Dengue virus regulates type I interferon signalling in a strain-dependent manner in human cell lines

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Outbreaks of dengue disease are constant threats to tropical and subtropical populations but range widely in severity, from mild to haemorrhagic fevers, for reasons that are still elusive. We investigated the interferon (IFN) response in infected human cell lines A549 and HepG2, using two strains (NGC and TSV01) of dengue serotype 2 (DEN2) and found that the two viruses exhibited a marked difference in inducing type I IFN response. While TSV01 infection led to activation of type I antiviral genes such as EIF2AK2 (PKR), OAS, ADAR and MX, these responses were absent in NGC-infected cells. Biochemical analysis revealed that NGC but not TSV01 suppressed STAT-1 and STAT-2 activation in response to type I IFN (α and β). However, these two strains did not differ in their response to type II IFN (γ). Although unable to suppress IFN signalling, TSV01 infection caused a weaker IFN-β induction compared with NGC, suggesting an alternative mechanism of innate immune escape. We extended our study to clinical isolates of various serotypes and found that while MY10245 (DEN2) and MY22713 (DEN4) could suppress the IFN response in a similar fashion to NGC, three other strains of dengue [EDEN167 (DEN1), MY02569 (DEN1) and MY10340 (DEN2)] were unable to suppress the IFN response, suggesting that this difference is strain-dependent but not serotype-specific. Our report indicates the existence of a strain-specific virulence factor that may impact on disease severity.

INTRODUCTION

Dengue virus belongs to the family Flaviviridae of positive-strand RNA viruses, of which there are four serotypes (DEN1–4). Dengue virus infection in humans causes a spectrum of clinical symptoms ranging from acute self-limited febrile dengue fever (DF) to dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). DHF is characterized by abnormalities in haemostasis and increased vascular permeability. It may progress to the life threatening DSS, which is a result of hypovolaemic shock associated with haemoconcentration. Dengue virus is found in the tropical and subtropical regions across the globe, affecting 50 million people in five continents. It is estimated that one-third of the world’s population is at risk of contracting Dengue virus.

Several hypotheses for the reason for the range in severity of pathogenesis of dengue virus infection have been proposed, with antibody-dependent enhancement thought to play a central role (reviewed by Halstead, 2003), although viral virulence has also been suggested (Messer et al., 2003; Rico-Hesse et al., 1997). An alternative hypothesis proposes that the initial innate immune response and in particular the type I interferon (IFN) response may determine the subsequent response and clinical outcome. Release of IFN-α appears to be critical for early immune response and resistance to virus infection in mice (Shresta et al., 2004). This is enforced further by the fact that replication was inhibited when different cell types were treated with IFN prior to viral exposure (Diamond et al., 2000), illustrating that viral replication is essential for the establishment of a solid infection in the host, thus paving the way for further host-to-host transmission and securing viral persistence. It is now evident that most viruses have evolved means to downregulate IFN responses. Hepatitis C virus (HCV), hepatitis A virus and rice stripe virus inhibit IFN production by acting on upstream components of IFN production such as RIG-1 and IRF-3 (Gale & Foy, 2005; Haller et al., 2006). Others such as mumps and nipah viruses encode genes that prevent phosphorylation and translocation of the signal transducer and activator of transcription (STAT), resulting in lower expression of IFN-stimulated gene factors (ISGs) (Kubota et al., 2002; Shaw et al., 2004).

A supplementary table and a supplementary figure are available with the online version of this paper.
The IFN response to dengue infection is not well understood. It has been observed that dengue virus inhibits IFN-α signalling by STAT-2 downregulation in myeloblastoma cell line K562 transfected with dengue replicons (Jones et al., 2005). IFN protects cells from de novo dengue infections but has no effect on established infection, indicating that dengue replication yields IFN antagonists (Diamond et al., 2000). This observation has been proven in cell lines infected with dengue that show inhibition in both type I and type II IFN signalling via NS4B non-structural protein (Munoz-Jordan et al., 2005, 2003). Tyk2 activation was also found to be reduced in dengue-infected dendritic cell lines (Ho et al., 2005). All these studies indicate that the IFN response is somehow impaired by dengue infection, thus facilitating establishment of dengue infection in the host.

We aimed to study the host response to dengue infection by utilizing microarrays and quantitative PCR (qPCR) by TaqMan Low Density Array (TLDA) (Applied Biosystems) to assess the differential gene expression regulation of two closely related DEN2 strains (NGC and TSV01) in A549 cells. Although A549 and HepG2 are transformed lung carcinoma and hepatoma cell lines which might have different IFN responses compared with primary cells, they have the advantage of being highly susceptible to infection. The transcript levels of the IFN pathway genes in these two strains of DEN2 are significantly different. Biochemical dissection revealed that the strains had different effects on STAT-1 and STAT-2 phosphorylation upon IFN-α stimulation. We extended the IFN response analysis to a panel of clinical isolates from Malaysia and Singapore and found that the ability of dengue virus to inhibit the IFN signalling pathway was strain-dependent. This report demonstrated that there is a differential host type I IFN response in human cell lines to dengue virus and that potential virulence factors may be influential in determining the host response, which affects the clinical outcome of dengue infection.

METHODS

Cells and viruses. All cell lines were obtained from ATCC and maintained in RPMI 1640 (BHk-21, C6/36), minimal essential medium (HepG2) or Ham’s F12K (A549) (all GibcoBRL) all supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (GibcoBRL) at 37 °C with 5% (v/v) CO2. C6/36 cells were cultured at 28 °C. Infection was performed with 2% FBS for all cell lines except C6/36, in which 5% FBS was used. DEN2 strains TSV01 (GenBank accession number AY037116) and NGC (GenBank accession number M29095) and clinical isolates were propagated in C6/36. The Malaysian strains 2569 (DEN1), 10340 (DEN2), 22713 (DEN4), 22563 (DEN2) and 10245 (DEN1) were obtained from University of Malaya, Kuala Lumpur, Malaysia, and had been isolated from outbreaks that occurred between 1995 and 2002. Virus stocks were prepared by single passage in C6/36. For virus growth analysis, culture supernatants were recovered between 1995 and 2002. Virus stocks were prepared by single passage in BHK-21 cells in triplicate. Heat-inactivated virus was prepared by incubating virus at 55 °C for 1 h.

Antibodies and plasmids. The following primary antibodies were used to probe the blots: mouse anti-Envelope antibody 4G2 (ATCC), rabbit anti-phosphothreonine 701 STAT1 (Cell Signalling), rabbit anti-phosphothreonine 689 STAT2 (Upstate), rabbit anti-STAT1 (Santa Cruz), rabbit anti-STAT-2 (Santa Cruz) and mouse anti-FLAG (M2; Sigma). NS4B and NS5 antibodies were generated in-house using full-length protein as an antigen. Secondary antibodies were horseradish peroxidase (HRP)-conjugated anti-mouse and anti-rabbit (Pierce) and fluorescein isothiocyanate (FITC)- and tetramethylrhodamine B isothiocyanate-conjugated anti-mouse and anti-rabbit (Molecular Probe).

TLDA and IFN-β Taqman RT-PCR. Thirty-five genes from the IFN pathway were chosen for study and the array was ordered from Applied Biosystems. Total RNA was extracted using RNeasy Mini RNA extraction kit (Qiagen). cDNA was prepared using High-Capacity cDNA Archive kit (Applied Biosystems) on the ABI 9700 PCR Machine (Applied Biosystems). The amount of cDNA was measured on an ND-1000 spectrophotometer (Nanodrop Technologies). RT-PCR mix was prepared using Taqman one-step RT-PCR kit (Applied Biosystems) and RT-PCR was performed on an ABI PRISM 7900HT (Applied Biosystems). All protocols were performed according to manufacturers’ recommendations. The raw data of four independent biological repeats were analysed using the SDS2.2 program (Applied Biosystems), using 18S-RNA as an external control. The significance of gene expression was analysed by EXCEL-SAM version 2.23A (Trustees of Leland Stanford Junior University). False discovery rate (q value) was set as less than 0.05 and the results were filtered with a fold change of 2. For qPCR of IFN-β, total RNA was extracted and cDNA was prepared as above using 1 μL RNA. cDNA (2 μL) was used in a Taqman PCR assay (Applied Biosystems) with human IFN-β primers (Applied Biosystems; ABF/GenBank accession numbers of the target sequence are GL_4504602 S/ NM_002176.1).

Transfection, immunostaining, immunoblotting. A549 cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. Cells (5 x 10⁴) were plated into a well of a 24-well plate the day before transfection. Lipofectamine 2000 (1.5 μL) and DNA (1 μg) were mixed in OPTI-MEM (Invitrogen) without serum or antibiotics for 5 h. The transfection mixture was then removed and cells were cultured for the time period that is indicated in the figure legends. For immunostaining, cells were fixed in cold methanol and stained with primary and secondary antibodies (above) in PBS–BSA (0.1%) and visualized using a Leica DM 4000 fluorescent microscope. For immunoblotting, cell lysates were harvested in modified RIPA buffer (50 mM Tris/HCl, pH 7.4, 1% NP-40, 0.25% sodium-deoxycholate, 150 mM NaCl, 1 mM EDTA, supplemented with protease and phosphatase inhibitors from Sigma); protein concentrations were normalized for immunoblotting. Cell lysates were then separated by SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was incubated with primary antibodies and HRP-conjugated secondary antibodies. Membranes were developed with an enhanced chemiluminescence reaction and exposed to Kodak BioMax XAR film for visualization.
RESULTS

DEN2 strain TSV01 does not inhibit STAT-1 activation

Contradictory results on the IFN response to dengue infection have been reported. While dengue NS4B has been shown to inhibit both type I and type II IFN signalling (Munoz-Jordan et al., 2003), other studies have shown that dengue infection downregulates type I but not type II IFN signalling (Ho et al., 2005; Jones et al., 2005). Therefore, we sought to characterize the IFN response to dengue infection. To investigate this, TSV01 NS4B was transfected into A549 cells and 24 h after transfection, the cells were serum-starved for 12 h and treated with 500 U IFN-β ml⁻¹ for 30 min. Surprisingly, TSV01NS4B did not inhibit phosphorylation of STAT-1 upon stimulation with IFN-β (Fig. 1a), contradicting previous reports. We also looked at A549 cells transfected with NS4B for activation of STAT-1 via its nuclear localization upon stimulation with IFN-β. This demonstrated that STAT-1 nuclear translocation was normal in cells treated with IFN in the presence of NS4B (Fig. 1c).

The IFN suppression studies were performed with overexpressed NS4B, which might not represent the normal physiological situation. We therefore repeated these studies in a whole virus infection setting. A549 cells were infected with TSV01 with an m.o.i. of 10 and treated for 30 min with 500 U IFN-β ml⁻¹ at 72 h post-infection. As seen in Fig. 1(b), TSV01 infection did not inhibit STAT-1 phosphorylation in IFN-treated cells.

Infection with NGC and TSV01 strains induces different IFN responses

Ongoing differential gene expression studies in our laboratory suggested that genomic variations of dengue strains had a role in determining the host response. For example, significance analysis of microarrays (SAM) on HepG2 cells infected for 48 h with an m.o.i. of 10 of either TSV01 or NGC, both serotype 2 viruses, revealed that 88 genes were differentially expressed between cells infected with these two strains. The most important difference was in the genes of the IFN-mediated innate immunity pathway (Hibberd et al., 2006).

In order to elucidate the difference in the IFN response to infection with NGC and TSV01, we chose 35 type I IFN-specific genes and performed qPCR by customized TLDA. A549 cells were infected for 48 h with live and heat-inactivated NGC and TSV01 (m.o.i. of 10), and infection was confirmed with plaque assays from culture supernatants (data not shown). The transcription levels of the genes of infected samples were compared with samples infected with heat-inactivated virus (Table 1). Some of the host response genes were similarly induced by infection with both strains, such as the chemokine CXCL10 that was highly expressed in dengue-infected patients (Fink et al., 2007); however, compared with NGC, TSV01 elicited higher levels of expression of all three IFN response pathway genes, including MX1, OAS1/2/3 and EIF2AK2 (also known as PKR). There were two genes whose transcript levels were higher in NGC than TSV01, IL6 and RSAD2, but expression levels of the remaining 15 IFN-
induced genes were all lower in NGC than in TSV01-infected cells. These results suggest that viral genomic variations generate significant differences in innate immune responses to infection.

### Differential ability of dengue viral strains to suppress type I IFN signalling

NGC elicited very little IFN response compared with TSV01. Previous IFN antagonism studies (Munoz-Jordan et al., 2003) used DEN2 strain 16681, which shows more sequence similarity to NGC than TSV01. We therefore hypothesized that NGC but not TSV01 would inhibit STAT-1 activation upon stimulation with type I IFN. We also included the clinical isolate SG167 (DEN1) from a recent outbreak of dengue virus infection in Singapore as a control, since both TSV01 and NGC are high-passage laboratory strains and might have acquired mutant phenotypes that are not physiologically relevant. HepG2 cells were infected at an m.o.i. of 5 with NGC, TSV01 and SG167. Cells were serum starved for 12, 24 h post-infection, and treated with 500 U IFN-α ml⁻¹ for 30 min.
Fig. 2(a) shows that treating mock-infected cells with IFN-α induced STAT-1 phosphorylation; this was also seen in cells infected with both TSV01 and SG167. However, NGC infection inhibited STAT-1 phosphorylation upon IFN stimulation. This experiment was repeated by treating A549 cells with IFN-α at 24, 48 or 72 h to determine whether TSV01 and SG167 inhibited signalling at an advanced time point. This demonstrated that STAT-1 phosphorylation was inhibited by NGC as early as 24 h post-infection but it was not inhibited by TSV01 or SG167, even after 72 h of infection (Fig. 2b).

**Dengue infection does not inhibit type II IFN signalling**

IFN-α/β signalling requires phosphorylation and subsequent heterodimerization of both STAT-1 and STAT-2, so we looked at STAT-2 activation during A549 infection. Again, NGC inhibited STAT-2 phosphorylation upon treatment with IFN-α, but TSV01 and SG167 did not (Fig. 3a). Interestingly, we did not observe a significant reduction in STAT-2 protein level in NGC-infected cells compared with TSV01- or SG167-infected cells at early time points, but only after 72 h of infection (Fig. 3b). It is possible that the STAT-2 degradation reported by Jones et al. (2005) is a relatively late event in viral survival.

STAT-1 activation and homodimer formation is common to both type I and type II IFN signalling pathways. As NGC inhibited STAT-1 phosphorylation upon IFN-α/β stimulation, we next investigated whether NGC would also inhibit STAT-1 activation in response to IFN type II signalling. A549 cells, which have been shown to express IFN-γ receptor (Luo & Ross, 2005), were infected with of TSV01, NGC and SG167 (m.o.i. of 5) and treated with 500 ng IFN-γ ml⁻¹ for 30 minutes at 48 h post-infection. As Fig. 3(c) shows, activation of STAT-1 was the same in dengue virus-infected cells (all three strains) compared to mock-infected cells. Our results clearly demonstrate that dengue virus manipulates the type I but not type II IFN response to infection in a viral strain-dependent manner. This is in agreement with the report of Ho et al. (2005) that dengue virus antagonizes the IFN-α but not the IFN-γ response, and that the virus probably manipulates the host at the Tyk2 level.

![Fig. 2.](image-url)
Varying the initial input of the virus does not alter the IFN signalling profile of different strains

Infection of A549 cells with NGC, TSV01 and SG167 showed that there were variations in the level of virus they produced. Supernatants from A549 cells infected with TSV01 and SG167 contained less virus by plaque assay compared with supernatant from NGC (see Supplementary Table S1, available in JGV Online). We therefore wanted to verify that the STAT-1 signalling inhibition is not affected by this difference in viral load.

A549 cells were infected with an m.o.i. of 1 of NGC and of 10 of each of TSV01 and SG167 and stimulated with IFN-α. Plaque assay of culture supernatants was used to determine virus production, and showed that an m.o.i. of 1 of NGC yielded a similar number of plaques to an m.o.i. of 10 of SG167 and, to a lesser extent, of TSV01 (Fig. 3e). However, infection with NGC at an m.o.i. of 1 inhibited STAT-1 phosphorylation, whereas neither TSV01 nor SG167 inhibited STAT-1 activation even at an m.o.i. of 10 (Fig. 3d). By immunofluorescence staining of viral envelope protein, we determined that there was no major difference in the percentage of NGC- and TSV01-infected cells after infection with an m.o.i. of 5 of each virus for 36 h (Fig. 3f). This suggested that the viral strain-dependent signalling profiles are independent of the viral load and percentage of infected cells at the point when the cell is stimulated with IFN. These results point towards the presence of a strain-dependent virus-specific factor that regulates this response.

Differential response to IFN signalling in dengue clinical isolates and strain-dependent inhibition of IFN-stimulated genes

We extended our study to a panel of other low-passage clinical isolates. We used clinical isolates MY02569 (DEN1, DHF), MY10245 (DEN1, DF), MY10340 (DEN2, DF), MY22563 (DEN2, DF) and MY22713 (DEN4, DF) to infect A549 cells at m.o.i. of 1 for 36 h and stimulated these cells with IFN-α. As seen in Fig. 4(a), MY10245, MY22563 and MY22713 inhibited STAT-1 phosphorylation in response to IFN treatment, whereas MY10340 and MY20569 did not. Note that plaque assay from culture supernatants of these infections validated our earlier observation that
strain-dependent IFN antagonism is not directly related to viral load. For example, MY10245 and MY10340 gave a comparable number of plaques but differed in their ability to inhibit IFN signalling (Fig. 4b). These results also confirmed that the difference in IFN signalling profiles of dengue viral strains occurred across at least two serotypes (DEN1 and DEN2).

The antiviral effects of IFN are exerted through proteins such as MX, OAS, ADAR and EIF2AK2. Therefore, we looked at the strain-dependent ability of dengue virus to suppress the antiviral genes downstream of Jak/STAT signalling. We used the same TLDA approach as before but in this case the infected cells were stimulated with IFN in order to assess the downstream signalling. We also included SG167 (which did not suppress IFN signalling) and MY22713 (that did suppress IFN signalling) in our study. A549 cells were infected with TSV01, SG167, NGC and MY22713 (m.o.i. of 10), and IFN-α (1000 U ml⁻¹) was added to the medium 18 h post-infection. Samples were collected 24 h later for RNA extraction and TLDA analysis. Heat-inactivated viruses were used as controls in this experiment and four biological repeats were used for analysis and cut offs were employed as described earlier.

Table 2 shows that IFN stimulated genes such as IRF9, MX1, OAS1/2/3, EIF2AK2, ISG20, G1P2/3 and IFI35/44. IFIT1 and IFITM1, which provide the antiviral activity, are suppressed by NGC infection (negative values in Table 2) but not by TSV01. SG167 largely behaved like TSV01 and did not suppress IFN-stimulated genes, whereas MY22713 suppressed a few, but not all, of the genes suppressed by NGC. These results provide evidence that different strains of dengue virus counter the type I IFN antiviral activity to different extents.

**Differential regulation of IFN induction by different strains of dengue**

Our results indicated that there are at least two subsets of dengue strains: inhibiting strains that reduce type I IFN signalling, exemplified by NGC and MY22713, and non-inhibiting, strains that do not reduce type I IFN signalling, exemplified by TSV01 and SG167. We speculated that the non-inhibiting strains have evolved other ways of antagonizing the IFN response. It has been shown that HCV infection fails to trigger the dsRNA signalling pathway in Huh-7 cells but does not inhibit the Jak/STAT signalling pathway (Cheng et al., 2006). Our TLDA results (Table 1) suggest that TSV01 did seem to induce less IFN-β than NGC. In order to validate this observation further, we infected A549 cells with NGC and TSV01. As shown in Fig. 5(a), NGC induced greater amounts of IFN-β than TSV01. Moreover, the transcript level of IFN-β was even lower in TSV01 compared with heat-inactivated TSV01, suggesting that TSV01 further suppressed IFN-β induction. The same difference between NGC and TSV01 was observed in the presence of external IFN-β 18 h post-infection, ruling out differences in the feedback loop of IFN induction.

MAVS is a mitochondrial antiviral protein, the gene for which is upstream of IFN-β activation genes, and its overexpression leads to enhanced IFN-α/β production (Seth et al., 2006, 2005). Since TSV01 weakly induces IFN-β transcription, we hypothesized that overexpression...
of MAVS would sensitize the cells towards defence against the infection. A549 cells were transfected with FLAG-tagged MAVS plasmid; 24 h after transfection, cells were infected with m.o.i. of 5 of NGC and TSV01. Supernatants were collected after 24 h to calculate virus titre and cell lysates were analysed for the presence of MAVS and NS5 protein (Fig. 5b). As seen in Fig. 5(c), MAVS could totally prevent TSV01 infection (no plaques), whereas NGC infection was only about 50% reduced by overexpression of MAVS. This shows that MAVS is involved in host defence against dengue and that NGC differs from TSV01

**Table 2.** Transcription of antiviral genes upon stimulation by exogenous IFN is suppressed by NGC but not by TSV01

A549 cells were infected with live and heat-inactivated TSV01, SG167, NGC and MY22713 strains and stimulated with IFN-β 18 h post-infection. RNA was extracted 24 h after IFN treatment and TLDA was performed. The values are the relative transcript levels of the genes comparing live virus with heat inactivated virus. Negative values indicate lower levels of transcript after live virus infection, hence suppression. –, Indicates non-significant values (q ≧ 0.05).

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*Pathway/function information and GenBank accession numbers for the genes are given in Table 1.

Fig. 5. Differential IFN transcript induction by TSV01 and NGC. (a) A549 cells were infected with TSV01 or NGC or the heat-inactivated viruses (indicated by ‘HI’) (m.o.i. of 5) in the absence of IFN-β for 24 h (black bars) or with IFN-β added 18 h post-infection (white bars). Cells were analysed for IFN-β by qRT-PCR. The fold change was calculated using uninfected A549 cells as a control. Data shown are means ± s.d. (b) A549 cells were transfected with FLAG-tagged MAVS and were mock-infected or infected with either NGC or TSV01 (m.o.i. of 5) 24 h post-transfection. Cells were harvested after 24 h and immunoblotted with FLAG and NS5 antibodies. (c) Infection supernatants from (b) were titrated to measure the decrease in viral replication by plaque assay (data shown are the means of three assays).
in MAVS-mediated antiviral activity. Overall, our results show that, while the strains that inhibit STAT-1 induce IFN and are resistant to IFN production, the non-inhibiting strains might suppress IFN induction and are sensitive to IFN production.

**DISCUSSION**

This report demonstrates that the type I IFN responses in A549 and HepG2 cells are dependent on the dengue viral strain. Interestingly, patient studies as early as the 1950s have indicated the presence of viral virulence factors that cause severe dengue (Bravo et al., 1987; Gubler et al., 1981; Pandey & Igarashi, 2000; Sabin, 1952). Both phylogenetic studies (Leitmeyer et al., 1999; Messer et al., 2003; Rico-Hesse et al., 1997) and epidemiological studies of dengue in the Pacific Islands (Fagbami et al., 1995; Gubler et al., 1978), Indonesia (Gubler et al., 1981) and the Indian subcontinent (Messer et al., 2002) have pointed to specific viral genotypes as being capable of producing DHF epidemics in a population with variable immune status (Cologna et al., 2005). Several studies demonstrated that DEN2 strains of the south-east Asian genotype are more pathogenic compared with the American genotype of DEN2, suggesting the presence of important biological differences among viral genotypes (Kochel et al., 2002; Rico-Hesse et al., 1997; Watts et al., 1999).

Our study provides the molecular basis for the contribution of viral factors that modulate the host cell response and, conceivably, modulate the clinical outcome of dengue infection. We showed that NGC and TSV01, two DEN2 strains that are very similar to each other at the amino acid level, elicit very diverse patterns of type I IFN response to infection in human cell lines. We used two different cell lines in the study in order to avoid cell-line-specific observations: HepG2, a hepatocytic cell line, and A549, a lung carcinoma cell line but neither cell line was the same as primary cells in the in vivo infection situation. Therefore, the observations that we obtained in the cell line study cannot be readily compared with nor be linked to clinical outcome. We also found low-passage clinical isolates that exhibited such strain-dependent variation in the type I IFN response to infection. Based on our results with the type I IFN signalling and TLDA experiments, there seem to be two groups of dengue virus: one that can inhibit the IFN signalling and its antiviral effects (suppressive strains) and the other that cannot (non-suppressive strains) (Fig. 6). This strain-dependent modulation of IFN response seems to be independent of the viral serotype and it occurred in both HepG2 and A549 cell lines. As described in Fig. 6, we predict that the suppressive strains of dengue inhibited STAT1/2 activation, which resulted in the inhibition of the antiviral pathway genes, including MX1, OAS1/2/3, EIF2AK2 and various IFN-induced genes such as G1P2/3, IFI35/44, IFI1 and IFITM1. However, this strain classification is based on cell line observation and cannot be directly extrapolated to the clinical outcome of an infection, which

**Fig. 6.** IFN pathway and dengue virus infection. The binding of IFN-α and -β to their receptors triggers a signal transduction and transcriptional response mediated by the tyrosine kinases, Jak1 and Tyk2. The signal transduction event involves the activation of the transcription factors STAT-1 and STAT-2 via phosphorylation. Phosphorylated STAT-1/2 complex recruits IRF-9, forming a complex known as the IFN-stimulated gene factor 3 (ISGF-3) that translocates to the nucleus and binds to IFN-stimulated response element (ISRE) in the promoter region of IFN-stimulated genes (ISGs). This process initiates transcription of hundreds of different ISGs with antiviral activity. Dengue viruses were grouped into IFN suppressive strains and IFN non-suppressive strains. The IFN suppressive strains inhibited Jak2, STAT-1 and STAT-2 activation, and resulted in suppression of the expression of the genes in all three IFN-induced antiviral pathways: MX1, OAS1/2/3, EIF2AK2, ISG20, G1P2, G1P3, IIF35, IIF44, IIF1 and IIFTM1.
is a combined consequence of both viral and host factors. In fact, with the exception of MY02569 that was isolated from a DHF patient, all of the other strains were isolated from DF patients.

Pre-treatment but not post-infection treatment of cells with IFN has been shown to inhibit dengue (Diamond et al., 2000). However, the effectiveness of IFN in countering dengue infection has been demonstrated by observing monkeys infected with dengue (Ajariyakhajorn et al., 2005). Such inconsistencies in the literature can be explained by the presence of IFN-insensitive and -sensitive dengue virus strains, as demonstrated in this study. TLDA analysis confirmed that NGC and MY22713, which inhibit STAT-1 phosphorylation, elicit more IFN-β production compared with TSV01 and SG167, which do not inhibit STAT-1 phosphorylation. Furthermore, overexpression of MAVS inhibited TSV01 but not NGC infection. These results reveal that the sensitivity of dengue strains to type I IFN are varied, while our signalling data provides evidence for the mechanism of such sensitivity.

Earlier studies that compared south-east Asian DEN2 strains with those from South America identified mutations in the viral 3’ UTR, as well as prM, E, NS4B and NS5, that correlate to the differences in viral survival and replication fitness (Alvarez et al., 2005; Edgil et al., 2003). We found that NGC and TSV01 did not exhibit similar infectivities in A549 cells, but when the viral load of TSV01 was increased to an m.o.i. of 10 and compared with an m.o.i. of 1 of NGC, the IFN signalling profiles of these strains remain unaltered. Moreover, MY10340 and MY10245, which show similar levels of infectivities, differ in their ability to inhibit IFN signalling. Our data clearly demonstrate that the differences in viral load alone cannot explain the differences in the IFN inhibition profiles of viral strains. This leads us to believe that a viral virulence factor might account for the differential response of dengue strains to type I IFN.

NS4B of dengue is thought to be the IFN antagonist but NS4B cloned from TSV01 did not antagonize the IFN response. Munoz-Jordan et al. (2003) showed that NS4B suppresses IFN signalling, using NS4B of a DEN2 infectious clone (pD2/IC-30P-A), which is identical to that of NGC and differs from that of TSV01 by four amino acids (F14L, A19T, I48V, L112F; see Supplementary Fig. S1, available in JGV Online). It is interesting to note that three of these changes in the NS4B sequence should be in the region that is important for IFN antagonism (aa 1–125) (Munoz-Jordan et al., 2003). While it is tempting to think that NS4B is the critical IFN antagonist, membrane topology studies of NS4B (Miller et al., 2006) present a logistical challenge to this hypothesis, because this region has been shown to reside in the endoplasmic reticulum lumen. Moreover, while it has been shown that the 2K fragment of NS4B is essential for IFN antagonism, the existence of uncleaved 2K–NS4B in the viral life cycle is debatable. Full genome sequencing and alignment studies of the clinical isolates are currently being performed. Genetic complementation studies will then be needed to validate these viral factors, which could be used as markers for severe dengue.

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REFERENCES


