Tumour necrosis factor alpha (TNF-α) stimulation of cells with established dengue virus type 2 infection induces cell death that is accompanied by a reduced ability of TNF-α to activate nuclear factor κB and reduced sphingosine kinase-1 activity

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INTRODUCTION

Dengue virus (DENV) is a member of the genus Flavivirus of the family Flaviviridae that causes disease ranging from acute febrile illness (dengue fever) to life threatening dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) (Clyde et al., 2006; Halstead, 2007; Martina et al., 2009). These latter forms of disease involve vascular leakage and are thought to be immune-mediated via T-cell activation (Kurane et al., 1991), the action of vasoactive factors such as enhanced metalloproteinases (Luplerdlop et al., 2006), reduced vascular endothelial growth factor (Srikiatkhachorn et al., 2007), endothelial cell expression of platelet activating factor receptor (Souza et al., 2009) and elevated cytokines and chemokines (Hober et al., 1993). TNF-α in particular can induce vascular leakage and elevated levels are found in DENV-infected patients, with higher levels...
associated with serious forms of disease (Green & Rothman, 2006; Hobert et al., 1993; Kittigul et al., 2000). Furthermore, there is a genetic association between a high-expressing TNF-α allele and susceptibility to DHF (Perez et al., 2010). Vascular haemorrhage in mice can be induced by injection of high-titre infectious DENV (Chen et al., 2007; Shresta et al., 2006; Yen et al., 2008). This process is less severe in TNF-α-deficient mice (Chen et al., 2007) and can be blocked by the addition of anti-TNF-α antibodies (Atrasheuskaya et al., 2003; Shresta et al., 2006) implicating TNF-α as being an important mediator of DENV-induced haemorrhage in these models. However, cells of the monocyte–macrophage lineage, which are important targets for DENV infection, produce elevated levels of TNF-α in response to infection (Carr et al., 2003; Chen & Wang, 2002; Espina et al., 2003), and TNF-α can have antiviral effects (Herbein & O'Brien, 2000) including inhibiting the replication of viruses such as hepatitis B virus (Gilles et al., 1992) and inhibiting infection by cytomegalovirus (Pavić et al., 1993) or the flavivirus West Nile virus (WNV) (Cheng et al., 2004; Shrestha et al., 2008). We have previously used an in vitro monocyte-derived macrophage (MDM) model of DENV type 2 (DENV-2) infection to show that the antiviral activity of TNF-α is only effective if added prior to established infection and neither the addition of exogenous TNF-α nor blocking of endogenous TNF-α with siRNA or antibody after an established infection affects DENV-2 replication or progeny virus production (Wati et al., 2007). Instead, TNF-α signalling is altered with DENV-2-infected MDM not able to fully activate the transcription factor NF-κB in response to TNF-α (Wati et al., 2007). NF-κB is an important mediator of TNF-α-stimulated pro-survival signals and the release of inflammatory cytokines, but in some situations TNF-α stimulation can also result in the induction of cell death (Chen & Goeddel, 2002; Hiscott et al., 2001; Li & Lin, 2008).

Here, we investigate TNF-α stimulation of DENV-2-infected cells in relation to cell death and pro-survival signals. Our results show that TNF-α stimulation of DENV-2-infected cells during productive infection leads to enhanced death by caspase-3-mediated apoptosis. This is accompanied by cellular changes that cause a predisposition to cell death: reduced ability of TNF-α to induce IkB-α degradation and activation of NF-κB, and reduced SphK1 activity. Importantly, the ability of TNF-α to activate NF-κB changes throughout the course of DENV-2 infection, and inhibition can be mediated by the DENV-2 capsid (CA), but enhancement can be mediated by the DENV-2 NS5 protein. These results have new implications for the role of TNF-α in DENV pathogenesis.

**RESULTS**

**TNF-α stimulation of DENV-2-infected cells is associated with enhanced caspase-3-mediated apoptosis**

We utilized HEK-293 cells as a laboratory cell line model of DENV-2 infection and primary MDM as a natural *in vivo* DENV-2 target cell. We have previously characterized a DENV-2–MDM infection model (Pryor et al., 2001; Wati et al., 2007) and based on this we chose 48 h post-infection (p.i.) as a time point of high-level DENV-2 production after the decline in the DENV-2-induced endogenous TNF-α release. We first characterized DENV-2 infection in HEK-293 cells. High levels of infectious virus were released at 18–48 h p.i. (Fig. 1a), with high levels of intracellular DENV-2 antigens present including DENV-2 CA at 24 and 48 h p.i. (Fig. 1b). The morphology of DENV-2 infected cells was unaltered at 24 h p.i. but at 48 h p.i. cytopathic effect (CPE) was observed visually by comparison with mock-infected cells (Fig. 1c). We thus selected 24 h p.i. for subsequent experiments as representing healthy productively DENV-2-infected HEK-293 cells.

TNF-α can be a pro-survival or apoptotic stimulus, and using the above models we analysed the ability of TNF-α to induce death in DENV-2-infected cells. HEK-293 cells were infected with DENV-2 and at 24 h p.i. were stimulated with TNF-α. Cells were harvested, stained with annexin V as an indicator of cell death and analysed by flow cytometry. Mock-infected cells did not show annexin V staining and this staining was not affected by TNF-α stimulation [Fig. 2a(i)]. Similarly, DENV-2-infected cells without TNF-α stimulation showed no annexin V binding. However, TNF-α stimulation induced the appearance of an annexin V-positive population [Fig. 2a(ii)] that averaged 15 ± 4 % (mean ± SEM) of cells, but varied from 11 up to 26 % of the cell population [shown in Fig. 2a(ii)]. Additionally, the loss of poly (ADP-ribose) polymerase (PARP), another indicator of cell death, was assessed by Western blot analysis. Full-length PARP was detected in all cell lysates but levels were lower in DENV-2-infected cells stimulated with TNF-α (Fig. 2b). The 89 kDa PARP cleavage product could be detected in DENV-2-infected cells treated with TNF-α but was not routinely or readily visualized unless samples were overloaded (Fig. 2b), potentially this is because, as indicated by Annexin V staining (Fig. 2a), the majority of cells are not induced to die. We then determined whether the TNF-α-induced death of DENV-2-infected cells was occurring via caspase-3-mediated apoptosis. Using a quantitative fluorescent-substrate assay, caspase activity was low in mock- and DENV-2-infected cell lysates but was significantly enhanced in DENV-2-infected cells treated with TNF-α (Fig. 2c). Consistent with this *in vitro* activity assay, mock-infected cells with or without TNF-α treatment or DENV-2-infected cells alone showed no active caspase-3 by immunostaining and confocal microscopy [Fig. 2d(i, ii); further data not shown]. In contrast, cells staining strongly for active caspase-3 were observed in the DENV-2-infected population following treatment with TNF-α [Fig. 2d(iii)]. Enumeration of caspase-3-positive cells estimated these to represent 13 % of the population (*n* = 122) consistent with quantification by annexin V staining.

The activation of caspase-3 by TNF-α demonstrated above in DENV-2-infected HEK-293 cells was also assessed in...
MDM. DENV-2-infected cells in this MDM model represented 12–16% of the total cell population, consistent with previous work (Carr et al., 2003; Wati et al., 2007). Caspase-3-positive cells were not observed in mock-infected MDM, with or without TNF-α stimulation [Fig. 3(i); further data not shown]. In DENV-2-infected MDM most cells were caspase-3 negative [Fig. 3(ii)], although some strongly staining caspase-3-positive cells were present in DENV-2-positive cells of the infected MDM population. These represented 3% of DENV-2-infected cells, indicating a low level of activated caspase-3 induced by DENV-2-infection alone. Following exogenous TNF-α stimulation, dual caspase-3- and DENV-2-positive cells were readily detected and represented 15% of the DENV-2-infected cell population [Fig. 3(iii)]. The induction of activated caspase in TNF-α-stimulated DENV-2-infected MDM was reflected by an increase in caspase activity as measured by a quantitative fluorescent-substrate assay (data not shown). However, the level of induction of caspase activity in this assay was not significant, potentially owing to the low level of DENV-2-infected, caspase-3-positive cells relative to the total cell population. Thus following establishing DENV-2-infection in healthy virus producing cells, TNF-α stimulation of DENV-2-infected HEK-293 cells or primary MDM results in induction of caspase-3-mediated apoptosis.

We next assessed if this TNF-α-induced cell death affected infectious virus release. HEK-293 cells were infected with DENV-2, treated with TNF-α prior to infectious virus release (2 h) or after establishment of productive infection (24 h), and infectious-virus release was assayed 24 h later. High levels of progeny infectious DENV-2 were observed in supernatants sampled at both time points, and TNF-α treatment did not significantly alter this (Fig. 4a). Similarly, TNF-α did not affect infectious virus release from DENV-2-infected MDM (Fig. 4b), demonstrating no direct effect of TNF-α on virus production in vitro. However, parallel experiments validate the activity of TNF-α in our system (see Fig. 5).

**Reduced TNF-α-induced degradation of IκB-α and activation of NF-κB in DENV-2-infected cells, dependent on the stage of viral infection**

We next investigated whether the TNF-α-stimulated cell death of DENV-2-infected cells is accompanied by changes in cell survival pathways. The ability of TNF-α to activate NF-κB was analysed by quantification of NF-κB-driven luciferase reporter gene (LUC) activation. HEK-293 cells were transfected with an NF-κB-driven LUC construct then
infected with DENV-2 or mock infected. Cells were stimulated with TNF-α prior to lysis and quantification of LUC activity (Fig. 5a). At 2–30 h p.i., DENV-2 infection itself did not activate NF-κB (Fig. 5a) but DENV-2 activation of NF-κB was observed at 48–54 h p.i. (data not shown), consistent with the timing of the observations of virus-induced CPE (Fig. 1c) and previous reports of DENV-2-induced NF-κB activation and associated cell death (Marianneau et al., 1997). At 2–8 h p.i., TNF-α stimulated comparable levels of NF-κB-driven LUC production in both DENV-2- and mock-infected cells (Fig. 5a). In contrast, at 18–30 h p.i., TNF-α-stimulated

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**Fig. 2.** TNF-α stimulation of DENV-2-infected cells induces caspase-3-mediated cell death. HEK-293 cells were infected with DENV-2 and cells were stimulated at 24 h p.i. with TNF-α (25 ng ml⁻¹) in 0.1 % FCS for 18 h then processed as described in (a–d), below. (a) Cells were stained for annexin V and analysed by flow cytometry. (i), mock infection; (ii), DENV-2 infected—solid line, −TNF-α; dotted line, +TNF-α. (b) Cell lysates were collected and analysed for PARP by SDS-PAGE and Western blot analysis, and proteins detected by using chemiluminescence. Blots were reprobed for actin. (c) Cells were lysed, total protein quantified and an equivalent amount of protein analysed for caspase activity by cleavage of DEVD-AMC fluorogenic substrate. Activity is expressed as the fold increase in caspase-3 activity compared with mock-infected cells (pmol substrate cleaved min⁻¹). Values represent mean ± SEM, (n=3). *, significantly different for DENV-2 +TNF-α compared with −TNF-α, P<0.05, Student’s paired t-test. (d) Cells co-stained for DENV-2 antigens and activated caspase-3, with visualization of immune complexes by confocal microscopy with Alexa-488 and Alexa-647, respectively. (i), Mock infected, +TNF-α; (ii), DENV-2 infected, −TNF-α; (iii), DENV-2 infected +TNF-α; white arrow, DENV-2-infected, capase-3-positive cell.
LUC production was significantly lower in DENV-2-infected than in mock-infected cells (Fig. 5a).

TNF-α-stimulated activation of NF-κB requires degradation of IκB-α, a cytoplasmic inhibitor of NF-κB. Thus, HEK-293 cells were DENV-2-infected and stimulated with TNF-α and the loss of IκB-α analysed by Western blot analysis (Fig. 5b). Consistent with the NF-κB–LUC assays, TNF-α stimulated a reduction in IκB-α in both mock- and DENV-2-infected cells when added at 2 h p.i. but had less effect on IκB-α levels at 24 h p.i. in DENV-2-infected cells. At 18 h p.i., 20 min TNF-α stimulation of DENV-2- or mock-infected cells induced a comparable reduction in IκB-α between mock and DENV-2-infected samples (Fig. 5b). This is in contrast to the lower activation of NF-κB-mediated LUC transcription in DENV-2-infected cells measured over 18–24 h p.i. (Fig. 5a). These differences, between the functional assay of TNF-α-stimulated NF-κB-mediated LUC transcription (Fig. 5a) and the molecular analysis of loss of IκB-α (Fig. 5b) probably relate to the different time frames for the two assays, with the 6 h incubation required for the NF-κB-mediated LUC transcription assay confounded by the progressing course of DENV-2 infection and the associated changing responses to TNF-α. Together, these results suggest that the DENV-2-inhibition of TNF-α-stimulated NF-κB-mediated transcription occurs upstream of IκB-α degradation with the onset of the inhibition after 18 h but before 24 h p.i.

**DENV-2 infection is associated with reduced SphK1 activity**

Cellular SphK1 is an important mediator of pro-survival signals and reduced SphK1 activity is associated with TNF-α-induced cell death (Xia et al., 1999, 2002). Additionally, SphK1 is necessary for TNF-α-stimulated phosphorylation of IκB-α and activation of NF-κB (Alvarez et al., 2010). We therefore quantified the activity of SphK1 in DENV-2-infected HEK-293 cells lysates by in vitro phosphorylation of sphingosine. DENV-2 infection resulted in a significant, approximately 40%, reduction in cellular SphK1 activity (Fig. 5c).

**DENV-2 CA protein inhibits and NS5 enhances TNF-α-stimulated NF-κB activation but DENV-2 CA does not induce TNF-α stimulated cell death or inhibit SphK1 activity**

Alterations in TNF-α signalling have been reported to be induced by the hepatitis C virus (HCV) core (Marusawa et al., 1999; Shrivastava et al., 1998; Zhu et al., 1998) and NS5A and NS5B proteins (Choi et al., 2006; Park et al., 2002). In DENV-2-infected cells, TNF-α stimulation does not affect infectious virus release (Fig. 4a). However, TNF-α stimulation of DENV-2-infected MDM induces activated caspase-3 (Fig. 3).

![Fig. 3.](http://vir.sgmjournals.org)  
**Fig. 3.** TNF-α stimulation of DENV-2-infected MDM induces activated caspase-3. Monocytes were isolated from blood and MDM differentiated in vitro. MDM were DENV-2 or mock infected and at 48 h p.i. were treated with or without TNF-α (25 ng ml⁻¹) in 0.1% FCS for 18 h. Cells were then fixed, permeabilized and co-stained for activated caspase-3 and DENV-2 proteins with visualization of immune complexes by confocal microscopy with Alexa-647 and Alexa-488, respectively. (i) Mock infected + TNF-α; (ii) DENV-2 – TNF-α; (iii) DENV-2 + TNF-α; white arrow, DENV-2-infected, capase-3-positive cells.

![Fig. 4.](http://vir.sgmjournals.org)  
**Fig. 4.** TNF-α stimulation of DENV-2-infected cells does not affect infectious virus release. (a) HEK-293 cells or (b) MDM were infected with DENV-2 and at the indicated time point treated with or without TNF-α (25 ng ml⁻¹) in 0.1% FCS. 24 h later supernatant was sampled and infectious virus release quantified by plaque assay. Values represent mean ± SEM (n=3).
control non-responsive Renilla LUC reporter constructs, then infected with DENV-2. (a) Cells were treated with TNF-\(\alpha\) (60 ng ml\(^{-1}\)) in 0.1% FCS for 6 h, then lysed and firefly LUC quantified and normalized against Renilla LUC (\(n=3\)). Values represent mean ± SEM. (b) Cells were treated with TNF-\(\alpha\) (25 ng ml\(^{-1}\)) in serum free medium for 20 min then lysed and subjected to Western blot for I\(\kappa\)B-\(\alpha\) with detection of proteins by chemiluminescence. Blots were reprobed for actin. (c) HEK-293 cells were DENV-2 or mock infected and at 24 h.p.i. incubated in 0.1% FCS for 6 h prior to lysis. Lysates were normalized for total protein content and analysed for SphK1 activity by quantification of the transfer of \(32\)P to sphingosine in vitro. Results represent relative SphK1 activity ± average % deviation (\(n=4\)). *Significant difference compared with mock infected cells, \(P<0.05\), Student’s unpaired t-test.

Fig. 5. TNF-\(\alpha\)-stimulated NF-\(\kappa\)B activation, I\(\kappa\)B-\(\alpha\) degradation and cellular SphK1 activity is reduced in DENV-2-infected cells. HEK-293 cells were transfected with NF-\(\kappa\)B-responsive firefly LUC and control non-responsive Renilla LUC reporter constructs, then infected with DENV-2. (a) Cells were treated with TNF-\(\alpha\) (60 ng ml\(^{-1}\)) in 0.1% FCS for 6 h, then lysed and firefly LUC quantified and normalized against Renilla LUC (\(n=3\)). Values represent mean ± SEM. (b) Cells were treated with TNF-\(\alpha\) (25 ng ml\(^{-1}\)) in serum free medium for 20 min then lysed and subjected to Western blot for I\(\kappa\)B-\(\alpha\) with detection of proteins by chemiluminescence. Blots were reprobed for actin. (c) HEK-293 cells were DENV-2 or mock infected and at 24 h.p.i. incubated in 0.1% FCS for 6 h prior to lysis. Lysates were normalized for total protein content and analysed for SphK1 activity by quantification of the transfer of \(32\)P to sphingosine in vitro. Results represent relative SphK1 activity ± average % deviation (\(n=4\)). *Significant difference compared with mock infected cells, \(P<0.05\), Student’s unpaired t-test.

We next assessed whether DENV-2 CA protein, previously reported to induce cell death (Limjindaporn et al., 2007), and thus the corresponding DENV-2 CA or NS5 proteins were assessed for their ability to mediate the changes in TNF-\(\alpha\)-stimulated NF-\(\kappa\)B activation observed in DENV-2-infected cells. HEK-293 cells were co-transfected with LUC reporter constructs and DENV-2 CA or NS5 as GFP fusion proteins, and expression of the DENV-2 fusion proteins confirmed by fluorescence microscopy (Fig. 6a). GFP–CA was clearly evident although levels were lower than those seen for CA in DENV-2-infected cells (Fig. 1b). GFP–NS5 was present at high levels (Fig. 6a). Quantification of fluorescent cells by flow cytometry indicated that the proportion of transfected cells was 50–60% for all constructs including GFP–CA. At 48 h post-transfection cells were treated with or without TNF-\(\alpha\) and then lysed and subjected to a LUC caspase activity assay (Fig. 6b, c). Expression of DENV-2 CA protein alone had no effect on NF-\(\kappa\)B-driven LUC production, but DENV-2 CA significantly inhibited TNF-\(\alpha\)-stimulated NF-\(\kappa\)B-driven LUC production in comparison with cells expressing GFP alone (Fig. 6b). Expression of DENV-2 NS5 protein alone also had no effect on NF-\(\kappa\)B-driven LUC production, but in contrast to DENV-2 CA, NS5 enhanced TNF-\(\alpha\)-stimulated NF-\(\kappa\)B-driven LUC production (Fig. 6c). NS5 is primarily localized to the nucleus [see Fig. 6a(iv)] while the effects on TNF-\(\alpha\)-stimulated NF-\(\kappa\)B activation are likely to be through actions in the cytoplasm. We have previously reported that mutations within the NS5 nuclear localization signal (NLS) A region (NLS-A) result in enhanced cytoplasmic localization of NS5 while mutation of the nuclear export sequence (NES) enhanced NS5 nuclear localization (Pryor et al., 2007; Rawlinson et al., 2009). HEK-293 cells were co-transfected with LUC reporter constructs and the GFP fusion proteins for DENV-2 NS5 NLS mutants A1 + A2 or nuclear export mutant NES (Pryor et al., 2007), and expression confirmed [Fig. 6a(v, vi)]. Similar to wild-type NS5, these NS5 mutants still failed to inhibit TNF-\(\alpha\)-stimulated NF-\(\kappa\)B-driven LUC production (Fig. 6c).
was responsible for TNF-α-induced cell death or inhibition of SphK1 activity. HEK-293 cells were transfected to express DENV-2 GFP–CA or GFP as a control and at 48 h post-transfection were stimulated with TNF-α. Immunostaining for activated caspase-3 and analysis by confocal microscopy showed no evidence of caspase-3-mediated cell death in cells transfected to express CA with or without TNF-α stimulation and no colocalization of active caspase-3 in fluorescent GFP–CA expressing cells (Fig. 7a). Cell lysates were also analysed for caspase activity. Results confirmed those seen by immunostaining with no difference in caspase activity between CA- and GFP-transfected cells, with or without TNF-α stimulation (Fig. 7b). Additionally, HEK-293 cells were mock transfected or transfected to express GFP or GFP–CA and at 48 h post-transfection were lysed and analysed for SphK1 activity. Transfection of cells to express GFP–CA, did not result in a significant reduction in SphK1 activity (Fig. 7c). Thus, DENV-2 CA protein can inhibit TNF-α-stimulated NF-κB activation but CA alone is not responsible for the TNF-α-stimulated caspase-3-mediated cell death or the reduction in SphK1 activity seen in DENV-2-infected cells.

**DISCUSSION**

TNF-α is both an important inflammatory cytokine and an antiviral agent in a number of viral infections, including WNV, a member of the genus *Flavivirus* related to DENV. In both DENV and WNV infections, pre-stimulation of cells with TNF-α is protective against virus infection (Cheng et al., 2004; Shrestha et al., 2008; Wati et al., 2007). However, while WNV infection of TNF-α-deficient mice results in severe disease (Shrestha et al., 2008), DENV infection of TNF-α-deficient mice results in mild disease compared with wild-type mice (Shresta et al., 2006). This and additional data obtained during DENV infection of humans (Green & Rothman, 2006; Hober et al., 1993; Kittigul et al., 2000) and other in vivo models of DENV infection in mice (Atrasheuskaya et al., 2003; Chen et al., 2007; Shresta et al., 2006; Yen et al., 2008), implicates TNF-α as being a mediator of DENV-associated haemorrhage, rather than as an antiviral or protective factor. In this study, however, we have demonstrated that TNF-α stimulation of healthy DENV-2-producing HEK-293 or primary MDM cells induces caspase-3-mediated apoptotic cell death. We have linked the timing of TNF-α-stimulated cell death to the reduced ability of TNF-α to stimulate degradation of IkBz and activate NF-κB – a well described pro-survival cell stimulus. Furthermore, we have shown that the changes in TNF-α activation of NF-κB induced by DENV-2 infection are related to the stage of infection. Early in infection and prior to release of progeny virus TNF-α is able to activate NF-κB normally but at the onset of high level infectious virus release this ability is reduced. In addition to the association of TNF-α-stimulated cell death with a reduced ability of TNF-α to activate NF-κB, we have also shown reduced activity of the host-cell enzyme SphK1 in DENV-2-infected cells. SphK1 is a known mediator of TNF-α-stimulated NF-κB activation and cellular pro-survival signals (Alvarez et al., 2010; Xia et al., 1999, 2002). Recently, infection with bovine viral diarrhea virus (BVDV), another member of the family Flaviviridae, and specifically the BVDV NS3 protein have been shown to inhibit SphK1 activity (Yamane et al., 2009). Similarly, DENV-2 NS3 may be involved in the DENV-2-mediated reduction in SphK1 activity and in combination with DENV-2 inhibition of TNF-α-stimulated NF-κB activation, may predispose DENV-2-infected cells to TNF-α-induced cell death. This novel biological link between reduced SphK1 activity, altered TNF-α-stimulated NF-κB activation and cell death in DENV-2-infected cells warrants further investigation. Together these results extend our knowledge of the mechanisms of killing of DENV-2-infected cells to show, for the first time, enhanced sensitivity of DENV-2-infected cells to TNF-α-induced cell death associated with a reduction in the cellular pro-survival pathways, and that these effects are evident prior to virus-induced CPE and cell death induced by DENV-2 infection itself.

Although we observed TNF-α-induced cell death in DENV-2-infected cells, this did not affect the release of infectious virus. This is consistent with our published data describing TNF-α treatment of DENV-2-infected cells prior to the peak of progeny virus release (Carr et al., 2003; Wati et al., 2007) and further demonstrates that, after an established viral infection, TNF-α is not directly antiviral in inhibiting viral production per se. The co-localization of DENV-2 antigens and activated caspase-3 staining described here shows that the TNF-α-stimulated DENV-2-infected cells are induced to die (active caspase-3 positive) but are still producing viral antigens (DENV-2 positive), and thus the dying cells are probably contributors to infectious virus production in this in vitro system. The degree of TNF-α-induced death of DENV-2 infected cells in our in vitro system varied from 11–26 % which may be a significant contributor to cell death in the in vivo setting where these dying cells are likely to be actively cleared and impact on viraemia.

Our study also investigated the DENV proteins mediating these altered responses to TNF-α in DENV-2-infected cells. Although expression of neither DENV-2 CA nor NS5 protein alone influenced NF-κB activation, CA inhibited and NS5 enhanced TNF-α-stimulated NF-κB activation. Consistent with this, the DENV-2-induced inhibition of TNF-α-stimulated NF-κB activation occurs when intracellular DENV-2 CA levels are high. In contrast to DENV-2 CA and our observations in DENV-2-infected cells, the DENV-2 NS5 protein enhanced TNF-α-stimulated NF-κB activation. The related member of the Flaviviridae family, HCV, encodes separate NS5A and NS5B proteins that can both inhibit TNF-α-stimulated NF-κB activation (Choi et al., 2006; Park et al., 2002). However, even when the primary functional NLS of DENV-2 NS5 was mutated to enhance the cytoplasmic localization of NS5 (Pryor et al.
DENV-2 NS5 enhanced and did not inhibit TNF-α-stimulated NF-κB activation. The ability of DENV-2 NS5 to enhance TNF-α-stimulated NF-κB activation is consistent with prior reports of NS5 induction of IL-8 secretion that is at least in part mediated via activation of NF-κB (Medin et al., 2005). Thus, DENV-2 CA and NS5 have opposing effects on TNF-α-stimulated NF-κB activation.

While expression of DENV-2 CA inhibited TNF-α-stimulated NF-κB activation, in our study DENV-2 CA alone did not induce caspase-3-mediated cell death or lead to a significant reduction in cellular SphK1 activity. This may relate to the lower level of expression of transfected GFP–CA compared with that seen for the level of CA in a DENV-2-infected cell. The lack of DENV-2 CA-induced cell death contrasts with WNV CA protein which can induce cell death via activation of the caspase-9–caspase-3 cascade (Yang et al., 2002, 2008), and with the HCV core protein which has been reported to either inhibit TNF-α-stimulated NF-κB activation (Shrivastava et al., 1998) and induce cell death (Zhu et al., 1998, 2001), or to inhibit cell death by activation of the caspase inhibitors, inhibitor of caspase-activated DNase (Sacco et al., 2003) and FAS-associated death domain (FADD)-like interleukin-1β converting enzyme (Saito et al., 2006). DENV-2 CA can interact with the Daxx protein in vitro and induce Fas-mediated apoptosis of DENV-2-infected liver cells (Limjindaporn et al., 2007). Other DENV-2 proteins such as NS3, NS2B-NS3 protease (Shafee & AbuBakar, 2003) or membrane protein (Catteau et al., 2003) have been reported to induce apoptosis. Contributions from these additional cell-death stimuli may combine with the CA-mediated inhibition of TNF-α-stimulated NF-κB activation and the DENV-2-induced reduction in SphK1 activity described herein, to culminate in sensitization of DENV-2-infected cells to TNF-α-stimulated cell death.

The results and implications from this study are summarized in Fig. 8. During established, productive DENV-2 infection (i) cellular SphK1 activity is reduced, (ii) TNF-α-stimulated NF-κB activation is inhibited, which can be modulated by the DENV-2 CA and NS5 proteins and (iii) TNF-α-stimulated, caspase-3-mediated cell death is enhanced. The opposing effects of DENV-2 CA and NS5 on TNF-α-stimulated NF-κB activation and the changing levels of these two viral proteins throughout the viral replication cycle might determine the overall level of SphK1 activity, and hence the sensitivity of DENV-2-infected cells to TNF-α-stimulated cell death.

**Fig. 6.** DENV-2 CA protein inhibits and NS5 enhances TNF-α-stimulated NF-κB activation. HEK-293 cells were co-transfected with NF-κB-responsive firefly LUC, control non-responsive Renilla LUC reporter constructs and constructs expressing GFP-CA or NS5 fusion proteins or control GFP expression vector alone. At 48 h post-transfection cells were processed as described in (a–c).
TNF-α-induced cell death in DENV-2 infected cells

Fig. 7. Expression of DENV-2 CA protein does not inhibit SphK1 activity or predispose cells to TNF-α stimulated cell death. HEK-293 cells were transfected to express DENV-2 GFP–CA or GFP alone. At 48 h post-transfection cells were stimulated with TNF-α (25 ng ml⁻¹) in 0.1 % FCS for 18 h. (a) Cells were immunostained for activated caspase-3, with visualization of DENV-2 GFP–CA and activated caspase-3 (Alexa-647) by confocal microscopy. (i) −TNF-α, (ii) +TNF-α. (b) Cells were lysed, total protein quantified and an equivalent amount of protein analysed for caspase activity by cleavage of DEVD-AMC fluorogenic substrate. Activity is expressed as the fold increase in caspase-3 activity compared with GFP-transfected cells (pmol substrate cleaved min⁻¹). Values represent mean ± SEM (n=3). (c) Cells were lysed and analysed for SphK1 activity by quantification of phosphorylation of sphingosine. Results represent relative SphK1 activity ± average % deviation (n=3).

TNF-α-stimulated NF-κB activation. We propose that in the scenario where DENV NS5 dominates the effects of CA, TNF-α stimulation of DENV-infected cells would promote cell survival and production of NF-κB-driven inflammatory mediators, as is seen in early infection in vivo. In contrast, when the effects of CA dominate those of NS5, as would be expected late in infection, TNF-α stimulation of DENV-infected cells induces cell death without NF-κB activation, leading to the resolution of infection. If these late cellular changes leading to TNF-α-induced death of DENV-infected cells and reduced NF-κB activation do not occur this could lead to extended and higher production of NF-κB-driven inflammatory mediators, including TNF-α, that are known to be associated with the late onset of severe DHF and DSS. Thus, although high levels of circulating TNF-α may be linked to adverse effects and the severe pathology of DHF, we propose that TNF-α stimulation of DENV-infected cells plays a role in killing DENV-producing cells and has beneficial effects in resolving DENV infection.

METHODS

Cells and virus stocks. Monocytes were generated by adherence of PBMC isolated from blood from healthy donors at the Australian Red Cross Blood Service. Monocytes were cultured to differentiate into macrophages and DENV-2-infected as previously described (Pryor et al., 2001; Wati et al., 2007). Infections utilized Mon601, a clone of DENV-2 New Guinea C (Gualano et al., 1998) and virus was produced from in vitro transcribed RNA transfected into BHK-21, amplified in C6/36 and titred in Vero cells.

DENV-2 infection and plaque assay. Cells were infected at an m.o.i. of 1 (HEK-293) or 3 (MDM), for 90 min at 37 °C, as described previously (Carr et al., 2003; Pryor et al., 2001; Wati et al., 2007). At the indicated time points p.i. supernatants were collected, clarified by centrifugation and snap frozen at −80 °C. Plaque assays were performed in Vero cells and infectious virus quantitated as p.f.u. ml⁻¹.

Plasmid constructs. The NF-κB reporter plasmid pTK81NFxBLUC (Dr Greg Goodall, SA Pathology, Adelaide, SA 5000) was a modified version of pTK-LUC and contained five tandem repeats of an NF-κB-responsive element upstream of the TK promoter and the Photinus firefly luciferase (LUC) gene. The control plasmid contained constitutive Renilla LUC (pRL-TK; Promega). DENV-2 pEPI–GFP–CA was generated by PCR from Mon601. pEPI–GFP–NS5 and NS5 sequences with NLS-A (pEPI–GFP–NS5–A1 + A2) and the nuclear export sequence (pEPI–GFP–NS5–NES) mutated were as described previously (Pryor et al., 2007; Rawlinson et al., 2009).

Quantification of TNF-α-stimulated NF-κB. HEK-293 cells were co-transfected with 0.5 μg of each of two different LUC reporter
Flow plots and quantification were generated by analysis of data with immediately by flow cytometry (XL-Euterpe; Becton Dickinson). 30 min at room temperature. Stained cells were fixed and analysed infected with heat inactivated virus (80°C reporter plasmids alone) were infected with DENV-2 or mock using Superfect (Qiagen). At 24 h post-transfection cells (with LUC NS5, GFP–NS5 mutants or the respective pEPI–GFP vector control FCS medium and then treated with 50 ng ml−1 above. At 48 h post-transfection, cells were pre-incubated in 0.1% Pierce). Membranes were also reprobed with mouse anti-actin (1/2000 dilution) and goat anti-rabbit secondary IgG–HRP conjugate membranes. Membranes were probed with rabbit anti-PARP (Roche, Cruz Biotech; 1/500 dilution) with detection of complexes and above. Membranes were probed with rabbit anti-IκB-α (C-21, Santa 4% (v/v) goat–rabbit IgG–Alexa 488 and goat anti-rabbit IgG–Alexa 647 (Molecular Probes) secondary antibodies. Fluorescence was visualized by confocal laser scanning microscopy (Radiance 2100; Bio-Rad).

Caspase activity assays. Cells were lysed in caspase assay buffer (100 mM HEPES pH 7.4, 10 mM MgCl2, 1 mM Na3VO4, 10 mM NaF, 100 mM β-glycerophosphate, 0.05% (v/v) Triton X-100 and protease inhibitors (complete mini; Roche). Lysates were sonicated and total protein concentration quantified (Bio-Rad protein assay). Protein content was normalized and SphK1 activity was determined by [35S] ATP transfer to v-eIF4E-sphingosine under conditions [0.3 % (v/v) Triton X-100] where SphK2 activity is inhibited, as described previously (Roberts et al., 2004).

ACKNOWLEDGEMENTS

Thank you to Adrian Purins and Kegomoditswe Rengoqo (Bless) for technical assistance. Thanks are also due to the staff of the Dettmold Imaging Suite, IMVS, Gafar Sarvestini, Alan Bishop and Kate Pilkington for assistance with confocal imaging and flow cytometry.

REFERENCES

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