Toscana virus induces interferon although its NSs protein reveals antagonistic activity

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INTRODUCTION

Toscana virus (TOSV) is an arthropod-borne virus belonging to the genus Phlebovirus, family Bunyaviridae (Bishop, 1990; Elliott et al., 1991). Currently, the prospect of newly emerging viruses in this family is a cause of public concern. Among the viruses belonging to this genus, TOSV is responsible for aseptic meningitis, meningoencephalitis, and encephalitis associated with fever, myalgia and severe frontal headache with a benign course, followed by a medium to long convalescence (Nicoletti et al., 1991; Braito et al., 1998; Valassina et al., 1998b, 2000; Dionisio et al., 2001). Epidemiological studies have shown that TOSV is the most important aetiological agent of meningitis and meningoencephalitis in the Mediterranean area (Braito et al., 1998; Valassina et al., 1998a, 2000; Echevarría et al., 2003) during the summer, causing an increase in hospitalization rates and having an important impact on human health. However, asymptomatic infections and infections without central nervous system involvement of this virus (about 60%) have also been described (Braito et al., 1997). Many unanswered questions remain as to the factors responsible for virulence and why most people do not develop neurological symptoms after TOSV infection. Although studies aimed at understanding the molecular biology of these viruses are in progress, a deeper analysis of the markers of virulence and pathogenicity mechanisms are necessary in order to design adequate strategies for prevention. TOSV contains a tri-segmented, ssRNA genome of negative or ambisense polarity (Accardi et al., 1993). The L and M segments are of negative polarity and encode the large protein (L) (Accardi et al., 1993) and the envelope glycoproteins (Gn and Gc), respectively (Di Bonito et al., 1997; Grò et al., 1997), whereas the S segment utilizes an ambisense strategy and encodes two proteins, the nucleocapsid protein (N) and the non-structural protein (NSs) (Giorgi et al., 1991). Recent studies have shown that the NSs protein of members of the family Bunyaviridae has a role in evasion of the antiviral system in mammalian hosts (Weber et al., 2002; Blakqori et al., 2007; Le May et al., 2008). Upon virus infection, the type I interferon (IFN) system represents the basis of an early host response. Transcriptional induction of the IFN-β promoter involves activation of IFN regulatory factor-3 (IRF-3), activating protein 1 and nuclear factor kB (NF-kB). Among these transcription factors, IRF-3 is the most essential for the immediate-early induction of IFN-β expression (Sato et al., 2000). Bunyamwera virus (BUNV), La Crosse virus (LACV), the phleboviruses Rift Valley fever virus (RVFV) and Sandfly fever Sicilian virus (SFSV) express NSs proteins that are efficient inhibitors of type I IFN synthesis (Bridgen et al., 2001; Billecocq et al., 2004; Léonard et al., 2006; Blakqori et al., 2007; Jääskeläinen et al., 2007; Weber & Haller, 2007; Habjan et al., 2009). As the NSs proteins are involved in antagonism of host defence mechanisms, we investigated whether TOSV NSs could also have this role in the infected host. In this study, we showed
that the TOSV NSs protein indeed inhibits activation of IRF-3 and hence IFN induction. However, TOSV itself was none the less an elicitor of IFN production in infected cells, suggesting that TOSV NSs has a relatively weak activity against IFN induction by its parental virus.

RESULTS

NSs suppresses IFN-β promoter activation

We explored the possibility that TOSV NSs could suppress IFN-β gene expression, similar to other members of the family Bunyaviridae. To this aim, we generated a recombinant RVFV in which the parental RVFV NSs gene was replaced by TOSV NSs. Such an approach has led to the verification of SFSV NSs as an IFN-induction suppressor (Habjan et al., 2009). The new recombinant virus, termed rZHANSs::NSsTOSV, was able to block IFN induction to the same extent as SFSV NSs, as shown by RT-PCR for IFN-β (Fig. 1). Similarly, TOSV-infected cells previously transfected with the NSs plasmid showed a decrease in IFN-β transcripts, whilst TOSV was able to induce IFN-β transcripts, suggesting that NSs, when transiently overexpressed, can interfere with the transcriptional activation of the IFN-β promoter (Fig. 1). To support these data, HeLa cells were transfected with a TOSV NSs plasmid together with a firefly luciferase reporter plasmid under the control of the IFN-β promoter (p125-FFLuc) (Yoneyama et al., 1998), and stimulated by transfection with poly(I: C) for 24 and 48 h. All cell lysates were assayed for luciferase activity. As shown in Fig. 2(a),

![Fig. 1. Inhibition of IFN-β induction by TOSV NSs protein. 293T cells were mock infected or infected with the recombinant RVFV expressing TOSV NSs (rZHANSs::NSsTOSV) or SFSV NSs (rZHANSs::NSsSFSV). RVFV deleted of its NSs gene (rZHANSs) was also tested. Cells were also infected with TOSV or transfected with the NSs plasmid and then infected with TOSV. After 16 h, the expression level of the indicated genes was determined by RT-PCR analysis. Detection of γ-actin was used as a loading control.](image)

![Fig. 2. NSs protein suppresses IFN-β promoter activation. (a, b) HeLa cells were co-transfected with the IFN-β promoter-containing reporter plasmid p125-FFLuc (encoding firefly luciferase) and a Renilla luciferase-encoding plasmid along with TOSV NSs plasmid (shaded columns), RVFV NSs plasmid (filled columns) or empty vector (open columns), as described in Methods. Cells were then induced by transfection with poly(I: C) for 24 and 48 h. Cell lysates were assayed for firefly and Renilla luciferase activities (a). Error bars show the SD of the normalized data. The cell lysates from (a) were tested by immunoblotting with mouse anti-TOSV or RVFV NSs polyclonal serum or goat anti-β-actin polyclonal serum as a loading control (b). (c) HeLa cells were transfected with the IFN-β promoter-containing reporter plasmid p125-FFLuc and a Renilla luciferase-encoding plasmid. Cells were then infected with live TOSV (open columns) or UV-inactivated TOSV (hatched columns) and analysed at 8, 24 and 48 h.p.i. Cells transfected with the reporter plasmids and infected with SeV for 24 h were used as a positive control. Mock-infected cells were transfected with the reporter plasmids only. Error bars show the SD of the normalized data.](image)
TOSV NSs was able to inhibit IFN-β promoter induction by poly(I:C); this was more evident at 48 h, when the recombinant NSs was expressed in vitro at the highest amount (Fig. 2b). Similar results were obtained when the cells were transfected with plasmid expressing RVFV NSs, a known strong IFN antagonist (Billecocq et al., 2004) (Fig. 2a, b). In parallel, cells transfected with the firefly luciferase reporter plasmid p125-FFLuc were infected with live or UV-inactivated TOSV or with Sendai virus (SeV). IFN-β induction did not occur when cells were treated with UV-inactivated TOSV, but when cells were infected with live TOSV, IFN-β induction occurred in a range similar to that induced by SeV, (Fig. 2c), indicating that only the replicating virus was able to induce IFN-β. These data were also confirmed by the presence of IFN-β in the cell medium at 24 and 48 h after TOSV infection, with values ranging from 0.7 to 0.9 ng ml⁻¹ (data not shown). Moreover, it was observed that the TOSV replicative cycle was very fast and a significant number of genome copies were present as early as 8 h after infection (Fig. 3). In contrast, whilst the N protein and Gc envelope glycoprotein were produced early after infection (8–24 h), the highest level of NSs expression was recorded at 48–72 h after TOSV infection (Fig. 4), indicating that this protein accumulates in a sufficient amount for IFN counteraction into the cell, later during virus replication. This could be due to the fact that the TOSV NSs gene is transcribed using an ambisense coding strategy from the complementary anti-sense S segment, which first needs to be synthesized in infected cells. Therefore, when TOSV-infected cells were transfected previously with NSs plasmid, causing overexpression of the protein, IFN promoter inhibition was obtained (data not shown). Finally, TOSV growth did not seem to be greatly affected by cell IFN production; in fact, it was able to grow similarly in IFN-competent and -incompetent cells, such as HeLa and Vero cells, respectively, and there was no significant difference between virus genome copy number in these two cell types (Fig. 3).

### Effect of IFN on TOSV growth in cell culture

To determine further whether NS can contribute to resistance against IFN, we evaluated the growth of TOSV in IFN-treated cells. For this assay, we chose Vero cells because they do not synthesize IFN but are fully responsive to the treatment, thus excluding any additional effects on virus-induced IFN that would bias the results. Cells were pre-treated with IFN-β (1000 IU ml⁻¹) and infected with virus at an m.o.i. of 0.05. Levels of infectious virus were determined after 24 h. A weak decrease in virus titre (from 9.1 × 10³ to 6.2 × 10³ p.f.u. ml⁻¹) was observed in IFN-treated cells, indicating that TOSV is not very sensitive to the antiviral effect of IFN when it is added exogenously at high levels.

### TOSV NSs inhibits IRF-3 activation

The transcription factor IRF-3 plays a fundamental role in activating the IFN response following virus infection (Sato et al., 2000). Consequently, we investigated the activity state of IRF-3 under the influence of TOSV or its NSs protein. Among the earliest steps of IRF-3 activation, we considered its phosphorylation, which represents an important post-translational modification, leading to its cytoplasm-to-nucleus translocation. A high-molecular-mass band, which most probably represents the phosphorylated form of IRF-3, appeared in lysates of cells infected with TOSV, but was undetectable in cells previously transfected with TOSV NSs plasmid and successively infected with SeV (see Supplementary Figure S1, available in JGV Online). In addition, we analysed IRF-3 dimerization (Lin et al., 1998; Yoneyama et al., 1998), and its translocation from the cytoplasm to the nucleus (Lin et al., 1998). To analyse the effect of TOSV NSs on the nuclear accumulation of IRF-3, we transfected cells with a plasmid encoding EGFP–hIRF-3 (Basler et al., 2003) together with the NSs plasmid. Cells were then infected with SeV to induce nuclear translocation of IRF-3. Cells were fixed and expression of NSs was detected by immunofluorescence. As expected, GFP–IRF-3 was detected in the cytoplasm of mock-infected cells transfected with the NSs plasmid (Fig. 5a–c). Interestingly, GFP–IRF-3 was also cytoplasmic in NSs-expressing cells that had been infected with SeV (Fig. 5d–f), whereas GFP–IRF-3 accumulated in the nucleus following SeV infection in cells not transfected with the NSs plasmid (Fig. 5g–i). Quantification of this effect by counting SeV-infected cells containing nuclear GFP–IRF-3 revealed a marked decrease in nuclear IRF-3 in the presence of TOSV NSs (data not shown). However, in line with the reporter assays shown in Fig. 2, TOSV infection itself activated the nuclear translocation of GFP–IRF-3 (Fig. 5j–l). This discrepancy observed in cells overexpressing TOSV NSs and cells

**Fig. 3.** TOSV growth in HeLa (□) and Vero (●) cells. Virus titre was determined as p.f.u. ml⁻¹. The virus genome copy number per 100 ng RNA extracted from infected cells is shown in parentheses. [Image: graph showing TOSV growth over time]
infected by TOSV was further confirmed by immunoblot assays on a native gel to check the IRF-3 dimerization event. In uninfected control cells, and in cells transfected with TOSV NSs plasmid followed by infection with SeV, IRF-3 remained in its monomeric form (Fig. 6). In contrast, IRF-3 dimerization occurred in cells infected by TOSV or transfected with the empty vector and infected by SeV (Fig. 6).

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**Fig. 4.** Time course of expression of NSs in HeLa cells infected by TOSV. Cells were analysed at 8, 24, 48 and 72 h p.i. and tested by immunofluorescence assay using anti-NSs, anti-N and anti-Gc mouse sera. Fluorescence was analysed with a Diaplan microscope using a ×20 objective.

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**Fig. 5.** Nuclear translocation of IRF-3 in Vero cells. All cells were transfected with EGFP–IRF-3 expression vector. (a–f) Cells were co-transfected with TOSV NSs plasmid. Some (d–f) were later infected with SeV for 6 h. (g–l) EGFP–IRF-3-transfected cells were infected with SeV (g–i) or TOSV (j–l). Viruses were detected using the specific mouse antisera. Fluorescence was analysed with a Diaplan microscope using a ×40 objective.
Mx protein expression in TOSV-infected cells

To investigate further the IFN response to TOSV infection, we evaluated the presence of Mx protein, a well-established marker of IFN activity (Haller et al., 2007), in Madin–Darby canine kidney (MDCK) cells infected by TOSV. It is known that Mx proteins inhibit multiplication of viruses that replicate in the cytoplasm, such as rhabdovirus and bunyaviruses (Haller et al., 2007). In particular, it appears that MxA can bind to the bunyavirus nucleocapsid protein and create co-polymers that efficiently immobilize the viral components and eventually block their function (Reichelt et al., 2004). We observed by immunoblot analysis that TOSV infection induced Mx expression with a similar range of induction to that induced by transfection with poly(I:C) (Fig. 7). Mx expression increased with time, being more marked at 48 h than at 24 h p.i. Thus, despite the presence of an NSs protein that clearly counteracts IRF-3, TOSV activates the IFN response.

DISCUSSION

The IFN system represents a major component of the innate immune response. Cells recognize and respond to RNA virus infection through several nucleic acid sensors, including Toll-like receptor 3 (TLR3) and the cytoplasmic dsRNA sensors retinoic acid inducible gene 1 (RIG-I) and melanoma differentiation-associated gene 5 (MDA-5) (Gantier & Williams, 2007). As a reaction to virus infection, cells synthesize and secrete IFNs that initiate the production of antiviral proteins, thus establishing an antiviral state. Many DNA and RNA viruses have developed strategies to counteract this innate immune reaction by expressing proteins able to inhibit IFN induction or activation of dsRNA-dependent antiviral enzymes (Peters et al., 1989; Player & Torrence, 1998; Yoneyama et al., 1998; Gotoh et al., 2001; Samuel, 2001; Conzelmann, 2005; Haller et al., 2006; García et al., 2007; Peng et al., 2007; Boxer et al., 2009; Habjan et al., 2009). Among the members of the family Bunyaviridae, the BUNV and LACV orthobunyaviruses, the Sin Nombre, Tula and Puumala hantaviruses, and the RVFV and SFSV phleboviruses express a non-structural protein, NSs, that acts as an IFN antagonist (Stark et al., 1998; Weber et al., 2002; Billecoq et al., 2004; Le May et al., 2004; Blakqori et al., 2007; Jääskeläinen et al., 2007; Spiropoulou et al., 2007; Habjan et al., 2009; Ikegami et al., 2009), affecting host-cell gene expression, IFN synthesis and IFN action. In this study, we showed that TOSV NSs also prevents IFN induction. Interestingly, TOSV NSs is so far the only bunyaviral NSs shown to target IRF-3 or some upstream sensor involved in the signalling cascade leading to production of type I IFN. None of the other well-characterized NSs proteins interfere with IRF-3 or with other factors upstream of it, but they act in the nucleus at the level of RNA polymerase II transcription (Billecoq et al., 2004; Thomas et al., 2004; Léonard et al., 2006). This is in agreement with the cytoplasmic localization of TOSV NSs, which is different from the nuclear localization of the other NSs proteins (Weber et al., 2001; Le May et al., 2004). It appears that TOSV NSs is able to prevent dimerization of IRF-3 and hence activation of IFN transcription. In agreement with this, we observed that TOSV NSs does not appear to interfere with RNA polymerase II activity in co-expression experiments (data not shown). Despite the clear effect of TOSV NSs on IFN induction, TOSV itself is an IFN inducer and, like other such viruses, has a ssRNA genome, which most likely triggers signalling pathways leading to activation of the transcription factors NF-κB and the IRF families (Honda & Taniguchi, 2006; Kaisho & Tanaka, 2008). TOSV is, however, the first bunyavirus that has an evident IFN-inducing activity, although it expresses an active NSs. Astonishingly, TOSV NSs efficiently blocked IFN induction, even in the context of a recombinant RVFV, which is a strong IFN inducer without NSs. Like TOSV, RVFV is a rapidly multiplying phlebovirus. The failure of TOSV to suppress IFN induction could be due to the fact that NSs is not potent enough to counterbalance the amount of IFN-inducing virus molecules, e.g. viral ssRNAs containing 5′-triphosphate ends. This observation is not without precedent: SeV, which we used as an inducer

Fig. 6. Dimerization of IRF-3 in Vero cells. IRF-3 was analysed by immunoblotting in mock-infected or TOSV-infected cells, and in cells transfected with empty vector or with NSs-encoding plasmid before SeV infection. IRF-3 was detected by probing the membrane with a specific mAb. Monomers and dimers are indicated.

Fig. 7. Immunoblotting of MDCK cell lysates tested with goat anti-Mx serum in mock-infected cells, cells induced by transfection with poly(I:C) for 24 h and cells infected with TOSV for 24 or 48 h. The same samples were also tested with β-actin antibody as a loading control.
control, expresses a V protein able to block IFN induction (Gotoh et al., 2001; Kiyotani et al., 2007). Moreover, wild-type isolates of vesicular stomatitis virus, which expresses a matrix (M) protein acting as an efficient IFN antagonist (Waibler et al., 2007), were shown to induce extremely high levels of IFN (Marcus et al., 1998). It is thus possible that the fast replication of TOSV is able to trigger IFN in infected cells, indicating that NSs is active during the initial phase of IFN activation and not on IFN-inducible antiviral effector proteins. More information will be available with the development of a reverse genetics system to generate an NSs deletion mutant of TOSV, as conducted for other members of the family Bunyaviridae. In any case, the fact that TOSV, a human pathogen, is able to induce IFN, may explain why its pathogenesis is mostly mild. In fact, TOSV infection can cause neurological symptoms, but spread to the central nervous system is not a regular feature of its pathogenesis. In fact, among infected individuals, more than 60% show no clinical signs (Valassina et al., 1998a). The virally induced IFN-β might restrict virus replication soon after infection. It would be interesting to see whether the more severe cases of TOSV infection are due to a specific genetic set-up of the host, as shown in other viral infections (Ge et al., 2009), or to a defect in the immune response. Further studies are in progress to delineate the mechanism(s) by which NSs inhibits IRF-3 and to clarify its role in TOSV pathogenesis.

**METHODS**

**Cells, viruses and chemicals.** Vero, HeLa, 293T and MDCK cells were grown as monolayers in Dulbecco’s modified Eagle’s medium (DMEM; Lonza) supplemented with 5% heat-inactivated FCS (Lonza) and 100 U penicillin/streptomycin (Hyclone Europe) ml-1 at 37 °C. TOSV strain 1812 (isolated from the cerebrospinal fluid of a patient with meningitis) and SeV Cantell strain (ATCC VR-907) were propagated in Vero cells. The supernatant of Vero and HeLa cells infected with TOSV (plaque purified) at an m.o.i. of 0.05 was collected at 8, 24, 48 and 72 h after infection for virus titration. Briefly, these samples were diluted tenfold, titrated by plaque assay on a six-well plate of Vero cells and incubated at 37 °C for in 5% CO₂ for 1 h. The medium was then removed and 3.5 ml overlay medium containing 3% purified agar (Oxoid) was added. After 5 days, the cell sheet was stained with neutral red and the viral titre was determined as p.f.u. ml-1. To determine the IFN sensitivity of TOSV, Vero cells were treated overnight with 1000 IU human IFN-β (BioVision) ml-1 and then infected at an m.o.i. of 0.05. After 1 h adsorption, the cells were washed with PBS and maintained in DMEM. After 24 h, the supernatant was collected and assayed for virus titration as described above. The level of IFN-β was evaluated in the medium of treated or infected cells using a Human IFN-β ELISA kit (PBL Interferon Source).

**Plasmids.** TOSV RNA was extracted from the cell-culture supernatant using a QIAamp viral RNA mini kit (Qiagen). The complete NSs gene (GenBank accession no. EU327772) was amplified by RT-PCR from purified viral RNA with primers NSs Ban HH sense (nt 1–15: 5'-GGATCCCAACAAAAGACCTCCC-3') and NSs Xhol antisense (nt 993–1007: 5'-CTCGAGTCAAAAGGTGGTTGA-3') (restriction sites shown in bold; MWG Biotech). The reaction was carried out using a SuperScript III One-Step RT-PCR System with Platinum Taq (Invitrogen) by one cycle of reverse transcription at 50 °C for 30 min and 94 °C for 2 min, followed by 40 cycles of 1 min at 94 °C, 30 s at 60 °C and 1 min at 72 °C. The gene was cloned in either pCdNA3.1 plasmid at the BamHI/Xhol unique sites of the polylinker, downstream of the cyanogemavirus immediate-early enhancer/promoter, or pCdNA4 His MAX-A plasmid (Invitrogen), in frame with the 6 × His tag. This resulted in plasmids GC-222 and GC-310, respectively. A plasmid encoding RVFV NSs protein was generated as described previously (Billecocq et al., 2004). A reporter plasmid encoding firefly luciferase downstream of the complete IFN-β promoter (p125-FLuc) was kindly provided by Takashi Fujita (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan). Plasmids expressing human IRF-3 (pCAGGS-hIRF3) and IRF-3 fused to GFP (pEGFP-C1-hIRF3) were kindly provided by L. Martinez-Sobrido and A. Garcia-Sastre (Mount Sinai School of Medicine, New York, USA). Transfections were performed using Effectene reagent (Qiagen) following the manufacturer’s instructions.

**Expression of NSs protein.** HeLa cells were seeded in six-well culture plates at a density of 6 × 10⁵ cells per well and incubated at 37 °C in 5% CO₂. The cell monolayer was infected with TOSV (m.o.i. of 0.05) or transfected with 1 µg GC-310 plasmid or empty vector. At 8, 24, 48 and 72 h later, cells were collected and assayed for indirect immunofluorescence or Western blotting. Briefly, some cells were spotted on a slide and fixed for 10 min at room temperature with cold methanol/acetone. The cells were then incubated for 1 h at 37 °C with mouse anti-NSs, anti-Gc or anti-N polyclonal serum (diluted 1:20), followed by washing with PBS, fluorescein-labelled anti-mouse IgG (Sigma) was added for 1 h at 37 °C. Immunofluorescence was visualized using a Diaplant microscope (Leica Microsystems). In parallel, cell samples were lysed and separated by 12% PAGE under reducing conditions, transferred to a nitrocellulose sheet (Towbin et al., 1979) and tested for immunoreactivity with mouse anti-NSs polyclonal serum (diluted 1:100, produced in house).

**Generation of recombinant viruses.** For recovery of recombinant RVFV, we used our strain ZH548 pol-I/pol-II-based rescue system, which has been described in detail elsewhere (Habjan et al., 2008). Generation of the recombinant S segments was achieved using a modified S segment rescue plasmid (pHH21-RVFV-vN_TCS) where the NSs gene has been replaced by a cloning cassette containing two Aar restriction sites. Linearization of this rescue construct with AarI restriction sites. Linearization of this rescue construct with AarI allows exact in-frame insertion of genes via Ncol/Xhol-compatible ends. The cloning frame of TOSV NSs was amplified from the cDNA of infected cells using primers 5'-GACAGACGTCCTCAGATCGGAGCTGTCATC-3' and 5'-GACAGACGTCCTCAGATCGGAGCTGTCATC-3'. The PCR fragments were inserted into the cloning cassette of the modified S segment rescue plasmid pH21-RVFV-vN_TCS. These constructs were verified by DNA sequencing and used for the generation of rZHAhNSs:NSsTSOV. Recombinant viruses were generated by transfecting co-cultures of HEK293T and BHK-21 cells in six-well plates with 0.5 µg helper plasmids (pL18-RVFV-L and pL18-RVFV-N) together with 1 µg each of the L, M and S segment plasmids (pHH21-RVFV-VL, pHH21-RVFV-VM and pH21-RVFV-vN_NSSOV) using NanoFectin transfection reagent (PAAP Laboratories) as described previously (Habjan et al., 2009). Supernatants containing recombinant virus were collected at 5 days post-transfection and used to grow virus stocks.

**RT-PCR analysis.** Total cellular RNA was extracted from cells infected with the recombinant RVFV or TOSV using an RNeasy Mini...
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INF reporter gene assay. HeLa cells (2 x 10⁵) were seeded in 24-well plates. Cell monolayers were transfected with 0.02 µg p125-FLuc, 0.02 µg pEGFP-C1-hIRF3, and 0.5 µg empty vector or TOSV or RVFV NSs-expressing plasmid. The cells were then stimulated by transfection with poly(I:C) (20 µg mL⁻¹) and analysed at 24 and 48 h after induction. In parallel, cells transfected with the reporter plasmids, as described above, were infected with plaque-purified, live or UV-inactivated TOSV at an m.o.i. of 0.05 for 8, 24 and 48 h. Cells infected with SeV for 24 h were used as a positive control. Cell extracts for the measurement of luciferase activity were prepared and assays were performed according to the manufacturer’s instructions (Dual Luciferase reporter Assay System; Promega).

IRF-3 dimerization assay. Vero cells (5 x 10⁴) were transfected with 0.15 µg pCAGGS-HIF3 alone or in combination with 0.3 µg NSs expression plasmid. At 24 h post-transfection, cell monolayers were infected with SeV or TOSV at an m.o.i. of 10, or were mock infected. At 12 h p.i., cells were resuspended in lysis buffer containing 50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 % NP-40, and 1 mM DTT containing protease inhibitors (Complete Protease Inhibitor; Roche) and phosphatase inhibitors (Phosphatase Inhibitor Cocktail II; Calbiochem). A total of 50 µg protein was separated by SDS-PAGE and transferred to an Immobilon-P PVDF membrane (Millipore), followed by incubation in saturation buffer (PBS containing 5 % BSA and 0.05 % Tween 20). The membrane was first incubated with goat polyclonal serum to Mouse IgG for 1 h at 68 °C. The primer sequences for diagnostic RT-PCR analyses were as follows: human INF-β: 5’-GAAGGCGCCAGTG-ACCACCTA-3’ and 5’-CCCTAGGGTTTACCTGACT-3’; human γ-actin: 5’-GCCGGTGGATTTCCACTCTGACT-3’ and 5’-CATGGC-GGGGTGTTGAAGGTC-3’; and TOSV NSs: 5’-GACAGACGTC-TCACATGCAATCCAGAGCTGTCATC-3’. A real-time PCR assay for quantification of TOSV genome copies in infected Vero or HeLa cells was carried out as described previously (Pérez-Ruiz et al., 2007).

Western blot analysis of Mx protein. MDCK cells infected with TOSV (m.o.i. of 0.05) or induced by transfection with poly(I:C) were lysed in RIPPA buffer [50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 % NP-40, 1 mM EDTA] containing protease inhibitors (Complete Protease Inhibitor; Roche) and phosphatase inhibitors (Phosphatase Inhibitor Cocktail II; Calbiochem). The mean differences were analysed statistically using StatView statistical software (Abacus Concepts). P values were calculated using Student’s t-test. A P value of <0.05 was considered statistically significant.

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