Dengue virus inhibits interferon-α signaling by reducing STAT2 expression

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Running title: Inhibition of interferon signaling by dengue virus

Abstract words: 248

Text words: 4,014

1

ABSTRACT

Type 1 interferons (IFN-α/β) are key mediators of innate antiviral responses but have little effect on established replication of dengue viruses, which are mosquito-borne flaviviruses of immense global health importance. Understanding how the IFN system is inhibited in dengue virus-infected cells would provide critical insight into disease pathogenesis. In a recent study analyzing the ability of individual dengue virus-encoded proteins to antagonize the IFN response, non-structural (NS) protein 4B, and possibly NS2A and NS4A, were identified as candidate IFN antagonists. In monkey cells, NS4B appeared to inhibit both IFN-α/β and IFN-γ signal transduction pathways, which are distinct but overlapping (**Munoz-Jordan, J. L., G. G. Sanchez-Burgos, M. Laurent-Rolle, and A. Garcia-Sastre.** 2003. Proc Natl Acad Sci U S A **100:**14333-8). Here we examine the effects of dengue on the human IFN system using cell lines that are stably transfected with self-replicating subgenomic dengue RNA (replicons) and express all the dengue non-structural proteins together. We show that in replicon-containing cells dengue RNA replication and replication of encephalomyocarditis virus, an IFN-sensitive virus, are resistant to the antiviral effects of IFN-α. The presence of dengue replicons reduces global IFN-α-stimulated gene expression, and specifically inhibits IFN-α, but not IFN-γ, signal transduction. In cells containing replicons, or infected with dengue virus, we found reduced levels of signal transducer and activator of transcription (STAT) 2, which is a key component of IFN-α but not IFN-γ signaling. Collectively, these data show that dengue virus is capable of subverting the human IFN response by down-regulating STAT2 expression.

INTRODUCTION

Dengue viruses are mosquito-borne flaviviruses of immense global public health importance, causing tens of millions of human infections worldwide each year (11). Intensity of viral replication in the first days of infection determines clinical outcome, which ranges from benign febrile illness to life-threatening disease (dengue hemorrhagic fever) (39). During this critical early phase, prior to full recruitment of antigen-specific defenses, innate cellular antiviral mechanisms mediated by IFN-α/β are potentially the most important pathways of host defense limiting viral replication. Virus infection classically induces secretion of IFN-α/β, which bind to cell-surface IFN alpha receptors (IFNAR, comprising IFNAR1 and IFNAR2 subunits) on infected and nearby cells. Binding of IFN-α/β to IFNAR leads to activation of Jak1 and Tyk2 kinases via tyrosine phosphorylation (4). In turn, STAT2 and then STAT1 are phosphorylated and form heterodimers, which then associate with p48/IRF-9 to form ISGF3 complexes (12). ISGF3 complexes translocate to the nucleus and initiate transcription of interferon-stimulated genes (ISGs) by binding interferon-stimulated response elements (ISREs), leading to transcriptional up-regulation of hundreds of cellular genes and induction of an antiviral state (35).

Experimental evidence suggests that the IFN system may play an important role in limiting dengue virus replication, since knockout mice that lack type 1 IFN receptors develop severe infection after challenge with dengue virus (15, 34). Also, pre-treatment of cultured cells with IFN-α/β dramatically reduces dengue virus replication (5, 6). This occurs primarily through inhibition of translation of input strand dengue RNA, by an unknown mechanism (5). In contrast, IFN-α/β has little effect on dengue replication after viral replication has been established (5, 6),

suggesting that the IFN system can not fully engage in dengue-infected cells. In keeping with this observation, dengue virus can achieve high titres ($<10^9$ infectious doses per ml) in humans despite induction of high levels of circulating IFN-α (21, 36, 39). It seems likely therefore that dengue virus has evolved mechanisms to counter the IFN response, although not absolutely, which is a characteristic that may be shared by many pathogenic viruses (9, 42).

Muñoz-Jordan and colleagues recently published an *in vitro* study that analyzed the ability of individual dengue proteins to block the IFN system, and concluded that NS4B, and possibly NS2A and NS4A, may act as IFN-signaling inhibitors (25). They showed that NS4B, and dengue virus infection, blocked signal transduction in response to both IFN-β and IFN-γ in a monkey kidney cell line, suggesting that the target for NS4B-mediated inhibition of IFN signaling may be a component (possibly phosphorylated STAT1 (STAT1-P)) that is common to these distinct but overlapping signal transduction pathways (25). We adopted a complementary experimental approach specifically aimed at studying the effect of dengue virus replication downstream of translation of input strand RNA on the human IFN system. We first established human cell lines that continuously express self-replicating subgenomic dengue RNA (replicons). Flavivirus replicons express all the viral non-structural proteins together in a way that mimics expression during authentic viral infection, and have proved powerful tools for studying the functional roles of non-structural proteins in RNA and virus replication (16-18, 24). We show here that the presence of dengue replicons in human cell lines inhibits the antiviral effect of IFN-α by blocking early events in IFN-α signal transduction, resulting in reduced levels of STAT1-P. In contrast, STAT1-P levels in replicon-containing cells are increased rather than

reduced in response to IFN-γ. We show that steady-state levels of STAT2 are reduced in cells containing dengue replicons, which is consistent with the observed responses to IFN-α and IFN-γ. Reduced STAT2 levels are also found in cells infected with dengue virus, suggesting that dengue virus is capable of subverting the human IFN response by down-regulating STAT2 expression.

MATERIALS AND METHODS

Cell lines stably expressing dengue replicons. A series of cell lines that continuously express dengue replicons have been established in our laboratory (manuscript in preparation). In this study, K562 (human chronic myeloid leukemia) and THP-1 (human monocytic) cell lines stably expressing the dengue replicon ΔCprME-PAC2A were used (designated K562.ΔCprME-PAC2A and THP-1.ΔCprME-PAC2A respectively). The plasmid pDENΔCprME-PAC2A was used for *in vitro* transcription of ΔCprME-PAC2A replicon RNA. pDENΔCprME-PAC2A was derived from pDVWS601 (29), which contains a genomic length dengue virus type 2 (New Guinea C strain) cDNA clone, by the introduction of a large in-frame deletion in the structural region, retaining only the first 27 codons of the C gene and the last 24 codons of the E gene. In addition, pDENΔCprME-PAC2A contains an antibiotic selection cassette encoding puromycin N-acetyl transferase (PAC) followed by an artificial protein "cleavage" site (foot-and-mouth disease virus protein 2A) in place of the deleted structural genes (Fig. 1). Cells stably expressing dengue replicon RNA were generated by transfection with ΔCprME-PAC2A RNA and then propagation in RPMI containing 10% FBS and 3 μg/ml puromycin (Sigma). Cells were removed from puromycin selection and checked for replicon expression before use by indirect immunofluorescence for dengue NS1 protein, using a specific monoclonal antibody (5H5.4, (7)). K562 and THP-1 cells without replicons were continuously maintained in the same medium without puromycin.

"Cured" K562 cell line. K562 cells that were stably expressing ΔCprME-PAC2A were removed from puromycin selection and passaged continuously in RPMI containing 10% FBS and 500 μg/ml glycyrrhizic acid (Fluka Chemicals), which has

6

activity against RNA viruses through an unknown mechanism (3). At intervals, cells were checked for replicon expression by indirect immunofluorescence for dengue NS1 protein and RT-PCR for dengue RNA (see below). Once the cell line had been "cured" of the replicon, it was subsequently grown in RPMI containing 10% FBS, without glycyrrhizic acid, and checked for the continued absence of replicons as before.

Analysis of dengue RNA levels and NS1 expression. K562.ΔCprME-PAC2A cells were grown in the presence of 0, 10, 100, 1000 or 10000 IU/ml IFN-α2a (Roferon-A, Roche) for 24 hours, and then total cellular RNA was extracted in Trizol (Invitrogen). Extracted RNA was treated with RQ1 RNase-free DNase (Promega) and reverse transcribed with M-MLV reverse transcriptase (Promega) using random decamer primers. The PCR reaction was performed and analyzed on the Rotorgene (Corbett Research) using custom primers and a fluorescent probe specific for dengue NS1 (forward 5'CTGAAGTGTGGCAGTGGGATT, reverse 5'CTTCAAAGCTAGC TTCAGCTATCCA and probe 5'CACAGACAACGTGCACACATGGACAGA). The housekeeping gene GAPDH was analysed in the same samples using specific primers (forward 5'ACAGTCCATGCCATCACTGCC, reverse 5'GCCTGCTTCACCACCTT CTTG) and QuantiTect SYBR green (Qiagen). In parallel experiments, K562.ΔCprME-PAC2A cells were grown in the presence of 100 IU/ml IFN-α2a and cell-associated and secreted dengue NS1 protein were analysed by immunoblotting and ELISA, respectively, as previously described (14, 44).

EMCV *trans* rescue assay. K562 cells that did and did not contain dengue replicons were grown in RPMI containing 10% FBS with 0, 10 and 100 IU/ml IFN-α2a for 24

hours. Cells were washed in RPMI and 1×10^6 cells were then infected with 5×10^5 pfu of encephalomyocarditis virus (EMCV) in RPMI containing 2% FBS for 1 hour. Cells were washed and then cultured for a further 24 hours in RPMI + 10% FBS. Culture supernatants were then harvested, and serial dilutions were plated on to confluent A549 cells in a 96-well plate for 1 hour before replacing the inoculum with RPMI containing 10% FBS. After a further 24 hours, the A549 cells were fixed and stained with methyl violet, and the optical density in each well was read at 570 nm in an automated plate reader (27).

MxA and PKR gene expression. K562 and K562.ΔCprME-PAC2A cells were grown in the presence or absence of 100 IU/ml IFN-α2a for 6 and 24 hours. RNA extraction and reverse transcription were performed as described above. PCR reactions were performed and analyzed on the Rotorgene using SYBR green as before, using specific primers for the MxA gene (forward 5'AACAACCTGTGCAGCCAGTA, reverse 5'AAGGGCAACTCCTGAGAGTG) or PKR gene (forward 5'TCTCTGGCGGTCTTCAGAAT, reverse 5'ACTCCCTGCTT CTGACGGTA). The housekeeping gene GAPDH was analysed in the same samples as described above.

ISG expression profiling: macroarray analysis. K562 and K562.ΔCprME-PAC2A cells (2×10^7 per reaction) were treated with 100 IU/ml IFN-α2a for 24 hours before extracting total cellular RNA with Trizol. Radiolabelled cDNA was generated from 20 μg total RNA by reverse transcription with Superscript II (Gibco) in the presence of ^{32}P-dCTP. Residual RNA was hydrolysed by alkaline treatment at 70 °C for 20 min., and the cDNA was purified using G-50 columns (Amersham Pharmacia).

Before hybridisation to the macroarrays the labelled cDNA was mixed with 50 µg COT-DNA (Gibco) and 10 µg Poly-A DNA (Sigma), denatured at 95°C for 5 min., and hybridised for 1 hour to minimise non-specific binding. Preparation of the macroarrays (representing 150 genes, including many known to be stimulated by interferon), hybridisation of the radioactive cDNAs and scanning and analysis of the macroarrays were carried out as described previously (33).

Immunoblotting. K562 cells (2.5×10^5 per reaction) that did and did not contain replicon were stimulated with 100 IU/ml IFN-α2a or IFN-γ (R&D Systems) for 30 min. Unstimulated cells were included for comparison. Cells were harvested and lysed in 250 µl SDS loading buffer (0.0625 M phosphate, pH 7.0, 10% glycerol, 2% SDS, 0.001% bromophenol blue) pre-warmed to 60 °C. Ten µl of each sample was separated by SDS-PAGE electrophoresis, and proteins were then transferred to a polyvinylidene difluoride membrane (Hybond-P, Amersham). Mouse monoclonal antibodies to STAT1, phosphorylated STAT1 (Tyr-701) (both Zymed) or STAT2 (BD Transduction Laboratories), a rabbit polyclonal antibody to phosphorylated STAT2 (Upstate Biotechnology), a goat polyclonal antibody to IFNAR1 (Abcam), and a rabbit polyclonal to IFNAR2 (PBL Biomedical Laboratories) were used as primary antibodies. Detection was performed using the relevant horseradish peroxidase conjugated secondary antibodies (Jackson Immunochemicals) and enhanced chemiluminescence reagents (ECL+, Amersham).

FACS analysis. K562 and K562.ΔCprME-PAC2A (1×10^6 per reaction) were stained with anti-IFNAR2 antibody in RPMI containing 2% FBS at 4°C. Detection was performed using a phycoerythrin-conjugated donkey anti-rabbit secondary antibody

(Jackson Immunochemicals) and samples analysed on a Becton Dickinson FACScan. Data anaylsis was performed using WinMDI software.

Dengue virus infection of K562 cells. K562 cells were incubated with dengue virus type 2 (New Guinea C strain) at a multiplicity of infection of 4 and then grown in RPMI containing 10% FBS. After 48 hours, cells were air-dried onto glass slides and fixed in cold methanol:acetone (50:50 v/v). Cells were dual-labeled with mouse anti-dengue NS1 antibody (5H5.4) and rabbit anti-STAT2 antibody (C20, Santa Cruz Biotechnology). Fluorescein isothiocyanate-conjugated goat anti-rabbit and Texas Red-conjugated horse anti-mouse antibodies (both Vector Laboratories) were used for detection. Images were analysed on a BioRad Radiance 2100 confocal microscope. In parallel experiments, cells (2 x 10^6 per reaction) were lysed and analysed by immunoblotting as described above.

RESULTS

Dengue replicon RNA replication is resistant to IFN-α. Previous studies have shown that the antiviral effect of IFN-α on dengue virus infection in cell culture is markedly abrogated if treatment is delayed by a few hours after infection (5, 6), suggesting that dengue virus can counter the IFN response once replication has been established. We tested directly whether IFN could inhibit established dengue RNA replication in the form of the dengue virus replicon ΔCprME-PAC2A, which is stably maintained in K562.ΔCprME-PAC2A cells (manuscript in preparation). K562.ΔCprME-PAC2A cells were grown in the presence of 0, 10, 100, 1000 or 10000 IU/ml IFN-α2a for 24 hours, and dengue replicon RNA levels were measured by quantitative RT-PCR. In addition, the effect of 100 IU/ml IFN-α on levels of cell-associated and secreted NS1 protein were analysed by Western blotting and ELISA, respectively. Figure 2 shows that IFN-α had no significant effect on dengue replicon RNA levels or NS1 expression. These data confirm previous evidence suggesting that established dengue RNA replication is resistant to IFN-α. (5).

Antiviral action of IFN-α is blocked by dengue RNA replication. We next tested whether the presence of dengue replicons inhibits the general antiviral action of IFN in cells. K562 cells that did and did not contain replicons were first treated with IFN-α2a and then infected with an IFN-sensitive virus, EMCV. The basis of this technique is that inhibition of the antiviral action of IFN by dengue replicons results in rescue of EMCV replication, which is detected in a modified plaque assay on A549 cells. Figure 3 shows that, in the absence of IFN-α2a, EMCV replication was equal in cells that did and did not contain replicons. As expected, pre-treatment of K562 cells with

11

either 10 or 100 IU/ml IFN-α2a dramatically inhibited replication of EMCV. In contrast, pre-treatment of K562.ΔCprME-PAC2A cells with the same concentrations of IFN-α2a had no effect on EMCV replication (Fig. 3). In order to prove that dengue RNA replication inhibited the IFN response, the EMCV rescue assay was repeated using K562.ΔCprME-PAC2A cells that had previously been "cured" of the replicon by continuous growth in the presence of glycyrrhizic.acid. "Cured" K562 cells were negative for the presence of dengue NS1 protein and dengue RNA by indirect immunofluorescence and RT-PCR, respectively (manuscript in preparation). "Cured" K562 cells reverted to the IFN-responsive phenotype of the original K562 cells (data not shown). Taken together, these data show that the antiviral activity of IFN is blocked in the presence of dengue replicons.

IFN-α-induced gene expression is inhibited in replicon-containing cells. Different viruses have evolved a diverse range of molecular mechanisms that act on different cellular targets to inhibit IFN-mediated antiviral pathways (9, 13). In order to test whether the observed inhibition of the antiviral effect of IFN in replicon-containing cells was due at least in part to inhibition of IFN signal transduction, we first measured induction of MxA and PKR gene transcription. These genes are classical ISGs that contain an ISRE within the promoter region (20) and encode proteins that are key mediators of the antiviral effects of IFN (13). K562 cells that did and did not contain replicon were stimulated with IFN-α2a for 6 and 24 hours, and MxA and PKR gene transcription were analysed by quantitative RT-PCR. As expected, IFN induced transcription of both of these genes in K562 cells. In contrast, IFN induction of MxA and PKR gene transcription was dramatically inhibited in K562.ΔCprME-PAC2A cells (Fig. 4). Having shown that induction of two genes, MxA and PKR, is

inhibited in replicon-containing cells, we examined the global pattern of IFN-inducible gene transcription in cells that did and did not contain replicons using a custom macroarray. We found that the IFN response was profoundly suppressed in K562.ΔCprME-PAC2A cells when compared with K562 cells. Table 1 lists the ISGs that were most up-regulated (more than 4-fold) in K562 cells in response to IFN, and shows the comparative data from K562.ΔCprME-PAC2A cells. These data indicate that IFN-α-induced gene expression is inhibited in cells containing dengue replicons, although not absolutely, and suggest that IFN-α signal transduction is inhibited.

Dengue RNA replication inhibits early events in IFN-α signaling by down-regulating steady-state STAT2 levels. In order to determine whether early events in IFN signal transduction were inhibited in replicon-containing cells, we performed Western blot analysis of STAT1 phosphorylation in K562 cells that did and did not contain replicons. Steady-state levels of STAT1 were similar in each cell line (Fig. 5A). As expected, treatment with IFN-α induced STAT1 phosphorylation in K562 cells. Levels of phosphorylated STAT1 (STAT1-P) were significantly lower in K562.ΔCprME-PAC2A cells in response to IFN-α, but the response was restored to normal in "cured" K562 cells. In contrast, STAT1-P levels were not reduced in K562.ΔCprME-PAC2A cells in response to IFN-γ, which signals through a distinct but overlapping pathway (9); in fact, we observed a consistent increase in STAT1 phosphorylation in response to IFN-γ in K562.ΔCprME-PAC2A cells when compared with K562 cells (Fig. 5A). In order to ensure that these observations were not limited to the specific cell type used, these experiments were repeated using THP-1 cells that stably expressed ΔCprME-PAC2A, with similar results (Fig. 5B). These data imply

13

that early components of the IFN-α but not IFN-γ signal transduction pathway are targets for dengue virus inhibition.

We next performed Western blot analysis of STAT2 phosphorylation, which is a key step in IFN-α but not IFN-γ signaling (9). Figures 5A and B show that steady-state levels of STAT2 were very markedly reduced in both K562 and THP-1 cells containing dengue replicons when compared with parental cells. Levels of STAT2-P in response to IFN-α were also greatly reduced in both replicon-containing cell lines. "Cured" K562 cells reverted to the phenotype of parental K562 cells, with similar steady-state levels of STAT2, and STAT2-P in response to IFN-α (fig. 5A). In order to confirm that cells containing dengue replicons did not have generally reduced expression of proteins involved in the first part of the IFN-α signal transduction pathway, we examined levels of IFNAR1 and IFNAR2. Total IFNAR1 and IFNAR2 protein levels were assessed by Western blotting and were similar in K562 and K562.ΔCprME-PAC2A cells (Fig. 6A). Cell surface expression of IFNAR2 was measured by flow cytometry and was similar in K562 cells that did and did not contain replicons (Fig. 6B); reagents to examine cell surface expression of IFNAR1 by FACS analysis were not available. Similar levels of IFNAR1 and IFNAR2 were also found in THP-1 cells that did and did not contain replicons (data not shown). Taken together, these data show that the presence of dengue replicons specifically inhibits early events in IFN-α but not IFN-γ signal transduction by reducing STAT2 levels.

Dengue virus infection reduces STAT2 levels. In order to determine whether STAT2 expression is also reduced in dengue-infected cells, we infected K562 cells

with dengue virus type 2 and analysed STAT2 expression in individual cells using dual-label immunofluorescence. We observed infected cells with markedly reduced staining for STAT2 compared with neighboring, uninfected cells (fig. 7A and B). In order to assess STAT2 expression in the whole population of cells infected with dengue virus, we analysed STAT2 levels 48 hours post-infection by immunoblotting. At this time point, approximately 30-50% of cells that had been infected stained strongly positive for NS1 protein by immunofluorescence. Figure 7C shows that STAT2 levels were markedly reduced in cells infected with dengue virus when compared with mock-infected cells, whereas STAT1 levels were unchanged. Collectively, the data suggest that down-regulation of STAT2 expression is a key component of dengue virus countermeasures against the human IFN response.

DISCUSSION

In this study, we used human cell lines that stably express dengue replicons to show that dengue RNA replication inhibits early events in IFN signaling. This work extends a related study published by Muñoz-Jordan and colleagues during the progress of our research, showing that dengue virus proteins NS2A, NS4A and NS4B have the capacity to inhibit nuclear translocation of STAT1-P in response to IFN-β (25). We show here for the first time that dengue RNA replication not only reduces STAT1-P levels in response to IFN-α, but also results in marked reduction of STAT2-P levels as a consequence of reduced steady-state levels of STAT2. We first made this observation in dengue replicon-containing cells, and subsequently in cells infected with dengue virus. Since STAT2-P recruits STAT1 for phosphorylation in response to IFN-α (22), generating STAT1/STAT2 heterodimers, all our data can be explained if dengue virus specifically down-regulates STAT2 levels in order to counter the IFN response. In keeping with this, dengue RNA replication did not reduce STAT1 phosphorylation in response to IFN-γ, which is independent of STAT2 (22). In fact, dengue replicon RNA replication increased rather than decreased STAT1 phosphorylation in response to IFN-γ, consistent with previous data showing increased STAT1 phosphorylation (30) and IFN-γ-mediated gene transcription (1) in the context of reduced STAT2 levels. The mechanism underlying this effect and its biological significance are, as yet, unknown.

We conclude that dengue virus specifically inhibits IFN-α/β signaling by down-regulating expression of STAT2. Muñoz-Jordan and colleagues reported conflicting data showing inhibition of both IFN-α/β and IFN-γ-mediated STAT1 phosphorylation by dengue NS4B, and dengue virus infection, in LLCMK2 (monkey kidney) cells,

and suggested that common players involved in both signaling pathways (which does not include STAT2) were likely targets for IFN antagonism by dengue (25). The differences in our findings may reflect interspecies differences in IFN antagonism. Although the cell tropism of dengue virus in humans is not definitively known, the predominant targets are probably cells of hematopoietic origin, in particular dendritic cells, monocytes and macrophages, and also hepatocytes (2, 40, 41). In this study, we used human cell lines (K562 and THP-1) related to cells targeted by dengue *in vivo*, and our data provide the basis for further work to confirm the relevance of our observations in human dengue infection.

Despite the fact that IFN-α signaling was not completely blocked in dengue replicon-containing cells, supra-physiological concentrations of IFN-α2a had no significant effect on dengue replicon RNA replication or protein production. It is possible that dengue utilizes more than one mechanism to counter the IFN response, as has been suggested for other viruses including another flavivirus, hepatitis C virus (8, 28, 37, 38). Alternatively, inhibition of IFN signaling by reducing STAT2 levels may be sufficient for dengue replication to proceed faster than can be inhibited by the reduced IFN antiviral response. Future work will elucidate whether STAT2 levels are reduced as a consequence of down-regulation of STAT2 gene transcription or protein synthesis, or enhanced degradation of STAT2 protein. Further data are also needed to know if the capacity to reduce STAT2 levels is conserved among all dengue strains (including field isolates) and in any members of the *Flaviviridae* family other than dengue. Recent data suggest that Japanese Encephalitis Virus also inhibits IFN-α signal transduction but utilizes a different mechanism that results in reduced levels of phosphorylated Tyk2 (and hence STAT2-P) without altering expression of STAT2

(23). Two important human pathogens in the *Paramyxoviridae* family of enveloped, negative-strand RNA viruses, Respiratory Syncytial Virus and Human Parainfluenzae Virus Type 2, have been shown to subvert IFN antiviral responses by reducing STAT2 levels (26, 30). This effect is likely mediated through proteosome-mediated degradation of STAT2. However, other paramyxoviruses do not reduce steady-state STAT2 levels and instead have evolved a variety of different strategies to block IFN-α/β signaling (10, 19, 32, 43), which is a broadly effective strategy to counter the IFN response.

Future work is needed to define the precise interaction between components of the IFN signal transduction pathway and specific dengue proteins. Replicon-containing human cell lines are powerful tools for these studies because preliminary evidence suggests that several dengue non-structural proteins may act together to produce strong, species-specific inhibition of IFN (25). We were concerned to ensure that our observations did not reflect selection of an IFN-defective sub-population of cells during generation of our stable replicon-containing cell lines. Several lines of evidence mitigate against this conclusion and suggest that our results accurately reflect an important host-pathogen interaction that subverts the IFN response: replicon-expressing cells were propagated from a total population of transfected cells rather than individual cell clones; our observations were the same in two cell types, K562 and THP-1; and K562 cells that had previously expressed replicons and had been "cured" with glycyrrhizic acid reverted to the phenotype of parental K562 cells. Most importantly, the key observation made in our replicon model, namely reduction in STAT2 levels, led us to the same finding in cells infected with dengue virus. Collectively, the data suggest that down-regulation of STAT2 expression is an

important mechanism by which dengue virus subverts innate antiviral defences mediated by IFN.

Understanding the molecular basis of the race between dengue virus replication and the IFN response early in infection would represent a critical advance: the efficiency with which dengue virus evades the IFN response in humans is probably an important factor in early viral replication and hence disease pathogenesis. Further data will elucidate whether differences in IFN antagonism contribute to differences in pathogenicity observed between dengue strains (31). Understanding the molecular mechanisms that dengue virus utilizes to subvert innate immune responses mediated by IFN may also inform strategies for rational attenuation, in order to generate safer and more cost-effective dengue vaccine candidates.

ACKNOWLEDGMENTS

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FIGURE LEGENDS

Figure 1. Schematic showing construction of plasmid pDENΔCprME-PAC2A. **(A)** Dengue virus type 2 cDNA infectious clone (in plasmid pDVWS601 (29)), showing single open reading frame encoding 3 structural genes (C, core; prM, pre-membrane; E, envelope), 7 non-structural (NS) genes and flanking 5'- and 3'- untranslated regions (UTR). **(B)** pDENΔCprME: a large in-frame deletion has been introduced within the region encoding structural genes. **(C)** pDENΔCprME-PAC2A: an antibiotic selection cassette encoding puromycin N-acetyl transferase (PAC) and foot-and-mouth disease virus protein 2A has been cloned in place of the deleted structural genes.

Figure 2. Dengue replicon RNA replication is resistant to IFN-α. **(A)** K562.ΔCprME-PAC2A cells were grown in the presence of various concentrations IFN-α2a as indicated for 24 hours. Dengue replicon RNA levels were measured using quantitative PCR and normalised to GAPDH mRNA. **(B)** K562.ΔCprME-PAC2A cells were grown in the presence of 100 IU/ml IFN-α2a for 0, 6 and 24 hours. Cell lysates (2×10^5 cells per reaction) were separated by SDS-PAGE, and then dengue NS1 protein was analysed by immunoblotting. K562 cells were included as a negative control. **(C)** K562.ΔCprME-PAC2A cells were grown in the absence (black bars) or presence (white bars) of 100 IU/ml IFN-α2a for 24 and 48 hours. The cumulative concentrations of NS1 in the culture supernatants at each time-point were measured by ELISA.

Figure 3. Antiviral effect of IFN-α is blocked in dengue replicon-containing cells.
K562 cells (solid lines) and K562.ΔCprME-PAC2A cells (broken lines) were treated with 0 (●), 10 (□) or 100 (○) IU/ml IFN-α2a for 24 hours. Cells were then infected with EMCV, and after a further 24 hours supernatants were harvested and serially diluted on confluent A549 cells. After 24 hours, A549 cells were fixed and stained with methyl violet. The amount of staining was quantified by measuring the optical density (OD) of each well at 570 nm. More EMCV replication results in increased cell death and hence lower OD readings.

Figure 4. Induction of classical ISGs by IFN-α is inhibited in dengue replicon-containing cells. K562 cells (black bars) and K562.ΔCprME-PAC2A cells (white bars) were stimulated with 100 IU/ml IFN-α2a for 6 and 24 hours. **(A)** MxA and **(B)** PKR gene transcription were measured by real-time PCR and normalised to the housekeeping gene GAPDH.
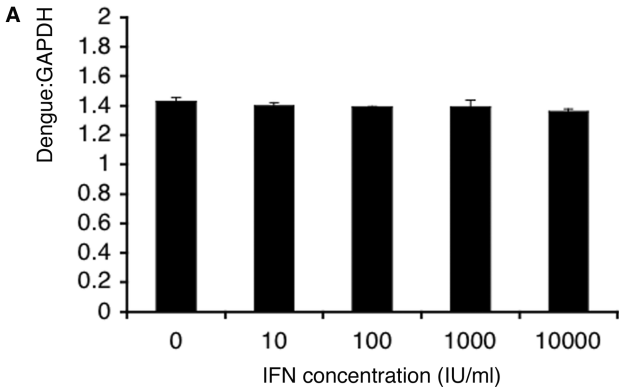
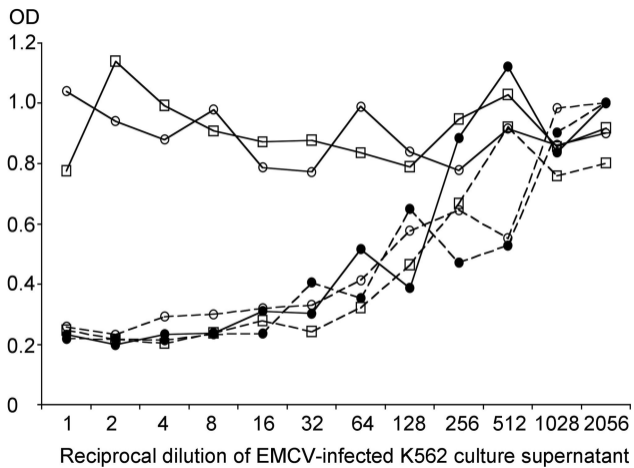**Figure 5. Dengue RNA replication inhibits STAT1 and STAT2 phosphorylation in response to IFN-α and reduces steady-state levels of STAT2. (A)** K562, K562. ΔCprME-PAC2A and "cured" K562 cells, or **(B)** THP-1 and THP-1.ΔCprME-PAC2A cells were treated with and without 100 IU/ml IFN-α or IFN-γ for 30 min., and then lysed in SDS loading buffer. Proteins were separated by SDS-PAGE and then analysed by immunoblotting using specific antibodies for phosphorylated STAT1, STAT1, phosphorylated STAT2 and STAT2 as indicated.
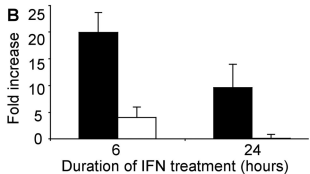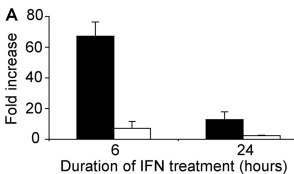
Figure 6. Dengue RNA replication does not affect IFNAR protein levels. (A) Cell lysates from K562 and K562.ΔCprME-PAC2A cells were separated by SDS-PAGE and then analysed by imunoblotting using specific antibodies for IFNAR1 and IFNAR2 as indicated. **(B)** K562 and K562.ΔCprME-PAC2A were stained with specific anti-IFNAR2 antibodies and analysed by flow cytometry. Cells stained with secondary antibody alone (gray-filled plot) were included as a negative staining control.
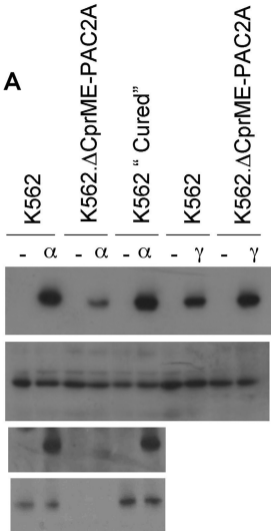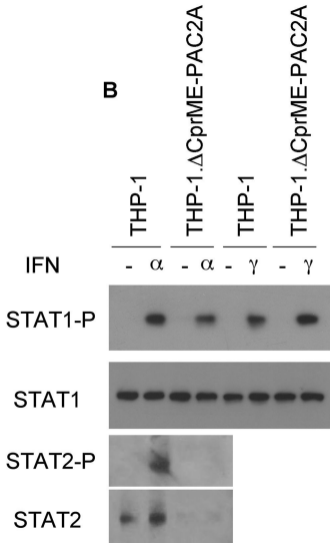
Figure 7. Dengue virus infection reduces STAT2 levels. K562 cells were infected with dengue virus for 48 hours and then dual-stained with **(A)** anti-dengue NS1 mouse monoclonal antibody followed by Texas Red-labeled secondary antibody to detect dengue-infected cells, and **(B)** anti-STAT2 rabbit polyclonal antibody followed by fluorescein-labeled secondary antibody to detect STAT2. Cells were visualised by confocal microscopy. The arrow shows an infected cell. **(C)** Cell lysates were separated by SDS-PAGE and then analysed by immunoblotting using specific antibodies for dengue NS1, STAT1 and STAT2 as indicated. Mock-infected cells were included for comparison.
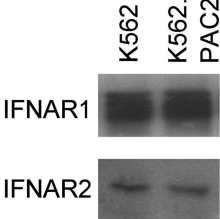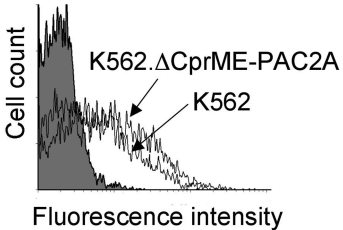
5'UTR C prM E NS1 NS2A NS2B NS3 NS4A NS4B NS5 3'UTR A

(C) B

(E) PAC 2A C

A

B

C

NS1

K562   K562.ΔCprME
        -PAC2A

Duration of IFN
treatment (hours)

OD

Reciprocal dilution of EMCV-infected K562 culture supernatant

A

	K562	K562.ΔCprME-PAC2A	K562 "Cured"	K562	K562.ΔCprME-PAC2A
IFN	− α	− α	− α	− γ	− γ

STAT1-P

STAT1

STAT2-P

STAT2

B

	THP-1	THP-1.ΔCprME-PAC2A	THP-1	THP-1.ΔCprME-PAC2A
IFN	− α	− α	− γ	− γ

STAT1-P

STAT1

STAT2-P

STAT2

A

K562 | K562.ΔCprME-PAC2A

IFNAR1

IFNAR2

B

Cell count

K562.ΔCprME-PAC2A
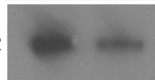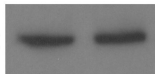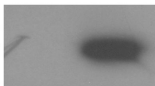
K562

Fluorescence intensity
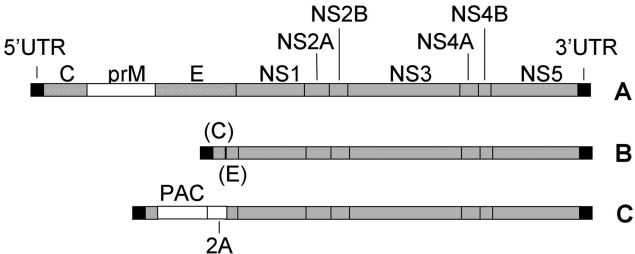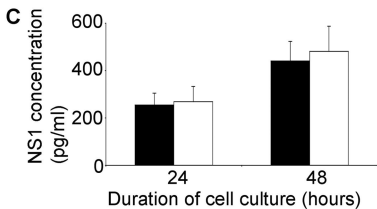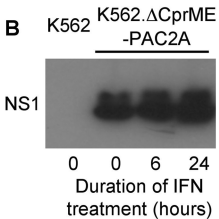
Table 1. ISG transcription in response to IFN-α in cells that do and do not contain dengue replicons. ISGs up-regulated more than 4-fold in K562 cells are shown in comparison to K562.ΔCprME-PAC2A cells.

	Fold induction	
	K562	K562.ΔCprME-PAC2A
IFN-alpha induced protein 27	55.8	11.5
VCAM-1	30.6	2.6
MxA	17.9	1.8
IFN-alpha induced protein (clone IFI-616)	12.9	1.1
met proto-oncogene (hepatocyte growth factor)	7.9	1.8
PSMB9	7.9	0.4
IFN-induced protein 17	7.5	1.3
Vipirin (cig5)	7.2	1.3
IL-15	6.5	1.1
9-27mrna	6.4	1.2
STAT1	6.2	1.1
STAT4	6.2	1.5
IFIT1	6.1	1.3
KIAA0284	5.6	1.5
STAT1 (91kDa)	4.9	1.8
IFN induced transmemberane protein 3	4.9	1.4
INDO	4.8	0.6
IL-6	4.8	0.6
IFN induced transmemberane protein 2	4.5	1.1
MAP2K4	4.5	1.0
IFI35	4.4	1.1
Homo sapiens STAT	4.1	1.6