. 6c). Since IFN-β is induced in SK1−/− iMEFs, a subsequent induction of ISGs was expected, but this was not observed for vaperin,
IFIT1 or CXCL10 in DENV-infected SK1\(^{-/-}\) iMEFs within the time frame of our experiment (up to 48 h p.i.) (Fig. 6a). Similarly, in WT 1\(^{+}\)MEFs, DENV infection induces mRNA for IFN-\(\beta\) and ISGs of viperin, IFIT1 and CXCL10. At 24 h p.i., DENV infection of SK1\(^{-/-}\) 1\(^{+}\)MEFs showed disparate results in relation to IFN-\(\beta\) in the four different SK1\(^{-/-}\) 1\(^{+}\)MEF extractions. Although DENV replication was enhanced in all four SK1\(^{-/-}\) 1\(^{+}\)MEF preparations, two SK1\(^{-/-}\) 1\(^{+}\)MEF lines showed reduced IFN-\(\beta\) mRNA (Fig. 6b) and two lines demonstrated enhanced IFN-\(\beta\) in comparison with WT 1\(^{+}\)MEFs (Fig. 6b, c). Similar levels of IFN-\(\beta\) mRNA and enhanced levels of IFN-\(\beta\) protein were observed in SK1\(^{-/-}\) 1\(^{+}\)MEFs in all cases at 48 h p.i. compared with WT 1\(^{+}\)MEFs (Fig. 6b, c). Similar to iMEFs, in all SK1\(^{-/-}\) 1\(^{+}\)MEFs at 24 h p.i. there are reduced levels of DENV-induced viperin, IFIT1 and CXCL10 mRNA compared with WT 1\(^{+}\)MEFs (Fig. 6b), regardless of IFN-\(\beta\) levels and even though in all four SK1\(^{-/-}\) 1\(^{+}\)MEFs DENV is replicating at a significantly higher level (Fig. 4). This recovers to normal by 48 h p.i. for CXCL10 but remains significantly reduced for viperin and IFIT1 (Fig. 6b).

The reduced ability to induce ISGs in the absence of SK1 was confirmed in cells with reduced SK1 activity through treatment with the SK inhibitor SK1-I. HEK293 cells were pre-treated or not with SK1-I and DENV-infected as in Fig. 3(c), and IFN-\(\beta\) and ISG mRNA were analysed as in Fig. 6. As previously observed in cells pre-treated with SK1-I, DENV infection was significantly reduced, but active DENV replication was still observed and analysis of ISGs demonstrated an induction of ISGs in DENV-infected HEK293 cells but at significantly lower levels in

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**Fig. 4.** DENV infection is altered when SK1 is absent. MEFs were generated from WT and SK1\(^{-/-}\) mouse embryos and utilized as primary cells (1\(^{+}\)MEFs; b, d) or immortalized (iMEFs; a, c). MEFs were infected with DENV at an m.o.i. of 1, and at the indicated time point p.i. culture supernatant was harvested and analysed for infectious virus release by plaque assay: (a) iMEFs; (b) 1\(^{+}\)MEFs. At 24 and 48 h p.i., RNA was extracted and DENV RNA quantified by RT-PCR: (c) iMEFs; (d) 1\(^{+}\)MEFs. Results represent DENV copy number, determined from a standard curve run in parallel, and were normalized against GAPDH quantified by RT-PCR from a defined amount of input RNA. Data represent mean±s.d of duplicate infections. Results were replicated in \(n=3\) independent biological experiments and rethawing of iMEF lines and \(n=4\) independently isolated 1\(^{+}\)MEF preparations. •Significantly different, \(P<0.05\), Student’s unpaired \(t\)-test.
Fig. 5. Innate host responses are induced in DENV-infected WT but not SK1-/- iMEFs. RNA was extracted from uninfected (UI) or DENV-infected WT and SK1-/- iMEFs at 24 h p.i. and subjected to microarray analysis. (a) Forty-five genes of interest were selected, and normalized intensity array data are shown as a heat-map representation. Each vertical cluster depicts four lanes from quadruplicates of each biological treatment. Viperin, IFIT1, CXCL10 and IRF7 labels are in red text. (b) Selected genes of interest were validated by RT-PCR. Results represent mean ± SE of three replicates. Relative gene expression was determined by the \( \Delta C_T \) method and data are normalized against GAPDH. *Significantly different from uninfected control, \( P < 0.05 \), Student’s t-test.
**Fig. 6.** DENV infection of SK1−/− MEFs shows reduced induction of ISG mRNA. WT and SK1−/− MEFs were DENV-infected at an m.o.i. of 1, and at 2, 24 and 48 h p.i. RNA was harvested and analysed by RT-PCR for IFN-β, CXCL10, IFIT1 and viperin. (a) Results represent mean ± SE of three replicates (iMEFs) (a); and mean ± SD of duplicates from n=2 different 18 MEF isolates (b). 18 MEF data were reproduced in a further two 18 MEF preparations (data not shown). The two 18 MEF infections that induced high levels of IFN-β mRNA are shown as 24B in the top, IFN-β, panel. Relative gene expression was determined by the ΔΔCt method and data are normalized against GAPDH. (c) IFN-β was quantified in cell culture supernatants from DENV-infected WT and SK1−/− iMEFs (left panel) and 18 MEFs (right panel) by ELISA. Results shown represent mean ± SD from n=4 values from n=2 biological replicates (iMEFs) and n=2 replicates from one 18 MEF clone. Similar IFN-β protein levels in DENV-infected 18 MEFs were reproduced in a further 18 MEF isolation while two additional 18 MEF isolations showed similar results, with the exception of reduced IFN-β at 24 h p.i. in SK1−/− 18 MEFs (data not shown). *Significantly different from 2 h control from each cell type, **Significantly different from WT MEF at the same time point p.i., P<0.05, Student’s t-test.
cells pre-treated with SK1-I (Fig. S1). Together, these results demonstrate a reduced ability to induce the ISGs for CXCL10, IFIT1 and viperin when SK1 is reduced or absent, independent of the level of DENV infection, and for 1°MEFs also independent of the level of IFN-β. This is summarized in Fig. 8(a).

**Basal antiviral pathways are increased in uninfected SK1−/− iMEFs**

The data in Figs 5 and 6 collectively demonstrate a dysregulation in the induction of ISGs in response to DENV infection when SK1 is reduced or absent. Of note, however, was the higher basal levels of a number of antiviral factors in uninfected SK1−/− than in WT iMEFs in the normalized RNA expression data (Fig. 5a) as well as RT-PCR array validation (Fig. 5b). We next specifically assessed the levels of IFN-β, IFIT1, viperin, CXCL10, OAS1, IRF7 and RNase L in uninfected SK1−/− compared with WT iMEFs by RT-PCR. As expected, RNA levels for all these genes were very low in uninfected WT iMEFs, particularly for IFN-β, viperin and RNase L (Fig. 7a). There was no significant change above basal variation in IFN-β or RNase L in uninfected SK1−/− compared with uninfected WT iMEFs (Fig. 7a). The elevated levels of viperin and IFIT1 suggested in uninfected SK1−/− (Fig. 5) were not significant when analysed in further replicate experiments and compared with a second WT iMEF line (Fig. 7). In contrast, a clear and significant increase in CXCL10, OAS1 and IRF7 in uninfected SK1−/− compared with uninfected WT iMEFs was observed (Fig. 7a), representing a 1.5–2.5-fold increase in RNA levels in SK1−/− iMEFs above those in WT iMEFs (Fig. 7b). These results demonstrate increased levels of known antiviral factors in this SK1−/− iMEF line, which correlates with the reduced ability of these cells to support DENV replication (Fig. 4). The levels of IFN-β, IFIT1, viperin, CXCL10, OAS1, IRF7 and RNase L were also quantified in uninfected SK1−/− and WT 1°MEFs. The levels of these RNAs were again very low and no reliable or significant changes in WT compared with SK1−/− 1°MEFs were observed (data not shown). Thus, the differing susceptibility of SK1−/− iMEFs and SK1−/− 1°MEFs to DENV infection correlates with differences in the basal levels of a number of innate antiviral factors in the SK1−/− iMEF line. This is summarized in Fig. 8(b).

**DISCUSSION**

SK is a host cell enzyme that is involved in a number of processes central to regulation of cell proliferation and survival, and thus has been a focus for anti-cancer therapy (Pyne *et al.*, 2011). More recently, the use of S1P receptor modulators has shown promise in alleviating viral inflammatory diseases of the lung (Oldstone & Rosen, 2014). With this interesting data and the growing availability of therapeutics and agents targeting SK, S1P and S1P receptors, this is an expanding area of potential importance to treatment of viral inflammatory disease, such as DENV. Accumulating evidence suggests a role for SK1 in promoting viral infection. In contrast however, to prior studies where overexpression of SK1 increased HCMV (Machesky *et al.*, 2008), IAV (Seo *et al.*, 2010) and measles virus infection (Vijayan *et al.*, 2014), overexpression of SK1 did not influence DENV infectious virus release. This suggests that elevating SK1 in cells is of no additional benefit to DENV above any normal role of endogenous SK1. The difference between our study and those previous most likely relates to virus-specific requirements for SK, or the use of a transient compared with an inducible SK1 expression system. Additionally, in five different cell lines the levels of endogenous SK1 prior to infection negatively correlated with the level of DENV infection, suggesting endogenous SK1 may conversely be an inhibitor of DENV infection.

DENV infection of cells, however, was not accompanied by an early change in endogenous SK1, within the first 24 h of infection. This contrasts to studies that have shown increased SK1 mRNA in HCMV- and RSV-infected cells at 24 h p.i. (Machesky *et al.*, 2008; Monick *et al.*, 2004) and increases in p-SK1 during IAV (Seo *et al.*, 2013) and measles virus infection (Vijayan *et al.*, 2014). We also did not detect any significant or reproducible change in SK activity within the first 24 h p.i. We have previously defined the time-course for changes in SK1 activity in DENV-infected cells from 2 to 48 h p.i and shown a late reduction in SK1 activity in DENV-infected cells (>30 h p.i.) (Carr *et al.*, 2013a; Wati *et al.*, 2011). In the study herein, we have more closely examined the first 24 h following DENV infection, but failed to demonstrate any additional changes in SK1 activity. In contrast, we have recently described an increase in SK1 activity in DENV-infected human umbilical vein endothelial cells (HUVECs) within 24 h of infection (Calvert *et al.*, 2015). In this HUVEC infection model there is only a low percentage of DENV-infected cells and we believe the induction of SK1 is secondary to the induction of host cell responses in these cells, such as production of TNF-α. From our current data in HEK293 cells, where the majority of the culture is DENV-infected, we conclude that DENV does not directly induce major changes in SK1 mRNA, protein or activity early in infection.

We have demonstrated, however, that manipulating endogenous SK1 can promote initial DENV infection with a decrease in infection when SK1 is reduced by chemical pre-treatments of cells with SK1, an SK1 and SK2 competitive inhibitor, and also with SK1-I, an SK1-specific inhibitor. This demonstrates a specific requirement for SK1 and is consistent with prior reports of inhibition of viral replication by treatment of cells with chemical SK inhibitors (Machesky *et al.*, 2008; Seo *et al.*, 2010; Vijayan *et al.*, 2014).

Analysis of DENV infection in the setting of genetic ablation of SK1, represented by MEFs from SK1−/− embryos,
yielded differing results, depending on whether the cells were from immortalized or primary cultures. DENV infectious virus release and RNA replication was reduced in an SK1<sup>-/-</sup> iMEF line that was immortalized (with SV40 large T-antigen) but DENV infection was significantly enhanced in multiple isolations of SK1<sup>-/-</sup> uMEFs. Our results suggest that the difference in this phenotype of DENV susceptibility likely relates to basal levels of antiviral factors (IRF7, CXCL10, OAS1), which we observed to be elevated in the SK1<sup>-/-</sup> iMEF line but not uMEFs. We propose that elevated levels of factors such as IRF7, CXCL10 and OAS1 may be cytotoxic and not be viably maintained without the benefit of immortalization. Additionally, upregulation of an antiviral state has been reported to be induced by expression of polyomavirus large T-antigens in MEFs, such as from SV40 (Giacobbi et al., 2015). Since our results in SK1<sup>-/-</sup> SV40-transformed iMEFs were compared with two independent WT SV40-transformed iMEF lines, the antiviral state induced by SV40 immortalization of MEFs is unlikely to be the sole factor underlying the resistance of SK1<sup>-/-</sup> iMEFs to DENV infection and elevated levels of IRF7, CXCL10 and OAS1 seen in these cells. Consistent with our observation of elevated CXCL10 in this SK1<sup>-/-</sup> iMEF line, Harikumar et al. (2014) have described higher

Fig. 7. Uninfected SK1<sup>-/-</sup> iMEFs have elevated levels of antiviral RNA. (a) RNA was extracted from uninfected WT and SK1<sup>-/-</sup> iMEFs and basal levels of IFN-β, IFIT1, viperin, CXCL10, OAS1 and IRF7 were analysed by RT-PCR. (b) Fold change was determined by normalization of SK1<sup>-/-</sup> against WT data. Results represent mean±se of n=3 (SK1<sup>-/-</sup>) and n=5, respectively, from two independent iMEF WT lines. Relative gene expression was determined by the ΔC<sub>t</sub> method and data are normalized against GAPDH. *Significantly different from uninfected WT control, P<0.05, Student’s t-test.
basal levels of CXCL10 in SK1<sup>−/−</sup> mice. Increased levels of the chemokines CXCL1, CXCL2 and IL-8 in placental decidual cells of SK1<sup>−/−</sup> and SK2<sup>−/−</sup> mice have also been reported (Mizugishi <em>et al.</em>, 2015), while prior studies have demonstrated increased levels of CXCL1, CXCL10, IL-8 and CCL20 in SK1 siRNA-treated cells (Adada <em>et al.</em>, 2013). Thus, the literature supports a link between SK1 and basal levels of at least some chemokines, such as CXCL10, and the links we describe here between SK1 and basal levels of CXCL10, IRF7 and OAS1 in this SK1<sup>−/−</sup> iMEF line expand the potential interactions between SK1 and the host innate response. Nevertheless, it is possible that the elevated levels of IRF7, CXCL10 and OAS1 are unique to this SK1<sup>−/−</sup> iMEF line. Regardless of the biological differences between SK1<sup>−/−</sup> iMEFs and 1<sup>°</sup>MEFs, our observation has yielded a useful model to investigate the role of SK1 in infection, independent of the level of DENV replication in cells.

We compared the global gene expression profile in WT and SK1<sup>−/−</sup> MEFs, using iMEFs at 24 h p.i. since in these cells there is active DENV infection but there is a clear difference in the level of virus production between WT and SK1<sup>−/−</sup> cells. Global gene expression analysis showed expected DENV-induced responses in WT iMEFs, including induction of chemokines, pathogen recognition pathways and ISGs (Conceição <em>et al.</em>, 2010; Fink <em>et al.</em>, 2007; Liew & Chow, 2006; Suthar <em>et al.</em>, 2013; Warke <em>et al.</em>, 2003). Interestingly, DENV infection of WT iMEFs also induced mRNAs for a number of SP100-related, PARP-related and Schlafen proteins, which may be of future interest. In contrast, array and RT-PCR analysis confirmed that, following DENV infection of SK1<sup>−/−</sup> iMEFs at 24 h p.i., although these cells contained DENV RNA and were producing infectious virus, there were no significant changes in RNA expression in comparison with uninfected SK1<sup>−/−</sup> iMEFs.

Viperin (Helbig <em>et al.</em>, 2013; Jiang <em>et al.</em>, 2010), OAS1 (Lin <em>et al.</em>, 2009; Perelygin <em>et al.</em>, 2002), IRF7 (Chen <em>et al.</em>, 2013) and CXCL10 have previously been ascribed antiviral functions against DENV infection. We further analysed and compared the kinetics of the induction of these genes in iMEFs and 1<sup>°</sup>MEFs. Our results demonstrated a significant
induction of IFN-β mRNA and protein in both WT and SK1−/− iMEFs or 1*MEFs, but, remarkably, there was no or poor subsequent induction of the ISGs for viperin, IFIT1 or CXCL10 in either type of SK1−/− MEF. Thus, cells lacking SK1−/− (both iMEFs and 1*MEFs) are refractory or delayed in the induction of ISGs in response to DENV infection. Further, these results argue that the poor induction of ISGs in cells lacking SK1 is not due to an inability to produce IFN-β but is due to defects downstream of IFN-β actions, such as IFN receptor levels, IFN signalling pathways or defects in pathways independent of IFN-β that can induce ISGs. Together, the profile of enhanced basal level of antivirals described above and lack of induction of ISGs correlate with reduced susceptibility of SK1−/− iMEFs to DENV infection, where the high levels of basal antiviral factors in uninfected SK1−/− iMEFs protect them from DENV infection, regardless of the subsequent inability to induce ISGs (Fig. 8b). In contrast, SK1−/− 1*MEFs showed enhanced infection with DENV, and this correlates with normal levels of basal antiviral factors but reduced ability to induce ISGs early in DENV infection (Fig. 8a).

In relation to mechanisms, there is precedence in the literature for a role for SK1 in cytokine/chemokine and pathogen recognition signalling processes. Both S1P and SK1 reportedly bind to TRAF2 and promote TNF-α-stimulated NFκB activation (Alvarez et al., 2010). Additionally, TRAF2 has been reported to bind to the IFN-α/β receptor (IFNAR) 1 subunit and mediate activation of NFκB, and induction of an antiviral response that protects against vesicular stomatitis virus or encephalomyocarditis virus infection in vitro (Yang et al., 2008). The lack of SK1 may also compromise this IFNAR--TRAF2 antiviral signalling pathway, thus increasing susceptibility to DENV infection. SK1 is also required for LPS stimulation of TLR4-mediated IL-6 production (Pchejetski et al., 2011). More recently, it has been demonstrated that IRF1-mediated IL-1-stimulated production of CXCL10 is reduced by chemical reduction in SK1 or in SK1−/− mice (Harikumar et al., 2014). Our gene expression array data also suggest a significant down-regulation of IRF1 (twofold) in uninfected SK1−/− compared with WT iMEFs (data not shown). This change in IRF1 remains to be confirmed by RT-PCR but may contribute to the poor induction of CXCL10 in the absence of SK1, as seen in this study and previously suggested (Harikumar et al., 2014). Together this supports a broader role for SK1 as a signalling mediator for pathogen response pathways.

Our data clearly suggest that SK1 itself does not promote DENV infection and that without SK1 cells are refractory to induction of ISGs. Thus, SK1 may have previously undescribed roles in innate antiviral responses and pose a novel link between growth, inflammatory and host IFN response pathways in the cell.

**METHODS**

**Cell culture.** Infections utilized HEK293 cells and HEK293-c18 cells (ATCC). The same results were obtained in both HEK293 lines and the cells are referred to as HEK293 herein. DOX (Sigma-Aldrich)-inducible WT-, LCK- and G82D-SK1 constructs in HEK293 cells were generated as previously (Pham et al., 2014). SV40 large T-antigen iMEFs were generated from WT and SK1−/− embryos from pregnant mice. WT and SK1−/− C57BL/6 mice were as previously described, with SK1−/− MEFs shown to have less than 10 % of the total SK activity of WT (Allende et al., 2004). Mice were used in accordance with animal ethics 181/12 approved by the SA Pathology/CALHN Animal Ethics Committee. Primary MEFs were generated from WT and SK1−/− embryos from each of four different pregnant mice, and frozen stocks were generated at passage 3. All MEF cultures were used in infection studies within 2 weeks of thawing and were mycoplasma-negative by routine in-house PCR screening.

**Chemicals.** SK1 (Sigma-Aldrich) and SK1-I (Tocris Bioscience) were resuspended in DMSO and stored at −80 °C.

**DENV infections and plaque assay.** Cells were infected at an m.o.i. of 1, or as indicated in individual experiments, for 90 min. Studies utilized Mon601, a full-length DNA clone of the DENV-2 strain New Guinea C (Gualano et al., 1998). Baby hamster kidney (BHK) in vitro-transcribed Mon601 RNA was transfected into BHK-21 cells, and supernatant was harvested and amplified in C6/36 insect cells. Virus stocks and cell culture supernatants were titrated by plaque assay on Vero (African Green monkey kidney) cells and quantified as p.f.u. ml−1.

**Real-time RT-PCR.** Total RNA was extracted from cells using Trizol (Gibco), DNase-treated and subjected to reverse transcription using random hexamers (NEB). cDNA was subjected to real-time PCR using primers as shown in Table S3. For DENV, quantitative RT-PCR was performed by amplification of a Mon601 DNA copy number standard curve in parallel, and results were normalized against total RNA by quantification of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). All other PCRs included high and low copy number controls to show linearity of amplification, and results were analysed by the △Ct method, with normalization against GAPDH. Reactions were performed using a SYBR Green reaction mix (iTaq; Bio-Rad) in a Corbett Rotorgene 6000.

**SDS-PAGE and Western blotting.** Cells were lysed in extraction buffer [EB, 50 mM Tris pH 7.4, 150 mM NaCl, 2 mM NaVO4, 10 mM NaF, 1 mM EDTA, 10 % (v/v) glycerol, 0.05 % (v/v) Triton X-100, 10 mM β-glycerophosphate, 1 mM DTT] with protease inhibitors (Roche Complete Mini) and protein levels were quantified (Bio-Rad protein assay). Fifty micrograms of total protein was subjected to SDS-PAGE, proteins were transferred to nitrocellulose, and membranes were serially incubated with antibodies for p-SK1 (ECM Biosciences), total SK1 (central region; ECM Biosciences) and tubulin (Abcam). Bound antibody complexes were detected by fluorescence of IR800- and IF680-conjugated secondary antibodies (Li-Cor Biosciences), using the Odyssey Infrared Imaging System (Li-Cor Biosciences). Membranes were stripped (Alpha Diagnostic) in between antibody detections.

**SK1 activity assay.** Cells were lysed in EB and protein concentration was determined, as described above. SK1 activity was determined by 32P transfer from ATP to sphingosine under conditions specific for SK1, as previously described (Pittman et al., 2012). SK1 activity was normalized per microgram of total protein.

**Global gene expression analysis.** WT and SK1−/− iMEFs were left uninfected or were DENV-infected and at 24 h.p.i. RNA was extracted using Trizol and purified (Zymo Research). RNA integrity was assessed using an Agilent BioAnalyser. Microarray analysis was performed on quadruplicate samples from two independent infection experiments. Arrays were performed on the Affymetrix TG3000.
system using the Affymetrix GeneChip mouse gene 2.0 ST array at the Adelaide Microarray Centre in accordance with standard procedures and the manufacturer’s protocols. Raw intensity data were normalized for background correction and used for visual heat-map profile generation [TM4 MeV multiple experiment viewer (http://www.tm4.org/mev.html); Dana Faber Cancer Institute]. Further statistics were performed using the Partek Genomics Suite with robust multi-array (RMA) data normalization. Significant mRNA changes between uninfected and DENV-infected cells were determined by ANOVA with false discovery rate set at a q-value of <0.05. Fold change in mRNA levels was determined on RMA-normalized data from calculation of least squared means with a cut-off of greater than twofold difference between uninfected and DENV-infected cells considered as significant.

Quantification of IFN-β. Cell culture supernatants were assayed for IFN-β using Legend Max Mouse IFN-β pre-coated ELISA plates (BioLegend), according to the manufacturer’s instructions. All samples were assayed at least twice.

Statistics and data analysis. All experiments were performed with a minimum of duplicate samples with a minimum of n=2 biological replicates or as stated in figure legends. Statistical differences were determined by Student’s unpaired t-test with P<0.05. Correlations were assessed by Pearson product moment correlation coefficient.

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