Critical role of RIG-I and MDA5 in early and late stages of Tulane virus infection

Preeti Chhabra,1 Priya Ranjan,2,* Theresa Cromeans,3 Suryaprakash Sambhara2 and Jan Vinjé1,*

**Abstract**

Human noroviruses are a major cause of acute gastroenteritis worldwide, but the lack of a robust cell culture system or small animal model have hampered a better understanding of innate immunity against these viruses. Tulane virus (TV) is the prototype virus of a tentative new genus, Recovirus, in the family Caliciviridae. Its epidemiology and biological properties most closely resemble human norovirus. The host innate immune response to RNA virus infection primarily involves pathogen-sensing toll-like receptors (TLRs) TLR3 and TLR7 and retinoic acid-inducible gene I-like receptor RIG-I and melanoma differentiation associated gene 5 (MDA5). In this study, by using siRNA knockdown, we report that TV infection in LLC-MK2 cells results in an early [3 h post infection (h p.i.), P<0.05] RIG-I-dependent and type I interferon-mediated antiviral response, whereas an MDA5-mediated antiviral effect was observed at later (12 h p.i.; P<0.05) stages of TV replication. Induction of RIG-I and MDA5 was critical for inhibition of TV replication. Furthermore, pre-activation of the RIG-I/MDA5 pathway prevented TV replication (>900-fold decrease; P<0.05), suggesting that RIG-I and MDA5 ligands could be used to develop novel preventive and therapeutic measures against norovirus.

**INTRODUCTION**

The family *Caliciviridae* consists of five genera, namely *Norovirus*, *Sapovirus*, *Lagovirus*, *Vesivirus* and *Nebovirus* [1]. Of these, noroviruses and sapoviruses are important etiological agents of viral gastroenteritis in humans. Noroviruses are the leading cause of epidemic and sporadic acute gastroenteritis and they infect people of all ages worldwide [2]. Many aspects of human norovirus biology are poorly understood, primarily because of the lack of a robust *in vitro* cell culture system or small animal models [3]. Most of the data on norovirus biology has been obtained from cultivable animal caliciviruses [4].

Tulane virus (TV) is the prototype virus of a new proposed genus, *Recovirus*, in the family *Caliciviridae* [5]. The virus is non-enveloped with icosahedral symmetry and contains a linear single-stranded RNA molecule of ~6.7 kb in length [6]. The complete TV genome is organized into three ORFs, of which ORF1 encodes six nonstructural proteins, while ORF2 encodes the major structural protein (VP1) and ORF3 a minor structural protein (VP2) [5]. Compared to human noroviruses, TVs have a similar genome organization, are genetically diverse and consist of genotypes that show differential binding patterns to histo-blood group antigens [6, 7].

An effective innate immune defence is critical to protect a host against invading viruses [8]. In mammals, the host innate immune system relies on evolutionarily conserved pathogen sensors, also known as pattern-recognition receptors (PRRs), such as toll-like receptors (TLRs; namely TLR3 and TLR7), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs) and melanoma differentiation associated gene 5 (MDA5) [8]. Recognition and binding of PRRs to their cognate ligands results in conformational changes that triggers a type I interferon (IFN)-dependent innate immune activation [9]. RLRs play a key role in sensing the introduction of RNA viruses into a cell [10]. The RIG-I family consists of three helicases: RIG-I, MDA5 and laboratory of genetics and physiology 2 (LGP2) [11, 12]. While RIG-I and MDA5 are involved in
antiviral responses, the role of LGP2 is poorly understood, as both positive and negative effects on the regulation of antiviral innate immune response have been reported for this member of the RIG-I family. Recently, LGP2 has been shown to activate antiviral signalling by regulating MDA5–RNA interaction and filament assembly [15].

Although RIG-I and MDA5 induce type I IFN responses through the same signalling pathway, RNA viruses are recognized differently by RIG-I and MDA5 [14]. For example, RIG-I is essential for the production of type I IFN in response to paramyxoviruses, influenza virus and Japanese encephalitis virus, while MDA5 plays a critical role in protection against picornavirus infections [15, 16]. For rotaviruses, both RIG-I and MDA-5 as well as MAVS/IPS-1 and IRF3 receptors are responsible for activating IFNβ responses [17]. For murine norovirus, MDA5 is the main receptor whereas IRF3 and NF-kB are responsible for activating the IFNβ response after infection with feline calicivirus [18–20]. In this study, we investigated the role of RIG-I and MDA5 in LLC-MK2 cells following TV infection.

RESULTS
TV activates RIG-I and MDA5 at different times after infection

To access the role of RLRs, RIG-I and MDA5 in TV infection, we infected LLC-MK2 cells with TV (m.o.i. of 1.0) and studied the kinetics of TV replication and expression of RIG-I, MDA5 and IFNβ. The replication kinetics of TV showed an increase in viral mRNA as early as 3–8 h p.i. reaching maximum levels at 24 h p.i. (Fig. 1a). Cytopathic effect was detected as early as 12 h p.i. (data not shown). Quantitative RT-PCR analysis of RIG-I, MDA5 and IFNβ indicated a time-dependent increase (>80-fold at 24 h p.i.; P<0.05) in type I IFN expression with progression of TV infection (Fig. 1b). Furthermore, TV infection resulted in an early onset of RIG-I expression that peaked at 8 h (>10-fold increase over control; P<0.05) (Fig. 1c). We did not observe any significant difference in the level of MDA5 mRNA at this time point compared to the non-infected control. MDA5 levels were increased 12 h p.i. and stayed elevated (>sevenfold increase up to 24 h p.i. compared to the control; P<0.05) (Fig. 1d).

Early IFN response is mediated by RIG-I in TV replication

TV infection resulted in early induction of RIG-I, whereas MDA5 induction was observed at later stages of infection (Fig. 1). Intriguingly, type I IFN induction also coincided with the expression of RIG-I and MDA5. To evaluate the role of RIG-I and MDA5, we used gene-specific siRNA to knockdown RIG-I and/or MDA5 in LLC-MK2 cells followed by TV infection at an m.o.i. of 1 (Figs 2, 3). RIG-I knockdown cells infected with TV resulted in elevated levels of virus replication (>50-fold; P<0.05) that coincided with reduced IFNβ induction as early as 6 h p.i. (P<0.05), as analysed by quantitative reverse transcription (RT)-PCR and ELISA. However, at later time-points (12 and 24 h p.i.) we did not observe significant differences in virus replication in RIG-I knockdown cells as compared to controls (Fig. 2a–c). Fig. 2(d) shows the successful knockdown of RIG-I mRNA in TV-infected LLC-MK2 cells as analysed by quantitative RT-PCR; while siRNA knockdown of RIG-I did not alter MDA5 mRNA expression (Fig. 2e). RIG-I protein expression was evident at 6 h p.i., whereas MDA5 protein expression was visible at 12 h p.i. RIG-I siRNA completely abolished TV-induced RIG-I expression while MDA expression remained unchanged following RIG-I siRNA treatment (Fig. 2f). In contrast, elevated TV replication (Fig. 3a) was observed at 12 h p.i. (>400-fold increase in viral RNA expression) and 24 h p.i. (>800-fold increase in viral RNA expression) in MDA5 siRNA knockdown LLC-MK2 cells (P<0.05). This also coincides with decreased IFNβ expression in TV-infected LLC-MK2 cells at these time points when analysed by quantitative PCR (Fig. 3b) and ELISA (Fig. 3c). Analysis of RIG-I and MDA5 mRNA expression in MDA5 siRNA knockdown experiments suggest that MDA5 knockdown did not alter RIG-I expression kinetics following TV infection (Fig. 3d, e). These findings were further confirmed by immunoblot analysis of RIG-I and MDA5 protein expression (Fig. 3f). Interestingly, when both RIG-I and MDA5 mRNA expressions were knocked down simultaneously, there was a drastic elevation (>1000-fold; P<0.05) of TV replication and suppressed IFNβ induction (Fig. 4a, b, c). Fig. 4(d, e) show the successful knockdown of TV-induced RIG-I and MDA5 mRNA expression. Fig. 4(f) shows the RIG-I and MDA5 protein expression by immunoblot following MDA5 siRNA treatment. We also investigated the role of TLR3 and TLR7 in TV-infected LLC-MK2 cells. TLR3 and TLR7 were barely detectable by quantitative PCR in LLC-MK2 cells, and their expression level remained unchanged following TV infection (data not shown).

5’PPP-RNA inhibits the replication of TV in LLC-MK2 cells

To explore whether pre-activation of RIG-I and/or MDA5 can prevent TV replication, we used in vitro transcribed 5’PPP-RNA, which is a synthetic ligand that has been shown to activate RIG-I [21]. For RIG-I and MDA5 activation, we used poly I:C, which activates both RIG-I and MDA5 in a dsRNA length-dependent manner [10]. Pre-treatment of LLC-MK2 cells with RIG-I ligand 5’PPP-ssRNA not only delayed TV replication but also drastically reduced (>900-fold decrease; P<0.05) viral titres up to 24 h.p.i. compared with CIAP-RNA (negative control) transfected cells as analysed by mRNA quantitation in cell lysate as well as in culture supernatants (Fig. 5a, b). 5’PPP-RNA treatment also resulted in significantly increased levels of RIG-I (>sevenfold increase; P<0.05) and IFNβ (>70-fold increase; P<0.05) compared with untreated LLC-MK2 cells infected with TV that peaked at 12 h.p.i. and stayed at increased levels up to 24 h p.i. (Fig. 5c, d). 5’PPP-RNA treatment did not alter MDA5 expression levels at early time-points (up to 8 h), although at 24 h increased MDA5 levels in 5’PPP-RNA transfected cells was observed (Fig. 5e). The data obtained from poly I:C treatment...
(data not shown) that was used to pre-activate RIG-I and MDA5 were similar to the data from pre-activated RIG-I by 5’PPP-ssRNA. IFN-dependent 5’PPP-RNA-mediated inhibition of TV replication was further confirmed by pretreating LLC-MK2 cells with recombinant IFN-β and then infecting the cells with TV. IFN-β treatment not only inhibited TV replication (Fig. 6a), but also induced IFN-β, RIG-I and MDA5 mRNA expression, as measured by quantitative RT-PCR (Fig. 6b, c, d).

**RNA-Seq analysis**
We also performed high-throughput sequencing of cDNA (RNA-Seq) to evaluate the transcriptional profiles of TV-infected LLC-MK2 cells 24 h p.i. Fig. 7 shows the heat map of the top highly expressed selected genes that showed significant enrichment following TV infection in LLC-MK2 cells at 24 h. Among these highly expressed genes, two subclasses were predominant: IFN-stimulated genes (ISGs) and pro-inflammatory genes. Type I IFN-dependent antiviral response was evident from highly expressed IFNA and IFNβ, and others including IFN-dependent ISGs. Notably, we observed a significant increase in DDX60 [22], which binds to ssRNA, dsRNA and dsDNA, and can promote the binding of RIG-I to dsRNA and exhibits antiviral activity against hepatitis C virus and vesicular stomatitis virus (VSV). Apparently this gene has a role upstream of RIG-I and MDA5 in TV infection, as RIG-I and MDA5 siRNA knockdown completely abrogated type I IFN-dependent protection against TV. Expression levels of RIG-I and MDA5 from the RNA-Seq analysis were comparable to quantitative PCR data (not shown). Furthermore, consistent with the quantitative PCR data, TLR3 and TLR7 were undetectable in the RNA-Seq analysis. Pro-inflammatory response genes CXCL11 and CXCL10 showed high expression levels in infected cells, along with increased expression of the TMPRSS2 gene which encodes a protein that belongs to the serine protease family.

**DISCUSSION**
We investigated the role of cytosolic RNA sensors, RIG-I and MDA-5, during TV infection. Our results indicate that both RIG-I and MDA5 regulate the expression of IFN-β at different stages of the infection. While RIG-I presence is critical for inhibition of virus replication at early stages (3 h p.i.) of infection, MDA5 inhibits TV replication at later stages (12 h p.i.). Activation of both RIG-I and MDA5 was critical for optimal type I IFN induction and thus effective inhibition of TV replication. Furthermore, activation of RIG-I with 5’PPP-RNA inhibited TV replication, suggesting that evolutionarily conserved RIG-I activation can provide important insights into developing novel therapeutic strategies against infection.
TV infection resulted in an approximately 80-fold increase in IFNβ, which is a type I IFN, is the first defence mounted by the host during viral infection and plays a critical role in the synthesis of IFNa and related innate antiviral responses [23–25]. Interestingly, increased IFN levels following TV infection did not inhibit TV replication. We also observed increased cell death at 24 h p.i. One of the possible explanations could be late onset of IFN induction, as well as its magnitude as 5’PPP-RNA treatment resulted in a fivefold higher IFN level as compared to TV infection alone effectively reducing virus replication and enhancing cell survival. This was further confirmed by direct treatment of LLC-MK2 cells with IFNβ before TV infection, which completely blocked TV replication. Furthermore, the protective role of RLRs was evident from reduced IFN expression and enhanced TV replication levels when RIG-I and MDA5 were knocked down. Our data indicate that early onset of RIG-I expression was critical for TV replication inhibition; however, the nature of TV pathogen-associated molecular patterns (PAMPs) that bind to RIG-I is not known. The TV genome consists of a compact, positive-strand linear RNA molecule. While RIG-I is known to sense negative-strand viruses, positive-sense RNA viruses have also been reported to activate RIG-I-mediated host protection [26].

Apart from 5’PPP, RIG-I can also recognize viral RNA in the presence of 5’-bound VPg or endoribonuclease RNase L at the 3’ terminus [27, 28]. Weber et al. reported that full viral replication is not necessary to activate RIG-I, as viral RNA bound to nucleocapsids is capable of activating RIG-I by direct interaction [29]. Previous studies have shown that the mechanism of norovirus negative-sense RNA synthesis in the presence of VPg more efficiently induced RIG-I signalling [28, 30]. Whether such mechanisms are responsible for early activation of RIG-I in TV infection needs further investigation.

The MDA5 dependency of antiviral response against TV was observed at later stages of infection. Our data is in agreement with reports that suggest MDA5 is required for the control of murine norovirus (MNV-1) infection [18]. MDA5 recognizes long dsRNA; however, the putative recognition structure that activates MDA5 in TV infection is unclear. It is possible that ssRNA of the TV genome or replication intermediates may contain secondary structures and can activate MDA5. Although VPg is essential for MDA5 recognition of MNV-1 [18], it remains unclear if MDA5 recognizes the VPg-RNA complex or dsRNA produced during viral replication, as VPg removal prevents MNV-1 replication. Notably, TV shares many important features with MNV-1 and human norovirus.
including the same genetic organization and a similar capsid structure [31]. Identification of proteins that activate MDA5 will improve our understanding of the mechanism.

Our data also indicate that the presence of both RIG-I and MDA5 was required for an optimal antiviral effect. Initial activation of RIG-I induces type I IFN, which acts on other PRRs in an autocrine and paracrine manner [32]. RIG-I-induced type I IFN also upregulated the MDA5 expression required for robust IFNβ induction at later stages of TV replication. This finding is consistent with reports suggesting that both RIG-I and MDA5 are involved in positive-strand RNA virus replication [17, 33–36]. Because TV is an RNA virus, we also investigated whether TLR3 and TLR7, which recognize dsRNA and ssRNA respectively [37], are involved in antiviral host response. We did not observe significant changes in TLR3 or TLR7 expression in response to TV infection, suggesting that TLR3 and TLR7 may not have a direct role in a host’s response against TV. Our observation is consistent with data that suggest that TLR3 is not involved in the antiviral response against MNV-1 infection [18].

TV infection itself induced RIG-I- and MDA5-dependent IFNβ however, the magnitude of IFN induction was not enough to restrict TV replication. Although we observed enhanced TV replication following RIG-I and MDA5 knockdown. Further, the IFNβ level induced by 5’PPP-RNA treatment was multifold higher than that of TV infection. Several viruses, including hepatitis C virus, vaccinia virus, Ebola virus, astrovirus, feline calicivirus [20] and influenza virus have developed strategies to target and inhibit distinct steps in the early signalling events that lead to the type I IFN induction, indicating the importance of type I IFNs in the host’s antiviral response [8, 9, 12, 17, 21, 38]. For example, influenza A NS1 can target innate immune receptor RIG-I and its signalling components to suppress host innate immune response [39]. For MNV, production of virulence factor (VF1) antagonized the innate immune response by delaying the production of IFNβ [40]. A recent report demonstrated that MNV infection modifies the host response by limiting the translation of host IFN stimulating gene mRNA by exploiting the difference between norovirus VPg-dependent and cellular cap-dependent translation [41]. The actual mechanism that suppresses the host innate immune response to TV infection remains unclear and additional studies are needed to identify the role of VPg during TV infection as well as the role of apoptosis.

RNA-Seq analysis not only confirmed our quantitative PCR data, but also identified antiviral and pro-inflammatory genes that can be used as targets for developing novel drugs against TV infection. Increased expression of the TMPRSS2 gene, which encodes a protein that belongs to the serine protease family, was detected. This protein has been shown...
to be essential for spread and pathogenesis of influenza A virus via proteolytic cleavage and activation of hemagglutinin (HA) protein, which is essential for viral infectivity [42]. Further studies are needed to understand if this protein also facilitates TV entry.

Because the natural target cells for TV in rhesus macaques (Macaca mulatta) are duodenal enterocytes [43], our data from infected monkey kidney cells (LLC-MK2) may not mimic the actual innate immune response against TV. Overall, TV entry in LLC-MK2 cells activates RIG-I, which is responsible for early IFNβ response. Based on our data and current knowledge in this field, we postulate that TV has the ability to activate a RIG-I-dependent antiviral response and during the replication and transcription of the TV RNA genome, viral RNA replicases generate dsRNA in ample amounts, which then will activate MDA5 at the later stages of TV infection. Hence, both RIG-I and MDA5 along with IFNB (generated by RIG-I early on during infection) might contribute to a high level of IFNB expression during the late stages of TV infection.

Fig. 4. Effect of RIG-I and MDA5 double knockdown on TV replication. LLC-MK2 cells were transfected with control siRNA or RIG-I and MDA5 siRNA for 6 h followed by infection with TV for indicated time-points, and analysed for (a) TV RNA, (b) IFNβ, (d) RIG-I and (e) MDA5 mRNA by quantitative RT-PCR. IFNβ release in culture supernatants was analysed by ELISA (c). RIG-I and MDA5 protein expression were assayed by immunoblotting (f). Beta-actin was used as loading control.

5’PPP-RNA is derived from viral genomes and is not protected by a 5’ cap. In negative-sense RNA viruses, 5’PPP-RNA is sensed by cytosolic helicase RIG-I [44]. The C-terminal regulatory domain (CTD) of RIG-I binds to blunt-end 5’PPP-RNA and triggers innate antiviral immune responses [44], which results in increased production of type I IFN. Previously, we have shown in vitro transcribed 5’PPP-RNA that activates RIG-I and suppresses replication of influenza viruses irrespective of their genetic make-up, pathogenicity and drug-sensitivity status [21]. Using the same approach, we have reported that RIG-I activation prevents Ebola virus replication [38]. The antiviral immune responses of type I IFNa, β or γ have been studied for MNV and for the Norwalk RNA replication system [45–47]. A similar inhibitory effect of type I IFN has been reported for porcine sapovirus [48] and feline calicivirus replication [49]. However, the role of 5’-PPP has as far as we know not been studied in caliciviruses. In our study, the early onset of IFNβ after pre-treatment with 5’PPP-RNA successfully reduced the replication of TV. Our data suggest that evolutionarily conserved RIG-I can potentially be explored as an alternative preventive strategy for TV infections.
In summary, both RIG-I and MDA5 play a critical role in optimal type I IFN induction and effective inhibition of TV. However, the role of other PRRs, such as protein kinase R (PKR) and 2′-5′-oligoadenylate synthetase (OAS), are yet to be explored. We showed that synthetic 5′PPP-RNA successfully reduced the replication of TV by increasing type I IFN production and thus could be considered as a possible approach for therapeutic strategies against calicivirus infections. Future studies should focus on confirmation of our 5′-PPP-RNA data in recently reported cell culture systems for human norovirus including human B cells [50] and human intestinal organoids [51]. If our findings using TV can be confirmed, manipulation of the innate immune response may become one of the possible measures in the tool box for controlling human noroviruses.

**METHODS**

**Virus, cell and infectivity assay**

TV was propagated in LLC-MK2 cells cultured in Dulbecco’s modified Eagle’s medium (DMEM) plus 10% foetal bovine serum (FBS; HyClone, Logan, UT). Monolayers of cells were infected for 1 h at 37 °C at an m.o.i. of 0.001, as described previously [4]. The virus was harvested at 24–32 h p.i. when the cytopathogenic effect (CPE) was complete, clarified at 3000 g for 30 min and frozen in aliquots at −70 °C until use. Infectious TV was assayed by inoculation of 60 mm dishes of LLC-MK2 monolayer cells with 500 µl of 10-fold serial dilutions, incubation for 1 h at 37 °C with gentle rotation, and subsequent addition of an overlay with a medium consisting of MEM plus 2% FBS and 0.5% agarose. At 2 days p.i., a second agarose overlay containing 66 mg ml$^{-1}$ neutral red (Sigma, St. Louis, MO) was added and visible plaques were counted within 8 h. Frozen aliquots of TV had titres of 10$^7$ to 10$^8$ p.f.u. ml$^{-1}$.

**5′triphosphate RNA (5′PPP-RNA) and IFNβ treatment, RIG-I and MDA5 knockdown, and TV infection**

5′PPP-RNAs and negative control RNA (CIAP-RNA) were in vitro transcribed using T7 RNA polymerase as described previously [21]. Poly-IC was purchased from Sigma. To study the prophylactic potential of 5′PPP-RNA, 1×10$^6$ cells were transfected with 3 µg in vitro transcribed 5′PPP-RNA using Lipofectamine 2000 (Invitrogen, CA). This amount was chosen after dose-kinetics studies using 1, 2, 4 and 6 µg of RNA. After 24 h, cells were washed and infected with TV at an m.o.i. of 1. Cells were also treated with IFNβ (100 U ml$^{-1}$; ProSpec-Tany TechnoGene, Rehovot, Israel) prior to TV infection. Three sets each of monkey-specific siRNAs for both RIG-I and MDA5 and one set of scrambled siRNA were custom-synthesized by Dharmacon (GE Dharmacon Healthcare, Lafayette, CO). To determine the effect of siRNA knockdown, cells were infected with TV at an m.o.i. of 1 for 1 h prior to transfection by siRNA. All siRNA transfections were carried out with freshly prepared cocktails for RIG-I and MDA5 siRNAs using DharmaFECT transfection reagent in six-well plates. In each cocktail, all three sets of scrambled siRNA were transfected with 3 µg

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**Fig. 5.** Pre-activation of RIG-I inhibits TV replication. LLC-MK2 cells were transfected with 5′PPP-RNA or CIAP-RNA (2 µg ml$^{-1}$) as negative control for 24 h followed by infection with TV virus for indicated time-points, and analysed for (a) TV RNA in cells, (b) TV RNA in cell supernatants, (c) IFNβ, (d) RIG-I and (e) MDA5 mRNA by quantitative RT-PCR.

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**Table 1.** Tulane virus vRNA expression and IFNβ mRNA expression following treatment with 5′PPP-RNA. 

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<th>Time (h)</th>
<th>TV RNA in cells (fold increase over control)</th>
<th>TV RNA in supernatants (fold increase over control)</th>
<th>IFNβ mRNA expression (fold increase over control)</th>
<th>RIG-I mRNA expression (fold increase over control)</th>
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concentration of each siRNA was 33 nM). Three independent experiments were performed at different times with each treatment carried out in duplicate. Cell culture supernatants were collected and cells were harvested at different time-points after infection (0, 3 or 4, 6 or 8, 12 and 24 h) and stored at −80 °C for further analysis.

**Quantitative RT-PCR**

Total RNA was isolated from cells using the RNA easy kit (Qiagen, Valencia, CA) and SYBR Green real-time RT-PCRs were conducted for mRNA expression of RIG-I, MDA5, IFNs, TV and GAPDH using the SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Oligonucleotide primers used in this study are listed in Table S1 (available in the online Supplementary Material). Reverse transcription was carried out at 50 °C for 3 min. PCR was conducted at 94 °C for 15 s and 60 °C for 45 s for a total of 40 cycles. The threshold cycle number for the PCR product was normalized to that of GAPDH mRNA and the resulting value was converted to a linear scale. Data from three independent experiments were analysed and reviewed if the data point s showed a normal distribution without outliers.

**ELISA**

IFNβ in culture supernatants were analysed by using a rhesus monkey IFNβ ELISA kit according to manufacturer's instructions (LifeSpan Bioscience, Seattle, WA).

**Immunoblot**

Immunoblot detection of RIG-I, MDA5 and beta-actin were performed by using the method described previously [21]. Anti-RIG-I, anti-MDA5 and anti-beta actin antibodies were purchased from Cell Signaling Technology (Danvers, MA), Life Technologies (Carlsbad, CA) and Sigma (St. Louis, MO), respectively.

**RNA-Seq library preparation and sequencing**

Total RNA was extracted using the RNeasy Mini kit (Qiagen, CA). Libraries were prepared using the TruSeq RNA kit (Illumina, San Diego, CA) as per the manufacturer's instructions. Briefly, 1 μg total RNA was used for library preparation. The TruSeq method (low-throughput protocol) employs two rounds of poly-A-based mRNA enrichment using oligo-dT magnetic beads followed by mRNA fragmentation (120–200 bp) using cations at high temperature. First- and second-strand cDNA synthesis was performed followed by

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**Fig. 6.** IFNβ pretreatment inhibits TV replication. LLC-MK2 cells were treated with IFNβ (100 U ml⁻¹) followed by infection with TV for indicated time-points, and analysed for (a) TV RNA in cells, (b) IFNβ, (c) RIG-I and (d) MDA5 mRNA by quantitative RT-PCR.
end repair of the blunt cDNA ends. One single ‘A’ base was added at the 3’ end of the cDNA followed by ligation of a barcoded adapter unique to each sample. The adapter-ligated libraries were then enriched using PCR amplification. The amplified library was validated using a high sensitivity DNA chip on the Agilent Bioanalyzer. The libraries were further quantified on a Qubit 2.0 Fluorometer (Life Technologies, Grand Island, NY) using high sensitivity dsDNA assay. The libraries were normalized and multiplexed in a lane of the flowcell. PhiX was used as an internal control on each lane to monitor the error statistics. Cluster generation was performed on the V3 flowcell on the Illumina cBot. The clustered flowcell was then sequenced on the Illumina HiSeq1000 system employing a single-end 101 cycle run.

**Statistical analysis**

We used analysis of variance (ANOVA) using GraphPad PRISM 5 (GraphPad Software) and a value of $P \leq 0.05$ was considered significant when compared with appropriate controls.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

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