Originally published as:

Min-Hi Lee, Pritesh Lalwani, Martin J. Raftery, Markus Matthaei, Nina Lütteke, Sina Kirsanovs, Marco Binder, Rainer G. Ulrich, Thomas Giese, Thorsten Wolff, Detlev H. Krüger and Günther Schönrich
RNA helicase retinoic acid-inducible gene I as a sensor of Hantaan virus replication
(2011) Journal of General Virology, 92 (9), pp. 2191-2200.

DOI: 10.1099/vir.0.032367-0

RNA helicase retinoic acid-inducible gene I as a sensor of Hantaan virus replication

Min-Hi Lee[1,†], Pritesh Lalwani[1,†], Martin J. Raftery[1], Markus Matthaei[2], Nina Lütteke[1], Sina Kirsanovs[1], Marco Binder[3], Rainer G. Ulrich[4], Thomas Giese[5], Thorsten Wolff[2], Detlev H. Krüger[1] and Günther Schönrich[1]

[1]*Institute of Medical Virology, Charité – Universitätsmedizin Berlin, D-10098 Berlin, Germany*
[2]*Robert Koch Institute, D-13302 Berlin, Germany*
[3]*Department of Molecular Virology, University of Heidelberg, D-69120 Heidelberg, Germany*
[4]*Friedrich Loeffler Institute, Institute for Novel and Emerging Infectious Diseases, D-17493 Greifswald–Insel Riems, Germany*
[5]*Institute of Immunology, University of Heidelberg, D-69120 Heidelberg, Germany*

[†] *These authors contributed equally to this work.*

Abstract

Hantaan virus (HTNV) causes severe human disease. The HTNV genome consists of three ssRNA segments of negative polarity that are complexed with viral nucleocapsid (N) protein. How the human innate immune system detects HTNV is unclear. RNA helicase retinoic acid-inducible gene I (RIG-I) does not sense genomic HTNV RNA. So far it has not been analysed whether pathogen-associated molecular patterns generated during the HTNV replication trigger RIG-I-mediated innate responses. Indeed, we found that knock-down of RIG-I in A549 cells, an alveolar epithelial cell line, increases HTNV replication and prevents induction of 2′,5′-oligoadenylate synthetase, an interferon-stimulated gene. Moreover, overexpression of wild-type or constitutive active RIG-I in Huh7.5 cells lacking a functional RIG-I diminished HTNV virion production. Intriguingly, reporter assays revealed that in vitro-*transcribed HTNV N RNA and expression of the HTNV N ORF triggers RIG-I signalling. This effect was completely blocked by the RNA-binding domain of vaccinia virus E3 protein, suggesting that dsRNA-like secondary structures of HTNV N RNA stimulate RIG-I. Finally, transfection of HTNV N RNA into A549 cells resulted in a 2 log-reduction of viral titres upon challenge with virus. Our study is the first demonstration that RIG-I mediates antiviral innate responses induced by HTNV N RNA during HTNV replication and interferes with HTNV growth.*

Introduction

Human infections with hantaviruses are on the rise due to enhanced human contact with rodents, their main reservoir (Ludwig *et al.*, 2003; Schmaljohn & Hjelle, 1997; Ulrich *et al.*, 2002). Humans are infected after inhalation of aerosols from excreta shed by chronically infected rodents that do not show obvious symptoms. The outcome of human infection is variable and depends on the infecting hantavirus species. Pathogenic hantavirus species elicit highly lethal diseases, hantavirus cardiopulmonary syndrome (HCPS) or haemorrhagic fever with renal syndrome (HFRS) (Krüger *et al.*, 2001; Muranyi *et al.*, 2005). Hantaan virus (HTNV), the prototype member of the genus *Hantavirus* in the family *Bunyaviridae*, causes HFRS with a case fatality rate up to 15%.

Hantaviruses are enveloped and contain a tripartite ssRNA genome of negative polarity (Schmaljohn & Nichol, 2007). It consists of a small, medium and large segment that encode the nucleocapsid (N) protein, envelope glycoproteins (Gn and Gc) and RNA-dependent RNA polymerase (RdRp). The 5′ and 3′ termini of the hantaviral genome form a panhandle structure. HTNV replication starts with virion attachment to integrin β3 (CD61), a receptor for pathogenic hantaviruses (Gavrilovskaya *et al.*, 1998, 1999). Recently, decay-accelerating factor (CD55) and receptor for globular heads of C1q (gC1qR) have been defined as additional HTNV receptors (Choi *et al.*, 2008; Krautkrämer & Zeier, 2008). After entry by endocytosis and acidification of endosomes fusion between endosomal membrane and viral envelope takes place. After release of the viral nucleocapsid into the cytoplasm the RdRp initiates transcription from genomic RNA. This results in production of positive-sense RNA, i.e. viral mRNA and

antigenomic RNA (cRNA). The latter serves as a template for the generation of negative-sense genomic RNA (Jonsson & Schmaljohn, 2001). Hantaviral mRNA is transcribed in such a way that 3′-truncated molecules are generated preventing the formation of a panhandle structure. Like other important human pathogens belonging to the family *Bunyaviridae*, hantaviruses start synthesis of viral mRNA through the process of cap-snatching (Garcin *et al.*, 1995; Jin & Elliott, 1993; Mir & Panganiban, 2010).

The immune system is assumed to play a crucial role in the development of hantavirus-associated disease (Schönrich *et al.*, 2008). It detects virus through pattern recognition receptors (PRRs). Toll-like receptors (TLRs) and retinoic acid-inducible gene I (RIG-I)-like RNA helicases (RLHs), including RIG-I, represent two major classes of PRRs (Takeuchi & Akira, 2010). Whereas TLRs detect viral nucleic acid either at the cell surface or inside endosomes the RLHs survey the cytoplasm for virus-derived RNA (Eisenächer *et al.*, 2008). The RLHs are found in all nucleated cells (Gilliet *et al.*, 2008) and are involved in detection of many RNA viruses (Loo *et al.*, 2008). Negative-sense genomic RNA purified from hantavirus virions released into the supernatant do not activate RIG-I (Habjan *et al.*, 2008; Wang *et al.*, 2011). On the other hand, pathogenic hantaviruses have been shown to interfere with RIG-I signalling pointing towards the importance of RIG-I for the hantaviral life cycle (Alff *et al.*, 2006).

In this study, we show that RIG-I inhibits HTNV replication and mediates an HTNV-induced antiviral innate response. Furthermore, we identify expression of the HTNV N ORF as a possible stimulus of RIG-I signalling.

Results

Generation of a stable RIG-I knock down in human type II alveolar cells

The lung is the entry site for hantaviruses in humans. The alveolar epithelial cell line A549 supports hantavirus replication (Lee & Cho, 1981; Nam *et al.*, 2003; Stoltz *et al.*, 2007). Thus, we analysed these cells to test whether HTNV replication is regulated by RIG-I. Stable RIG-I knock-down (RIG-I KD) A549 cells were established by transfecting A549 cells with a retroviral vector expressing RIG-I-specific small hairpin RNA (shRNA). RIG-I was not detectable in RIG-I KD A549 cells after treatment with type I interferon (IFN) (Fig. 1a). In contrast, A549 cells transfected with a vector expressing non-target (nt) shRNA and parental A549 cells abundantly expressed RIG-I. Vesicular stomatitis virus (VSV), a known RIG-I stimulator (Kato *et al.*, 2006; Yoneyama *et al.*, 2005), replicated more efficiently in RIG-I KD than in nt shRNA or parental A549 cells (Fig. 1b). In conclusion, we have established a suitable experimental system to analyse the influence of RIG-I on hantavirus replication.

Generation of stable RIG-I KD A549 cells. (a) A549 cells expressing RIG-I-specific shRNA (lane 1, RIG-I KD), A549 cells expressing nt shRNA (lane 2) and parental A549 cells (lane 3) were treated with type I IFN (500 U ml^{-1}) before RIG-I expression was analysed by Western blot. (b) RIG-I KD, nt shRNA and parental A549 cells were infected with VSV (m.o.i. of 1). Supernatants were collected at different time points after infection as indicated. Virus titres were determined and are given as $TCID_{50}$ ml^{-1} on a log scale. The experiment was carried out twice with different stable RIG-I KD A549 cell clones giving similar results.

Increased virus replication in RIG-I KD A549 cells after HTNV infection

The RIG-I KD A549 cell lines did not differ from parental A549 cells with regard to surface expression of known cellular HTNV receptors. Fig. 2(a) shows that CD61 was strongly expressed on Vero E6 cells but hardly detectable on parental and RIG-I KD A549 cells. In contrast, CD55 was found on parental and RIG-I KD A549 cells, whereas Vero E6 cells only weakly expressed this HTNV receptor. Finally, gC1qR expression was hardly detectable on both cell types. Next, we examined whether knock-down of RIG-I influenced the growth pattern of HTNV. Fig. 2(b) demonstrates that HTNV replicated much more efficiently in RIG-I KD cells than in parental A549 cells over a time period of 7 days. Together, these loss-of-function experiments demonstrate that RIG-I negatively regulates HTNV replication in human type II alveolar cells.

Impaired HTNV replication in Huh7.5 cells expressing functional RIG-I

The physiological importance of RIG-I for restricting HTNV replication was confirmed in a gain-of-function experiment. For this purpose, we used Huh7.5 cells as they carry a mutated endogenous RIG-I allele which exerts a dominant-negative effect (Sumpter, *et al.*, 2005). The latter is partially compensated in transduced Huh7.5 cells that overexpress functional RIG-I, wild-type or constitutive active RIG-I (Binder *et al.*, 2007). The wild-type RIG-I molecule is maintained in an autoinhibited state and has to be stimulated by a ligand to undergo a conformational shift allowing innate signalling. In contrast, constitutive active RIG-I lacks the repressor domain, resulting in constitutive innate signalling (Saito *et al.*, 2007). Parental and transduced Huh7.5 cells did not significantly differ with regard to HTNV receptor surface expression (Fig. 3a). CD55 was weakly expressed and expression of CD61 and gCq1R was hardly detectable. HTNV replicated more efficiently in parental Huh7.5 cells as compared with transduced Huh7.5 cells expressing a functional RIG-I (Fig. 3b). The inhibitory effect of both wild-type or constitutive active RIG-I on HTNV replication was relatively weak probably because they had to overcome the dominant-negative effect of mutated endogenous RIG-I. Most importantly, however, the extent of HTNV growth inhibition was similar in Huh7.5 cells expressing wild-type or constitutive active RIG-I. Thus, HTNV efficiently stimulates wild-type RIG-I, resulting in downregulation of HTNV replication.

Lack of antiviral innate responses in stable RIG-I knock-down cells after HTNV infection

Next, we connected HTNV replication with the antiviral innate response. For this purpose, we tested expression of 2′,5′-oligoadenylate synthetase (OAS), an important IFN-stimulated gene that is induced by HTNV in A549 cells (Nam *et al.*, 2003). As shown in Fig. 4, hardly any OAS transcripts were detected in parental and RIG-I KD A549 cells that were uninfected but otherwise treated in the same way as HTNV-infected A549 cells (mock). Intriguingly, after HTNV infection parental but not RIG-I KD A549 cells upregulated OAS expression, demonstrating that HTNV-induced antiviral innate responses were severely hampered in the absence of RIG-I. However, both parental and RIG-I KD A549 cells strongly expressed OAS after treatment with type I IFN, showing that RIG-I KD A549 cells were in principle responsive to this signature cytokine of viral infections. In conclusion, increased HTNV replication in alveolar epithelial cells that lack RIG-I was linked to the inability to induce an antiviral innate response.

RIG-I stimulation by expression of the HTNV N ORF

We performed dual luciferase reporter assays in human HEK 293T cells to identify HTNV-derived RNA that triggers the RIG-I pathway. In line with a previous report (Habjan *et al.*, 2008), IFN-β promoter activity was not significantly induced by genomic HTNV RNA (data not shown). As genomic HTNV RNA is of negative polarity, it was still possible that positive-sense HTNV RNA triggers RIG-I signalling. To test this hypothesis, plasmids (pcDNA3) expressing either the N or G ORF were co-transfected with an IFN-β promoter-based RIG-I reporter system into human HEK 293T cells. It is important to note that pcDNA3-derived DNA alone does not stimulate IFN-β promoter activity in transfected HEK 293T cells (Ablasser *et al.*, 2009). Thus, transfection of empty pcDNA3 (vector) marks the background level caused by overexpression of the RIG-I reporter system. Intriguingly, RIG-I signalling was significantly stimulated by plasmids expressing the HTNV N ORF as compared with vector (Fig. 5a, left graph). In striking contrast, plasmids expressing the HTNV G ORF did not activate RIG-I (Fig. 5a, right graph). The NS1 ORF of influenza B virus, a RIG-I blocker (Wolff *et al.*, 2008), even diminished RIG-I activity below background level. Moreover, expression plasmids with inverted N ORF did not stimulate RIG-I after co-transfection with the RIG-I reporter system into HEK 293T cells, suggesting that negative-sense HTNV N RNA is not stimulatory (data not shown).

Next we verified that HTNV N RNA triggers IFN-β promoter activity via RIG-I. For this purpose uncapped HTNV N RNA was generated by *in vitro* transcription. After transfection of uncapped HTNV N RNA into HEK 293T cells RIG-I stimulation was significantly increased compared with the vector control (Fig. 5b, left graph). In striking contrast, *in vitro*-transcribed G RNA did not modulate RIG-I signalling as compared to the vector control (Fig. 5b, right graph).

Now, we compared HTNV N ORF expression plasmids to known stimulators of RIG-I signalling, poly(dA:dT) (Ablasser *et al.*, 2009; Chiu *et al.*, 2009) and VSV RNA (Kato *et al.*, 2006; Yoneyama *et al.*, 2005). Fig. 6(a) demonstrates that after transfection into HEK 293T cells 1 μg of HTNV N ORF expression plasmid was as efficient as 0.5 μg poly(dA:dT) in activating RIG-I signalling compared with the vector. In contrast, VSV RNA showed a higher RIG-I stimulatory capacity. Similar results were obtained using an NF-κB promoter-based reporter system (Fig. 6b). Altogether, these results show that HTNV N ORF expression significantly triggers RIG-I signalling.

HTNV N ORF expression stimulates RIG-I through dsRNA structures

It has been shown recently that dsRNA-like secondary structures play a pivotal role in RIG-I stimulation (Baum *et al.*, 2010; Fujita, 2009; Schlee *et al.*, 2009; Schmidt *et al.*, 2009). To test this hypothesis, we used a pcDNA4 vector expressing the C terminus (Δ83N, aa 84–190) of the vaccinia virus E3 protein (Valentine & Smith, 2010). Δ83N represents a well described dsRNA-binding domain that sequesters dsRNA (Chang & Jacobs, 1993; Watson *et al.*, 1991) and has been demonstrated to block the RIG-I stimulatory effect of poly(dA:dT) (Valentine & Smith, 2010). Intriguingly, Δ83N completely blocked the RIG-I stimulatory effect of HTNV N ORF expression (Fig. 7). In contrast, Δ83N did not abrogate the background level caused by the RIG-I reporter system (vector control). These findings indicate that dsRNA-like secondary structures of HTNV N RNA stimulate RIG-I.

Antiviral response after transfection of HTNV N RNA into A549 cells

To test the idea that HTNV N RNA induces an antiviral response through endogenous RIG-I A549 cells were transfected with *in vitro*-transcribed N RNA. Subsequently, cells were challenged with VSV and the viral titres were determined (Fig. 8). Indeed, transfection of *in vitro*-transcribed N RNA resulted in a 2 log-reduction upon VSV challenge compared to the control (mock transfection). Pre-treatment with poly(dA:dT) caused a similar reduction. As expected, pre-treatment with type I IFN reduced viral titres even more strongly. Importantly, treating *in vitro*-transcribed HTNV RNA with calf intestinal alkaline phosphatase (CIAP), which catalyses the hydrolysis of 5′-triphosphate groups from RNA, only slightly diminished the induction of an antiviral innate response. This suggests that the presence of 5′-triphosphate is less important for the RIG-I stimulation by HTNV N RNA. Taken together, these results show that HTNV N RNA induces an antiviral innate response that interferes with viral replication.

Discussion

After infection with HTNV, we found strongly enhanced virus replication in RIG-I KD A549 cells as compared with parental cells. This suggests that RIG-I acts as a cytoplasmic sensor of replicating HTNV as parental and RIG-I KD A549 cells did not differ with regard to hantavirus receptor expression. The role of RIG-I as a negative regulator of HTNV replication was confirmed by a gain-of-function experiment using Huh7.5 cells that lack functional RIG-I (Sumpter, *et al.*, 2005). In stably transduced Huh7.5 cells that overexpress wild-type or constitutive active RIG-I the sensitivity to RIG-I stimulatory virus is partially restored (Binder *et al.*, 2007). Similar to A549 cells Huh7.5 cells expressed CD55 as a HTNV receptor but not CD61 or gC1qR. Intriguingly, in HTNV-infected Huh7.5 cells expressing wild-type RIG-I and Huh7.5 cells expressing constitutive active RIG-I the extent of HTNV growth inhibition was comparable. In contrast to constitutive active RIG-I the wild-type RIG-I molecule has to be activated by a ligand to relay downstream signalling (Saito *et al.*, 2007), suggesting that HTNV stimulates wild-type RIG-I efficiently. Taken together, by using two different experimental approaches, a loss-of-function and a gain-of-function RIG-I system, we could demonstrate that RIG-I negatively regulates HTNV replication.

Confirming a previous report (Nam *et al.*, 2003), we found that HTNV-infected A549 cells upregulate OAS, an essential player during antiviral innate responses (Roberts *et al.*, 1976). In striking contrast, in RIG-I KD A549 cells no induction of OAS was observed despite strongly increased HTNV replication. Exogenous type I IFN induced OAS expression in parental and RIG-I KD A549 cells to the same extent, excluding an intrinsic defect in antiviral innate responses of RIG-I KD A549 cells. In conclusion, these results clearly show that the antiviral innate response against HTNV is severely impaired in the absence of RIG-I allowing HTNV to replicate more efficiently.

OAS represents a PRR on its own that is located in the cytoplasm as an inactive monomer (Chakrabarti *et al.*, 2011). It is activated by some form of dsRNA (Minks *et al.*, 1979) similar to TLR3. In addition, ssRNA has been shown to stimulate OAS (Hartmann *et al.*, 1998). Upon stimulation OAS generates 2′,5′-oligoadenylates from ATP (Kerr & Brown, 1978). Predominantly 2′,5′-oligoadenylate trimers and longer oligomers subsequently activate RNase L (Hovanessian *et al.*, 1979), a cellular endoribonuclease that cleaves cellular and viral RNA within single-stranded loop regions. Intriguingly, some of the resulting cleaving products stimulate RIG-I (Malathi *et al.*, 2007, 2010). Thus, RNA molecules derived from replicating HTNV may stimulate RIG-I indirectly through the OAS/RNase L pathway.

The nature of HTNV RNA that activates RIG-I is unclear. Genomic HTNV RNA did not significantly trigger RIG-I (data not shown) confirming observations by other investigators (Habjan *et al.*, 2008). This may be due to the prime-and-realign mechanism during initiation of hantaviral genome replication (Garcin *et al.*, 1995). It removes possible RIG-I stimulating 5′-triphosphates and creates complementary 5′- and 3′-ends forming a panhandle structure. Hantaviral N protein trimers specifically bind to the panhandle conformation to encapsidate the genomic RNA during replication (Mir *et al.*, 2006; Mir & Panganiban, 2004, 2005). In this way the likelihood of innate responses induced by genomic hantavirus RNA is further reduced because panhandle structures potentially activate RIG-I (Baum *et al.*, 2010; Fujita, 2009; Schlee *et al.*, 2009; Schmidt *et al.*, 2009). In contrast to genomic hantavirus RNA panhandles formed by hantavirus cRNA interact at much lower affinity with N protein trimers (Mir & Panganiban, 2005). Thus, they are more accessible to RIG-I as compared with genomic hantavirus RNA. It remains to be investigated, however, whether hantaviral cRNA is RIG-I stimulatory.

Intriguingly, N but not G ORF expression induced RIG-I signalling. The plasmid expressing NS1 protein of influenza B virus, a RIG-I inhibitor (Wolff *et al.*, 2008), even diminished RIG-I activity below background level. It is unlikely that the N protein itself triggers RIG-I signalling. The N-terminal caspase activation and recruitment domain (CARD) of RIG-I is masked by the RNA-binding C-terminal domain (Cui *et al.*, 2008; Takahasi *et al.*, 2008). Appropriate RNA structures, therefore, have to interact with this domain to induce conformational changes that expose CARD and allow downstream RIG-I signalling. Strongly supporting this view, the RNA-binding domain of vaccinia virus E3 protein (Chang & Jacobs, 1993; Watson *et al.*, 1991) completely blocked the RIG-I stimulatory effect of HTNV N ORF expression. Moreover, it has been demonstrated that N protein inhibits rather than stimulates innate responses (Levine *et al.*, 2010; Ontiveros *et al.*, 2010; Taylor *et al.*, 2009).

In vitro-transcribed N but not G RNA containing 5′-triphosphate activated RIG-I. This finding is explained by recent publications showing that 5′-triphosphate is not sufficient to trigger RIG-I signalling (Baum *et al.*, 2010; Fujita, 2009; Schlee *et al.*, 2009; Schmidt *et al.*, 2009). Thus, N but not G RNA may form dsRNA-like secondary structures that are crucial for RIG-I activation. These higher order structures may be bound and sequestered by the RNA-binding domain of vaccinia virus E3 protein explaining its inhibitory effect. As no dsRNA can be detected in HTNV-infected cells with dsRNA-specific J2 antibody (Wang *et al.*, 2011) dsRNA regions within HTNV N RNA may be smaller than 40 bp, the detection limit of J2 (Bonin *et al.*, 2000). In line with this view, crystallographic studies revealed that dsRNA of 14 bp and lacking 5′-triphosphate bind to RIG-I (Lu *et al.*, 2011). Alternatively, one dsRNA molecule per cell is sufficient to stimulate generation of type I IFN (Marcus & Sekellick, 1977) and RIG-I may be more sensitive in detecting dsRNA than dsRNA-specific antibodies.

Transfection of *in vitro*-transcribed HTNV N RNA into A549 cells resulted in a 2 log-reduction of viral titres upon VSV infection. This finding underlines the physiological importance of HTNV N RNA as a stimulus of antiviral innate responses. Intriguingly, a similar reduction was observed when the 5′-triphosphate groups of *in vitro*-transcribed HTNV N RNA were removed by CIAP treatment. Thus, dsRNA-like secondary structures rather than 5′-triphosphate seem to determine the RIG-I stimulatory capacity of HTNV N RNA. This explains why N mRNA triggers RIG-I signalling although the 5′-triphosphate is masked by a 7-methylguanosine cap. In accordance with this view, it has been recently shown that RIG-I detects synthetic short dsRNA without 5′-triphosphate (Kato *et al.*, 2008; Takahasi *et al.*, 2008).

The extent of RIG-I stimulation by N ORF expression plasmids was comparable to that observed with poly(dA:dT). This synthetic dsDNA is transcribed by DNA-dependent polymerase III into RNA molecules containing 5′-triphosphate ends that are sensed by RIG-I (Ablasser *et al.*, 2009; Chiu *et al.*, 2009). However, a role for DNA-dependent RNA polymerase III in our dual luciferase assays can be excluded for several reasons. The HTNV ORF lacks a long stretch of homopolymeric AT that is

required for recognition by RNA polymerase III. Moreover, RNA polymerase III transcribes AT-rich dsDNA in a promoter-independent fashion (Ablasser *et al.*, 2009). Accordingly, an expression plasmid with inverted N ORF should also stimulate RIG-I. However, this was not observed (data not shown).

In general viruses are detected by several PRRs at multiple checkpoints during the viral life cycle. For hantaviruses the following scenario could be envisaged. After receptor binding before viral entry there could be a first checkpoint for hantaviruses (Jiang *et al.*, 2008; Prescott *et al.*, 2005, 2007). Subsequently, after phagocytosis of HTNV-infected, apoptotic cells HTNV-derived dsRNA could trigger TLR3 in endosomal compartments (Handke *et al.*, 2009). We identified RIG-I, a cytoplasmic PRR, as a further checkpoint. It is likely that all PRRs stimulated by hantaviral components act synergistically to induce antiviral innate responses. Future studies have to elucidate the precise nature of hantaviral RNA structure(s) that trigger RIG-I activity. These efforts will finally lead to a better understanding of the molecular mechanisms that allow discrimination between 'self' and virus-derived RNA inside host cells.

Methods

Viruses.

HTNV (strain 76-118) was propagated in Vero E6 cells in a biosafety level 3 facility as described previously (Kraus *et al.*, 2004). The VSV Indiana strain (laboratory-adapted Mudd-Summers isolate) was propagated in Vero E6 cells. At 24 h post-infection (p.i.), supernatants were centrifuged at 2000 ***g*** to clear cellular debris, aliquoted and frozen at −80 °C.

Virus titration.

Hantavirus titres were determined as described previously (Heider *et al.*, 2001). Briefly, Vero E6 cells were infected by serial dilutions of supernatants for 1 h to allow absorbance of virus particles. Thereafter, cells were overlaid with medium containing 0.5% agarose. After 7 days of incubation at 37 °C the overlay was removed, cells were washed with PBS and fixed with methanol for 10 min. After incubation with a hantavirus N protein-specific polyclonal rabbit serum the SuperSignal West Dura Extended Duration Substrate kit (Pierce/Perbio) was used according to the manufacturer's instructions to visualize antigen-positive foci. Finally, the antigen-positive foci were counted to calculate virus titres (f.f.u. ml^{-1}).

VSV was titrated on Vero E6 cells in a 10-fold serial dilution in quadruplets. At 24 h p.i., cells were fixed with formaldehyde and stained with crystal violet. $TCID_{50}$ values were calculated by the Spearman–Kaerber method. For titration of supernatants from VSV-infected A549 cells plaque assays were performed. Cells were fixed with 1% paraformaldehyde and stained with crystal violet. Titres were expressed as p.f.u. ml^{-1}.

Cell lines.

HEK 293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FCS, 2 mM l-glutamine, penicillin and streptomycin (Gibco-Invitrogen). Vero E6 cells were grown in minimal essential medium supplemented with the same additives.

For generation of A549 RIG-I knock-down cell lines, pSM2c-derived plasmids containing RIG-I specific and nt shRNA expression cassettes (Expression Arrest human retroviral $shRNA^{mir}$ individual constructs; oligo ID V2HS 199776 and RHS1707, respectively) were obtained from Open Biosystems through BioCat and checked by sequencing using a U6 promoter-specific primer. A549 cells purchased from ATCC were reverse transfected (8th passage) in 100 mm cell culture dishes using 30 µl Lipofectamine 2000 (Gibco-Invitrogen) and 15 µg of the pSM2c-derived plasmids according to the manufacturer's protocol. After 12–18 days of puromycin selection (2 µg ml^{-1}) single clones were picked and transferred to 48-well plates, while the nt shRNA expressing clones were pooled. All cells were further passaged under puromycin selection (1 µg ml^{-1}).

Huh7.5 cells that are derived from a human hepatoma cell line (Blight *et al.*, 2002) express an endogenous RIG-I with a mutation (T55I) in the first caspase-recruiting domain. It binds viral RNA but is unable to transduce signals thereby acting as a dominant-negative inhibitor (Sumpter, et al., 2005). Transduced Huh7.5 clones overexpressing WT RIG-I or CA RIG-I have been generated previously (Binder *et al.*, 2007). Culture conditions for Huh7.5 parental cells and clones were according to Quinkert *et al.* (2005). All cell lines used were free of mycoplasma as tested by PCR-based VenorGeM mycoplasma detection kit (Minerva Biolabs).

Western blot.

Western blot analysis was performed as described previously (Lütteke *et al.*, 2010). RIG-I- and β-actin-specific antibodies were purchased from Alexis Biochemicals. For detection of primary antibodies an anti-rabbit or anti-mouse peroxidase-conjugated antibody (Amersham, GE Healthcare) was used.

RNA preparation.

Total viral RNA from supernatants of Vero E6 cells infected for 8 days (m.o.i. of 1) with HTNV or uninfected Vero E6 cells (mock) were isolated with TRIzol reagent (Gibco-Invitrogen) according to the manufacturer's instructions. Subsequently, RNA concentrations were determined by photometric analyses. VSV RNA was prepared from supernatants of Vero E6 cells infected for 24 h with VSV. Virus was ultracentrifuged at 25000 r.p.m. (SW 32Ti; Beckman Coulter) using a sucrose density gradient. RNA was isolated by TRI Reagent (Sigma Aldrich) according to the manufacturer's instruction.

Plasmids.

Expression plasmids derived from pcDNA3 and encoding HTNV N protein or G glycoprotein precursor molecule were used. The ORFs were cloned into pcDNA3 vector between sites *Eco*RI and *Xba*I. The nucleotide sequence of the entire ORF of each expression plasmid was confirmed by sequencing using the BigDye Terminator 3.1 kit (Applied Biosystems) and ABI Prism 3100 genetic analyser (Applied Biosystems). Data were evaluated with Sequencing Analyses 3.7 software.

***In vitro* transcription.**

The pcDNA3-derived expression plasmids were digested with *Apa*I (Fermentas). Subsequently, *in vitro*-transcription was performed using T7 polymerase (Fermentas) according to the manufacturer's protocol. After treatment with DNase, the RNA was purified by phenol/chloroform extraction or with RNAeasy Mini kit (Qiagen), washed with ice-cold 75% ethanol and immediately used for experiments. An aliquot of each *in vitro*-transcribed RNA sample was analysed by RNA gel electrophoresis to verify its integrity (data not shown). To test the influence of 5′-triphosphates, aliquots of *in vitro*-transcribed RNAs were treated with CIAP (Fermentas) for 3 h at 37 °C. Thereafter, the samples were purified by phenol/chloroform extraction as described above.

LightCycler quantitative RT-PCR.

The quantitative RT-PCR analysis was performed as described previously (Lütteke *et al.*, 2010).

Dual luciferase assay.

Duplicate wells of subconfluent HEK 293T cells were transfected with 50 ng IFN-β promoter luciferase reporter plasmid p125-Luc (Yoneyama *et al.*, 1996) or NF-κB-driven Firefly luciferase plasmid (Stratagene) and 5 ng of constitutively active renilla luciferase expression vector pRL-TK-Luc (Stratagene) using Lipofectamine 2000 (Gibco-Invitrogen). To assess the RIG-I stimulatory activities,

cells were co-transfected with 1 µg expression plasmids (HTNV N ORF or HTNV G ORF) or 500 ng of viral RNA generated by *in vitro* transcription, respectively, together with 100 ng of RIG-I expression plasmid (pEF-Bos-RIG-I Flag; Yoneyama *et al.*, 2004). For each sample, transfections were done in parallel without RIG-I expression plasmid to assess background activity due to possible stimulation of endogenous pattern recognition receptors. Cells were lysed at 24 h post-transfection in 1× passive lysis buffer (Promega) by incubation for 15 min at 25 °C with gentle agitation. Subsequently, firefly luciferase reporter activity was assayed 24 h after transfection and normalized to renilla luciferase activity. All luciferase assays were performed at least three times with similar results using the Dual-Luciferase Reporter Assay System (Promega) and a LB96V luminometer (Berthold). Empty pcDNA3 vector was used as negative control; a vector expressing the non-structural protein of influenza B virus (B/NS1) was used as positive control for inhibition (Dauber *et al.*, 2004). For every sample appropriate expression of RIG-I was verified by Western blot analysis (data not shown). Poly(dA:dT) (Enzo Life Sciences) was used as a positive control for RIG-I stimulation. For inhibition of RIG-I stimulation by HTNV N ORF expression Δ83N inserted into pcDNA4 (Valentine & Smith, 2010) was used.

Flow cytometry.

Flow cytometry was performed as described previously (Lütteke *et al.*, 2010). The following primary antibodies were used: anti-β3, clone C17 (Immunotools); anti-CD55/DAF, clone 143-30 (Southern Biotechnology); and anti-gC1qR, clone 74.5.2 (Chemicon). As secondary antibody PE-coupled goat anti-mouse antibodies (Dianova) were used.

Acknowledgements

We thank T. Kaiser (Deutsches Rheumaforschungszentrum) for assistance in flow cytometry, F. Weber (University of Marburg) for VSV, Å. Lundkvist (Swedish Institute for Infectious Disease Control) for HTNV (strain 76-118), C. Rice (The Rockefeller University) for Huh7.5 cells, H. Feldmann (NIH, Hamilton) for the HTNV GPC plasmid, A. Hegele (Charité – Universitätsmedizin) for generation of the pcDNA3-derived plasmids, T. Fujita (Kyoto University) for plasmid p125-Luc, G. L. Smith (Imperial College London) for Δ83N plasmid, and C. Priemer (Charité – Universitätsmedizin Berlin) for cultivation of Vero E6 cells. This work was supported by Deutsche Forschungsgemeinschaft (GraKo 1121 to M.-H.L., N.L., S.K. and P.L.; DFG 554/3-2 to T.W.).

References

Ablasser, A., Bauernfeind, F., Hartmann, G., Latz, E., Fitzgerald, K. A. & Hornung, V. (2009). RIG-I-dependent sensing of poly(dA:dT) through the induction of an RNA polymerase III-transcribed RNA intermediate. Nat Immunol 10, 1065–1072.

Alff, P. J., Gavrilovskaya, I. N., Gorbunova, E., Endriss, K., Chong, Y., Geimonen, E., Sen, N., Reich, N. C. & Mackow, E. R. (2006). The pathogenic NY-1 hantavirus G1 cytoplasmic tail inhibits RIG-I- and TBK-1-directed interferon responses. J Virol 80, 9676– 9686.

Baum, A., Sachidanandam, R. & García-Sastre, A. (2010). Preference of RIG-I for short viral RNA molecules in infected cells revealed by next-generation sequencing. Proc Natl Acad Sci U S A 107, 16303–16308.

Binder, M., Kochs, G., Bartenschlager, R. & Lohmann, V. (2007). Hepatitis C virus escape from the interferon regulatory factor 3 pathway by a passive and active evasion strategy. Hepatology 46, 1365–1374.

Blight, K. J., McKeating, J. A. & Rice, C. M. (2002). Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. J Virol 76, 13001–13014.

Bonin, M., Oberstrass, J., Lukacs, N., Ewert, K., Oesterschulze, E., Kassing, R. & Nellen, W. (2000). Determination of preferential binding sites for anti-dsRNA antibodies on double-stranded RNA by scanning force microscopy. RNA 6, 563–570.

Chakrabarti, A., Jha, B. K. & Silverman, R. H. (2011). New insights into the role of RNase L in innate immunity. J Interferon Cytokine Res 31, 49–57.

Chang, H. W. & Jacobs, B. L. (1993). Identification of a conserved motif that is necessary for binding of the vaccinia virus E3L gene products to double-stranded RNA. Virology 194, 537–547.

Chiu, Y. H., Macmillan, J. B. & Chen, Z. J. (2009). RNA polymerase III detects cytosolic DNA and induces type I interferons through the RIG-I pathway. Cell 138, 576–591.

Choi, Y., Kwon, Y. C., Kim, S. I., Park, J. M., Lee, K. H. & Ahn, B. Y. (2008). A hantavirus causing hemorrhagic fever with renal syndrome requires gC1qR/p32 for efficient cell binding and infection. Virology 381, 178–183.

Cui, S., Eisenächer, K., Kirchhofer, A., Brzózka, K., Lammens, A., Lammens, K., Fujita, T., Conzelmann, K. K., Krug, A. & Hopfner, K. P. (2008). The C-terminal regulatory domain is the RNA 59-triphosphate sensor of RIG-I. Mol Cell 29, 169–179.

Dauber, B., Heins, G. & Wolff, T. (2004). The influenza B virus nonstructural NS1 protein is essential for efficient viral growth and antagonizes beta interferon induction. J Virol 78, 1865–1872.

Eisenä cher, K., Steinberg, C., Reindl, W. & Krug, A. (2008). The role of viral nucleic acid recognition in dendritic cells for innate and adaptive antiviral immunity. Immunobiology 212, 701–714.

Fujita, T. (2009). A nonself RNA pattern: tri-p to panhandle. Immunity 31, 4–5.

Garcin, D., Lezzi, M., Dobbs, M., Elliott, R. M., Schmaljohn, C., Kang, C. Y. & Kolakofsky, D. (1995). The 59 ends of Hantaan virus (Bunyaviridae) RNAs suggest a prime-and-realign mechanism for the initiation of RNA synthesis. J Virol 69, 5754–5762.

Gavrilovskaya, I. N., Shepley, M., Shaw, R., Ginsberg, M. H. & Mackow, E. R. (1998). b3 integrins mediate the cellular entry of hantaviruses that cause respiratory failure. Proc Natl Acad Sci U S A 95, 7074–7079.

Gavrilovskaya, I. N., Brown, E. J., Ginsberg, M. H. & Mackow, E. R. (1999). Cellular entry of hantaviruses which cause hemorrhagic fever with renal syndrome is mediated by b3 integrins. J Virol 73, 3951–3959.

Gilliet, M., Cao, W. & Liu, Y. J. (2008). Plasmacytoid dendritic cells: sensing nucleic acids in viral infection and autoimmune diseases. Nat Rev Immunol 8, 594–606.

Habjan, M., Andersson, I., Klingström, J., Schü mann, M., Martin, A., Zimmermann, P., Wagner, V., Pichlmair, A., Schneider, U. & other authors (2008). Processing of genome 59 termini as a strategy of negative-strand RNA viruses to avoid RIG-I-dependent interferon induction. PLoS ONE 3, e2032.

Handke, W., Oelschlegel, R., Franke, R., Krüger, D. H. & Rang, A. (2009). Hantaan virus triggers TLR3-dependent innate immune responses. J Immunol 182, 2849–2858.

Hartmann, R., Norby, P. L., Martensen, P. M., Jorgensen, P., James, M. C., Jacobsen, C., Moestrup, S. K., Clemens, M. J. & Justesen, J. (1998). Activation of 29-59 oligoadenylate synthetase by singlestranded and double-stranded RNA aptamers. J Biol Chem 273, 3236–3246.

Heider, H., Ziaja, B., Priemer, C., Lundkvist, A., Neyts, J., Krüger, D. H. & Ulrich, R. (2001). A chemiluminescence detection method of hantaviral antigens in neutralisation assays and inhibitor studies. J Virol Methods 96, 17–23.

Hovanessian, A. G., Wood, J., Meurs, E. & Montagnier, L. (1979). Increased nuclease activity in cells treated with pppA2'p5'A2'p59 A. Proc Natl Acad Sci U S A 76, 3261–3265.

Jiang, H., Wang, P. Z., Zhang, Y., Xu, Z., Sun, L., Wang, L. M., Huang, C. X., Lian, J. Q., Jia, Z. S. & other authors (2008). Hantaan virus induces toll-like receptor 4 expression, leading to enhanced production of beta interferon, interleukin-6 and tumor necrosis factor-alpha. Virology 380, 52–59.
Jin, H. & Elliott, R. M. (1993). Non-viral sequences at the 59 ends of Dugbe nairovirus S mRNAs. J Gen Virol 74, 2293–2297.
Jonsson, C. B. & Schmaljohn, C. S. (2001). Replication of hantaviruses. Curr Top Microbiol Immunol 256, 15–32.
Kato, H., Takeuchi, O., Sato, S., Yoneyama, M., Yamamoto, M., Matsui, K., Uematsu, S., Jung, A., Kawai, T. & other authors (2006).
Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature 441, 101–105.
Kato, H., Takeuchi, O., Mikamo-Satoh, E., Hirai, R., Kawai, T., Matsushita, K., Hiiragi, A., Dermody, T. S., Fujita, T. & Akira, S. (2008). Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation associated gene 5. J Exp Med 205, 1601–1610.
Kerr, I. M. & Brown, R. E. (1978). pppA2’p5’A2’p5’A: an inhibitor of protein synthesis synthesized with an enzyme fraction from interferon-treated cells. Proc Natl Acad Sci U S A 75, 256–260.
Kraus, A. A., Raftery, M. J., Giese, T., Ulrich, R., Zawatzky, R.,
Hippenstiel, S., Suttorp, N., Krüger, D. H. & Schönrich, G. (2004). Differential antiviral response of endothelial cells after infection with pathogenic and nonpathogenic hantaviruses. J Virol 78, 6143–6150.
Krautkrä mer, E. & Zeier, M. (2008). Hantavirus causing hemorrhagic fever with renal syndrome enters from the apical surface and requires decay-accelerating factor (DAF/CD55). J Virol 82, 4257–4264.
Krüger, D. H., Ulrich, R. & Lundkvist A, A. (2001). Hantavirus infections and their prevention. Microbes Infect 3, 1129–1144.
Lee, H. W. & Cho, H. J. (1981). Electron microscope appearance of Hantaan virus, the causative agent of Korean haemorrhagic fever. Lancet 1, 1070–1072.
Levine, J. R., Prescott, J., Brown, K. S., Best, S. M., Ebihara, H. & Feldmann, H. (2010). Antagonism of type I interferon responses by new world hantaviruses. J Virol 84, 11790–11801.
Loo, Y. M., Fornek, J., Crochet, N., Bajwa, G., Perwitasari, O., Martinez-Sobrido, L., Akira, S., Gill, M. A., García-Sastre, A. & other authors (2008). Distinct RIG-I and MDA5 signaling by RNA viruses in innate immunity. J Virol 82, 335–345.
Lu, C., Ranjith-Kumar, C. T., Hao, L., Kao, C. C. & Li, P. (2011). Crystal structure of RIG-I C-terminal domain bound to blunt-ended double-strand RNA without 59 triphosphate. Nucleic Acids Res 39, 1565–1575.
Ludwig, B., Kraus, F. B., Allwinn, R., Doerr, H. W. & Preiser, W. (2003). Viral zoonoses – a threat under control? Intervirology 46, 71–78.
Lütteke, N., Raftery, M. J., Lalwani, P., Lee, M.-H., Giese, T., Voigt, S., Bannert, N., Schulze, H., Krüger, D. H. & Schönrich, G. (2010).
Switch to high-level virus replication and HLA class I upregulation in differentiating megakaryocytic cells after infection with pathogenic hantavirus. Virology 405, 70–80.
Malathi, K., Dong, B., Gale, M., Jr & Silverman, R. H. (2007). Small self-RNA generated by RNase L amplifies antiviral innate immunity. Nature 448, 816–819.
Malathi, K., Saito, T., Crochet, N., Barton, D. J., Gale, M., Jr & Silverman, R. H. (2010). RNase L releases a small RNA from HCV RNA that refolds into a potent PAMP. RNA 16, 2108–2119.
Marcus, P. I. & Sekellick, M. J. (1977). Defective interfering particles with covalently linked [±] RNA induce interferon. Nature 266, 815–819. Medline
Minks, M. A., West, D. K., Benvin, S. & Baglioni, C. (1979). Structural requirements of double-stranded RNA for the activation of 29,59-oligo(A) polymerase and protein kinase of interferon-treated HeLa cells. J Biol Chem 254, 10180–10183.
Mir, M. A. & Panganiban, A. T. (2004). Trimeric hantavirus nucleocapsid protein binds specifically to the viral RNA panhandle. J Virol 78, 8281–8288.
Mir, M. A. & Panganiban, A. T. (2005). The hantavirus nucleocapsid protein recognizes specific features of the viral RNA panhandle and is altered in conformation upon RNA binding. J Virol 79, 1824–1835.
Mir, M. A. & Panganiban, A. T. (2010). The triplet repeats of the Sin Nombre hantavirus 59 untranslated region are sufficient in cis for nucleocapsid-mediated translation initiation. J Virol 84, 8937–8944.
Mir, M. A., Brown, B., Hjelle, B., Duran, W. A. & Panganiban, A. T. (2006). Hantavirus N protein exhibits genus-specific recognition of the viral RNA panhandle. J Virol 80, 11283–11292.
Muranyi, W., Bahr, U., Zeier, M. & van der Woude, F. J. (2005). Hantavirus infection. J Am Soc Nephrol 16, 3669–3679.

Nam, J. H., Hwang, K. A., Yu, C. H., Kang, T. H., Shin, J. Y., Choi, W. Y., Kim, I. B., Joo, Y. R., Cho, H. W. & Park, K. Y. (2003). Expression of interferon inducible genes following Hantaan virus infection as a mechanism of resistance in A549 cells. Virus Genes 26, 31–38.
Ontiveros, S. J., Li, Q. & Jonsson, C. B. (2010). Modulation of apoptosis and immune signaling pathways by the Hantaan virus nucleocapsid protein. Virology 401, 165–178.
Prescott, J., Ye, C., Sen, G. & Hjelle, B. (2005). Induction of innate immune response genes by Sin Nombre hantavirus does not require viral replication. J Virol 79, 15007–15015.
Prescott, J. B., Hall, P. R., Bondu-Hawkins, V. S., Ye, C. & Hjelle, B. (2007). Early innate immune responses to Sin Nombre hantavirus occur independently of IFN regulatory factor 3, characterized pattern recognition receptors, and viral entry. J Immunol 179, 1796–1802.
Quinkert, D., Bartenschlager, R. & Lohmann, V. (2005). Quantitative analysis of the hepatitis C virus replication complex. J Virol 79, 13594–13605.
Roberts, W. K., Hovanessian, A., Brown, R. E., Clemens, M. J. & Kerr, I. M. (1976). Interferon-mediated protein kinase and low-molecularweight inhibitor of protein synthesis. Nature 264, 477–480.
Saito, T., Hirai, R., Loo, Y. M., Owen, D., Johnson, C. L., Sinha, S. C., Akira, S., Fujita, T. & Gale, M., Jr (2007). Regulation of innate antiviral defenses through a shared repressor domain in RIG-I and LGP2. Proc Natl Acad Sci U S A 104, 582–587.
Schlee, M., Roth, A., Hornung, V., Hagmann, C. A., Wimmenauer, V., Barchet, W., Coch, C., Janke, M., Mihailovic, A. & other authors (2009). Recognition of 59 triphosphate by RIG-I helicase requires short blunt double-stranded RNA as contained in panhandle of negative-strand virus. Immunity 31, 25–34.
Schmaljohn, C. & Hjelle, B. (1997). Hantaviruses: a global disease problem. Emerg Infect Dis 3, 95–104.
Schmaljohn, C. S. & Nichol, S. T. (2007). Bunyaviridae. In Fields Virology, 5th edn, pp. 1741–1789. Edited byD.MKnipe &P. M. Howley. Philadelphia: Lippi0ncott Williams & Wilkins.
Schmidt, A., Schwerd, T., Hamm, W., Hellmuth, J. C., Cui, S., Wenzel, M., Hoffmann, F. S., Michallet, M. C., Besch, R. & other authors (2009). 59-triphosphate RNA requires base-paired structures to activate antiviral signaling via RIG-I. Proc Natl Acad Sci U S A 106, 12067–12072.
Schönrich, G., Rang, A., Lütteke, N., Raftery, M. J., Charbonnel, N. & Ulrich, R. G. (2008). Hantavirus-induced immunity in rodent reservoirs and humans. Immunol Rev 225, 163–189.
Stoltz, M., Ahlm, C., Lundkvist, A. & Klingström, J. (2007). Lambda interferon (IFN-l) in serum is decreased in hantavirus-infected patients, and in vitro-established infection is insensitive to treatment with all IFNs and inhibits IFN-c-induced nitric oxide production. J Virol 81, 8685–8691.
Sumpter, R., Jr, Loo, Y. M., Foy, E., Li, K., Yoneyama, M., Fujita, T., Lemon, S. M. & Gale, M., Jr (2005). Regulating intracellular antiviral defense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I. J Virol 79, 2689–2699.
Takahasi, K., Yoneyama, M., Nishihori, T., Hirai, R., Kumeta, H., Narita, R., Gale, M., Jr, Inagaki, F. & Fujita, T. (2008). Nonself RNAsensing mechanism of RIG-I helicase and activation of antiviral immune responses. Mol Cell 29, 428–440.
Takeuchi, O. & Akira, S. (2010). Pattern recognition receptors and inflammation. Cell 140, 805–820.
Taylor, S. L., Frias-Staheli, N., García-Sastre, A. & Schmaljohn, C. S. (2009). Hantaan virus nucleocapsid protein binds to importin a proteins and inhibits tumor necrosis factor a-induced activation of nuclear factor kappa B. J Virol 83, 1271–1279.
Ulrich, R., Hjelle, B., Pitra, C. & Krüger, D. H. (2002). Emerging viruses: the case ‘hantavirus’. Intervirology 45, 318–327.
Valentine, R. & Smith, G. L. (2010). Inhibition of the RNA polymerase III-mediated dsDNA-sensing pathway of innate immunity by vaccinia virus protein E3. J Gen Virol 91, 2221–2229.
Wang, H., Vaheri, A., Weber, F. & Plyusnin, A. (2011). Old World hantaviruses do not produce detectable amounts of dsRNA in infected cells and the 59 termini of their genomic RNAs are monophosphorylated. J Gen Virol 92, 1199–1204.
Watson, J. C., Chang, H. W. & Jacobs, B. L. (1991). Characterization of a vaccinia virus-encoded double-stranded RNA-binding protein that may be involved in inhibition of the double-stranded RNAdependent protein kinase. Virology 185, 206–216.
Wolff, T., Zielecki, F., Abt, M., Voss, D., Semmler, I. & Matthaei, M. (2008). Sabotage of antiviral signaling and effectors by influenza viruses. Biol Chem 389, 1299–1305.
Yoneyama, M., Suhara, W., Fukuhara, Y., Sato, M., Ozato, K. & Fujita, T. (1996). Autocrine amplification of type I interferon gene expression mediated by interferon stimulated gene factor 3 (ISGF3). J Biochem 120, 160–169.
Yoneyama, M., Kikuchi, M., Natsukawa, T., Shinobu, N., Imaizumi, T., Miyagishi, M., Taira, K., Akira, S. & Fujita, T. (2004). The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. Nat Immunol 5, 730–737.

Yoneyama,M., Kikuchi, M., Matsumoto, K., Imaizumi, T., Miyagishi, M., Taira, K., Foy, E., Loo, Y. M., Gale, M., Jr & other authors (2005). Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. J Immunol 175, 2851–2858.

Figures

Figure 1. Generation of stable RIG-I KD A549 cells. (a) A549 cells expressing RIG-I-specific shRNA (lane 1, RIG-I KD), A549 cells expressing nt shRNA (lane 2) and parental A549 cells (lane 3) were treated with type I IFN (500 U ml^{-1}) before RIG-I expression was analysed by Western blot. (b) RIG-I KD, nt shRNA and parental A549 cells were infected with VSV (m.o.i. of 1). Supernatants were collected at different time points after infection as indicated. Virus titres were determined and are given as $TCID_{50}$ ml^{-1} on a log scale. The experiment was carried out twice with different stable RIG-I KD A549 cell clones giving similar results.

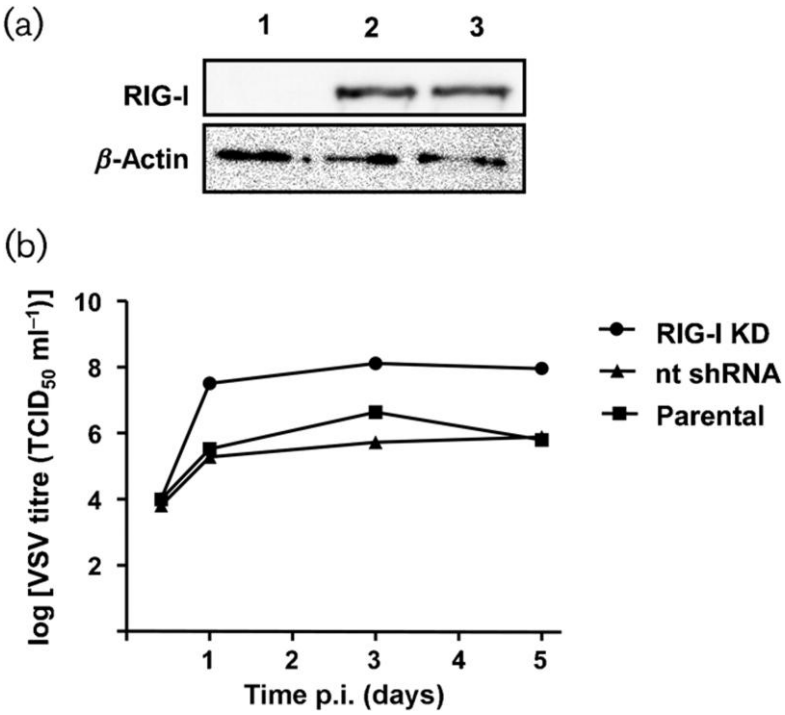

Figure 2. Receptor expression and growth kinetics in RIG-I KD A549 cells. (a) Vero E6, parental and RIG-I KD A549 cells were stained for CD61, CD55 or gC1qR (grey filled histograms). Cells incubated with secondary antibody alone served as a control (unfilled histograms). The *x*-axis and *y*-axis indicate fluorescence intensity (log scale) and cell counts, respectively. Numbers in the upper right-hand corner represent mean fluorescence intensities. The data shown are representative for two independent experiments. (b) Parental and RIG-I KD A549 cells were infected with HTNV (m.o.i. of 1). Supernatants were collected at different time points and virus titres were determined by chemiluminescence focus assay (f.f.u. ml^{-1}; log scale). The results represent one of three independent experiments carried out with different RIG-I KD A549 cell clones.

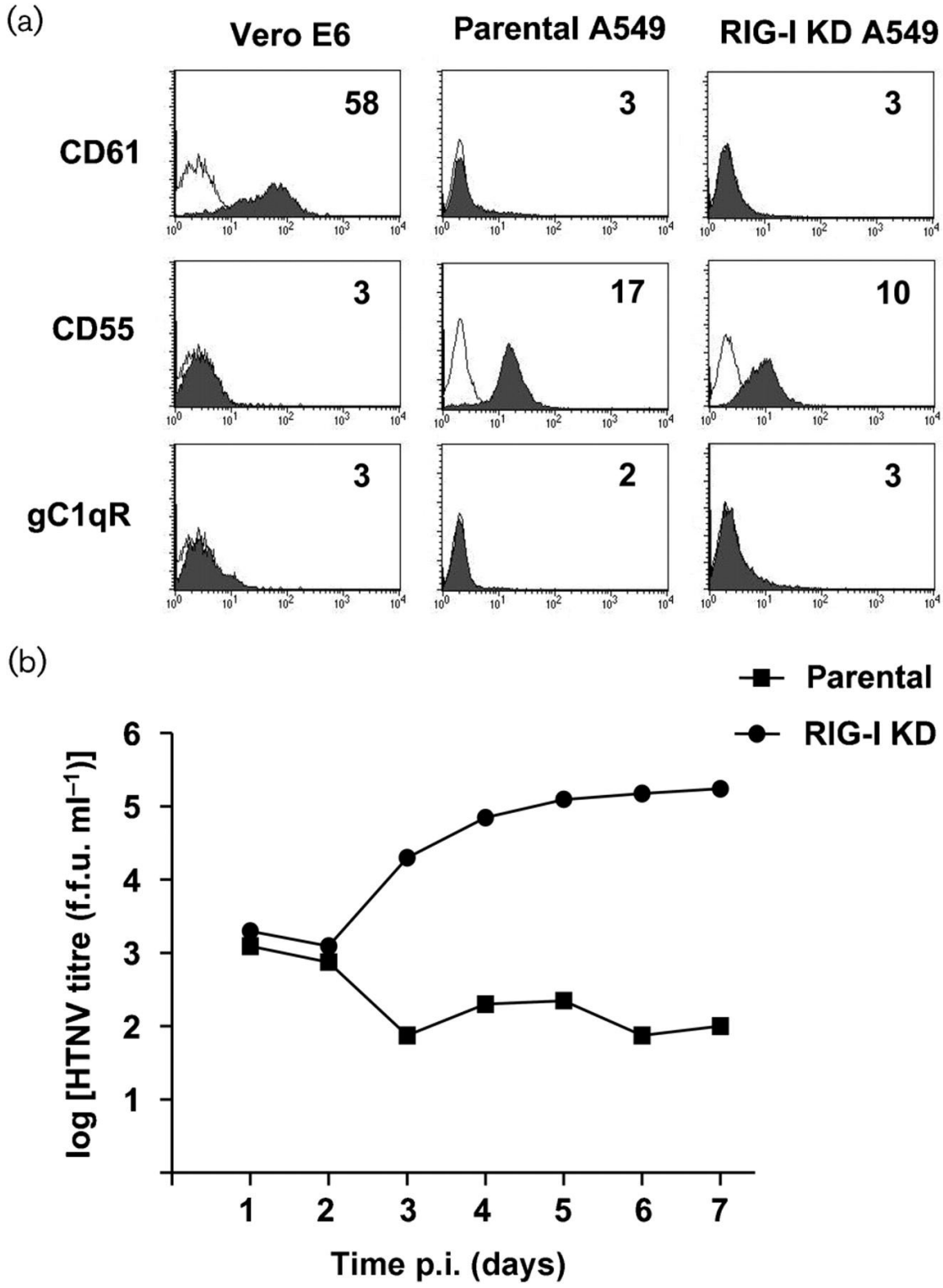

Figure 3. Receptor expression and growth kinetics in transduced Huh7.5 cells. (a) Parental cells and transduced cells expressing wild-type (WT) or constitutive (CA) RIG-I were stained for CD61, CD55 or gC1qR (grey filled histograms). Cells incubated with secondary antibody alone served as a control (unfilled histograms). The *x*-axis and *y*-axis indicate fluorescence intensity (log scale) and cell counts, respectively. Numbers in the upper right-hand corner represent mean fluorescence intensities. The data shown are representative for two independent experiments. (b) After infection with HTNV (m.o.i. of 1) supernatants from parental, WT RIG-I and CA RIG-I Huh7.5 cells were collected at different time points and virus titres were determined by chemiluminescence focus assay (f.f.u. ml^{-1}; log scale).

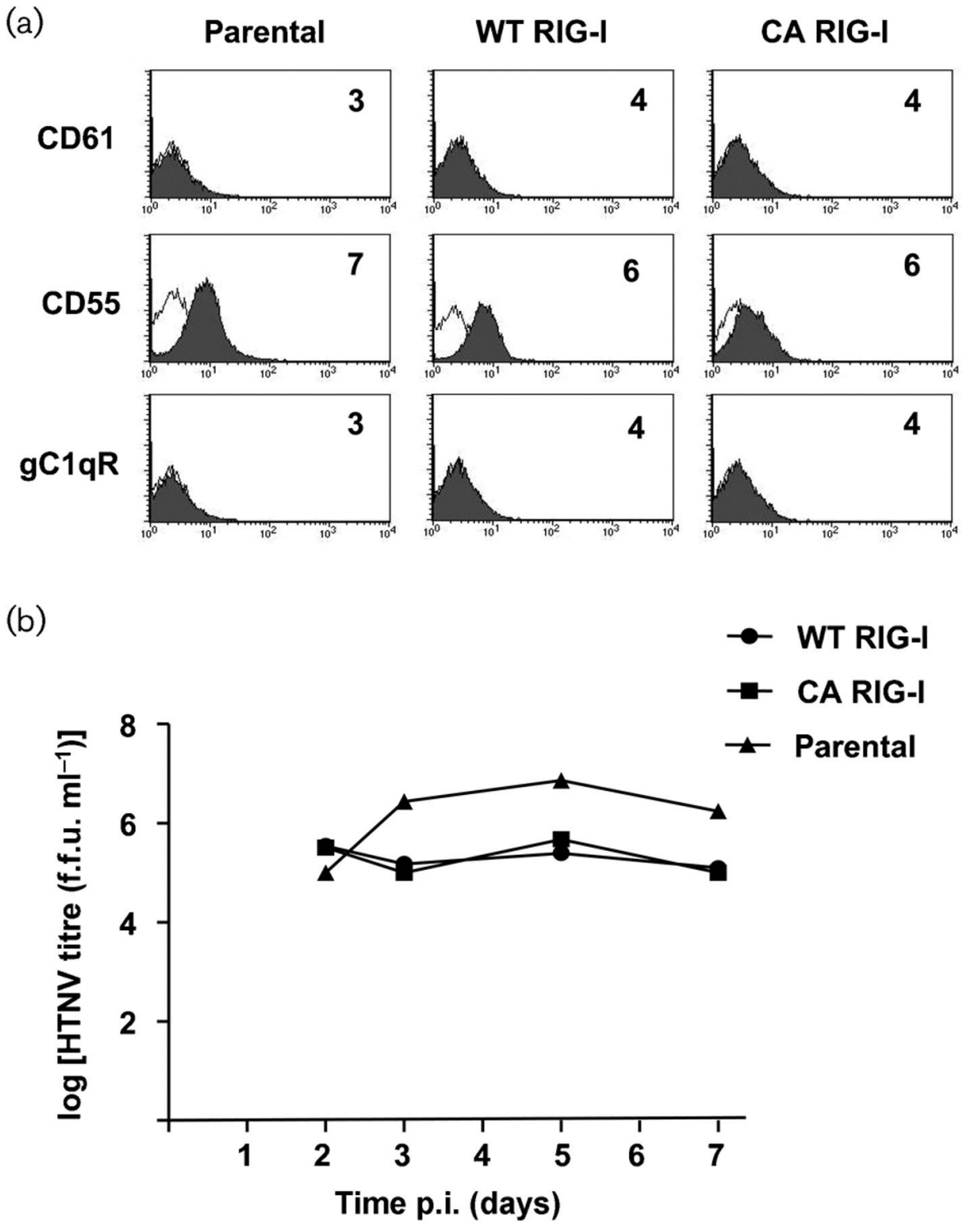

Figure 4. Antiviral innate response in RIG-I KD and parental A549 cells after HTNV infection. RIG-I KD and parental A549 cells as indicated were left uninfected (mock) or infected with HTNV (m.o.i. of 1.5). RNA was isolated at 4 days p.i. and the relative abundance of transcripts encoding 2′,5′-OAS was determined by quantitative RT-PCR (n=3, **P<0.005; Mann–Whitney U).

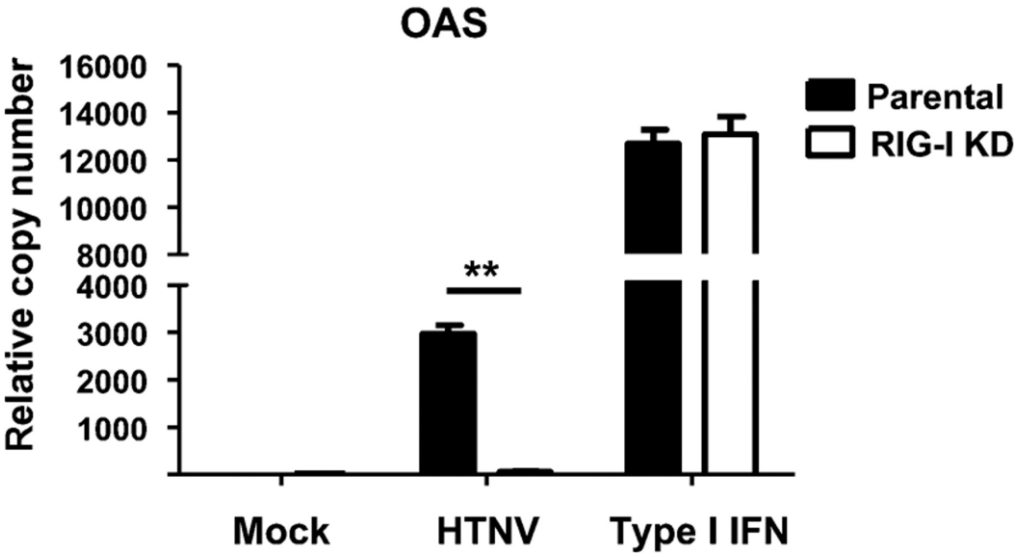

Figure 5. Induction of RIG-I signalling by HTNV-associated RNA. (a) Plasmids (1 µg) encoding HTNV N (N ORF, left graph) or HTNV G (G ORF, right graph) were transfected into HEK 293T cells together with an IFN-β promoter-based RIG-I reporter system (50 ng IFN-β promoter-dependent firefly luciferase reporter plasmid p125-Luc, 5 ng constitutively active renilla luciferase expression vector pRL-TK-Luc and 100 ng RIG-I expression plasmid). As controls the same amount of empty pcDNA3 (vector) or plasmid expressing NS1 of influenza B (Flu B/NS1) was transfected together with the RIG-I reporter system. (b) RNA (0.5 µg) derived from *in vitro* transcription of the HTNV N (left graph) or G (right graph) expression plasmid was transfected into HEK 293T cells together with the RIG-I reporter system. Results are shown as fold activation (mean±sd) of p125-Luc after normalization to renilla luciferase expression. They are derived from three independent experiments with transfections performed in duplicates (*P<0.05, Student's t-test).

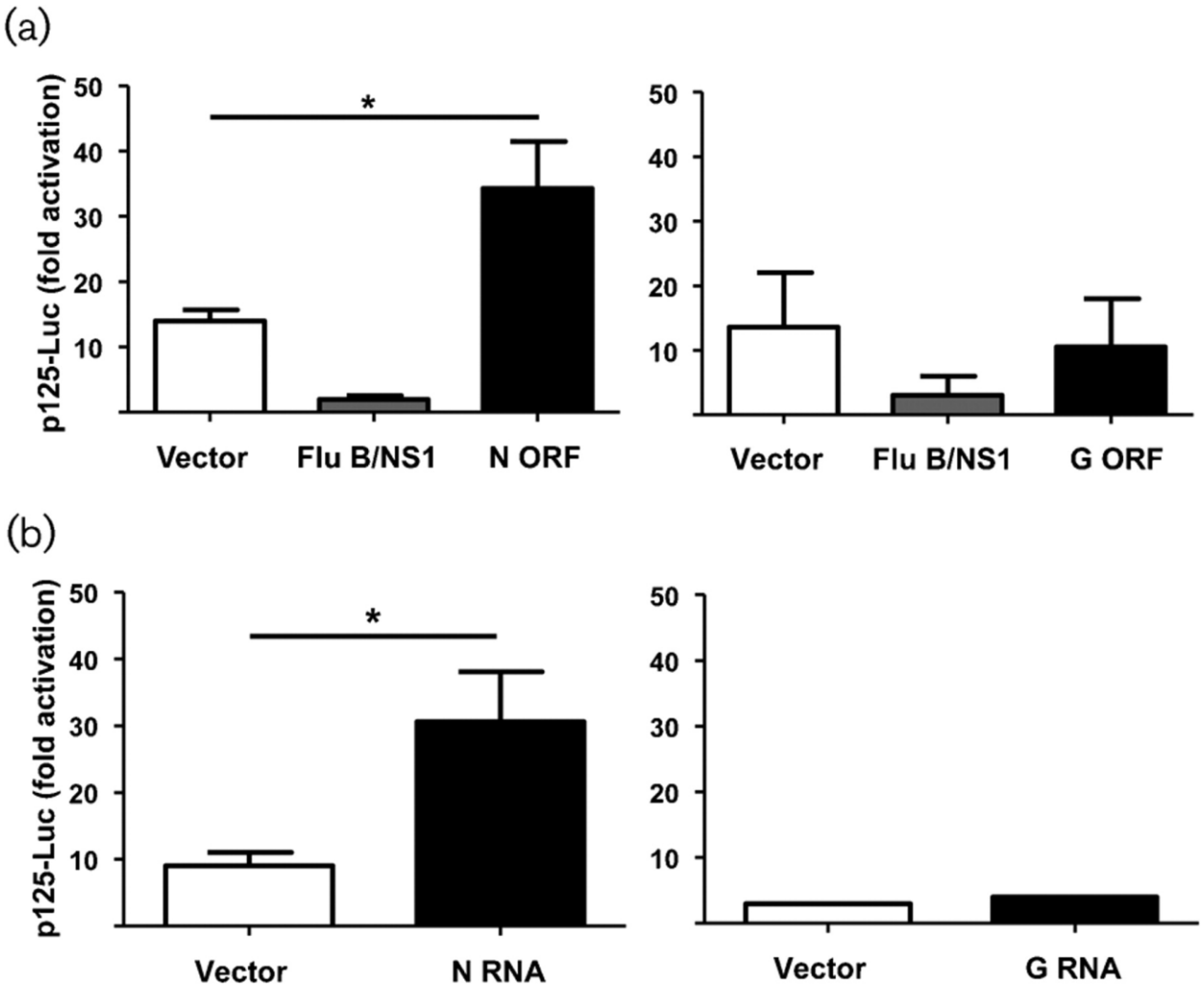

Figure 6. RIG-I stimulatory capacity of HTNV N ORF expression in dual luciferase assays. (a) Transfection of an IFN-β promoter-based RIG-I reporter system together with 1 µg empty pcDNA3 (vector), 1 µg HTNV N expression plasmid (N ORF), 0.5 µg poly(dA:dT) or 1 µg genomic VSV RNA into HEK 293T cells. (b) Transfection of an NF-κB promoter-based RIG-I reporter system together with 1 µg empty pcDNA3 (vector), 1 µg HTNV N expression plasmid (N ORF), 0.5 µg poly(dA:dT) or 1 µg genomic VSV RNA into HEK 293T cells. Results are shown as fold activation (mean±sd) of p125-Luc and pNF-κB-Luc, respectively, after normalization to renilla luciferase expression driven by a constitutive promoter. They are derived from three independent experiments with transfections performed in duplicate (**P*<0.05, ***P*<0.005; Student's *t*-test).

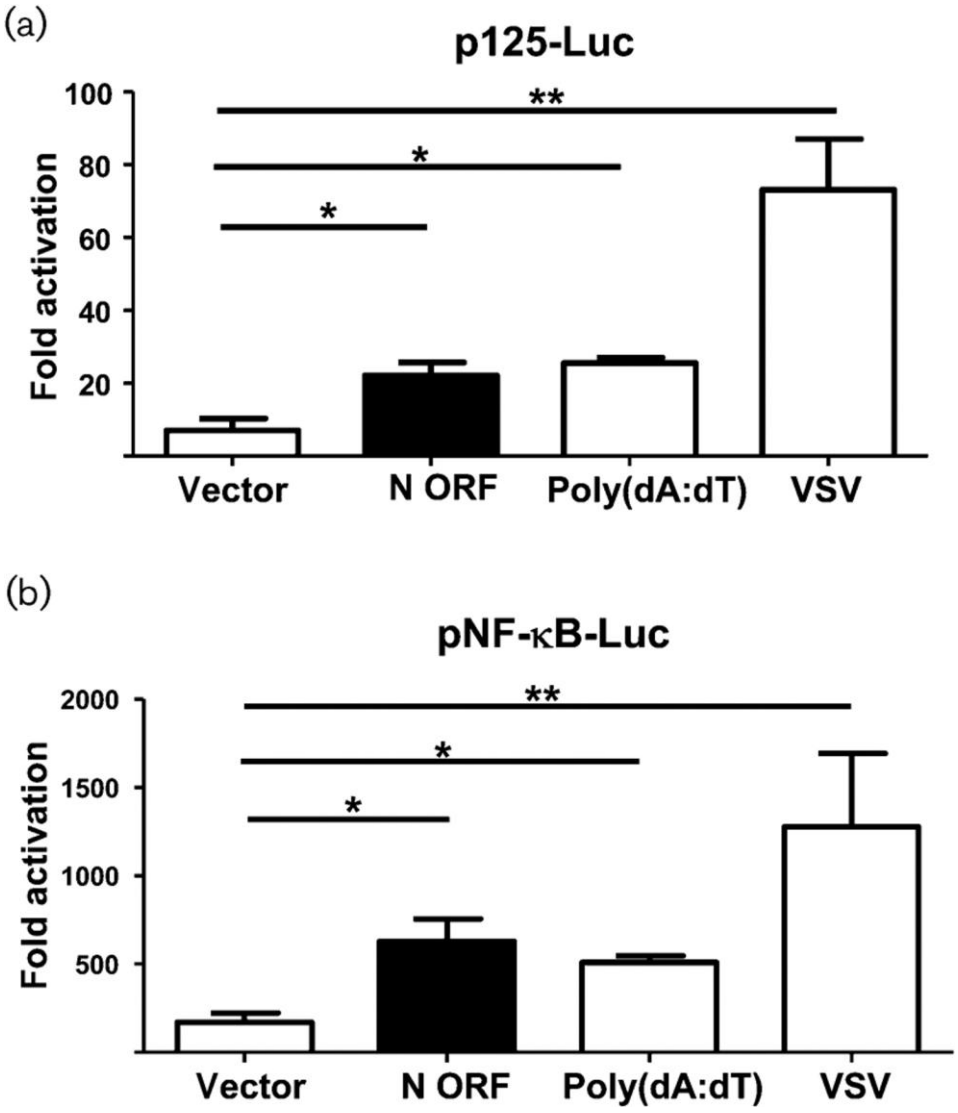

Figure 7. dsRNA-binding domain of vaccinia virus E3 protein (Δ83N) inhibits RIG-I stimulation by HTNV N ORF expression. 293T cells were co-transfected with 1 µg empty pcDNA3 (vector), 1 µg HTNV N expression plasmid (N ORF) or 0.5 µg poly(dA:dT) together with 1 µg Δ83N plasmid or empty pcDNA4 (vector) and IFN-β promoter-based RIG-I reporter system (50 ng p125-Luc, 5 ng pRL-TK, 100 ng RIG-I expression plasmid). Results are shown as fold change (mean±sem) of p125-Luc after normalization to renilla luciferase expression. They are derived from two independent experiments with transfections performed in triplicate. Statistical analysis was performed by an F-test to determine equal or unequal variance followed by a Student's *t*-test where appropriate (**P*<0.05).

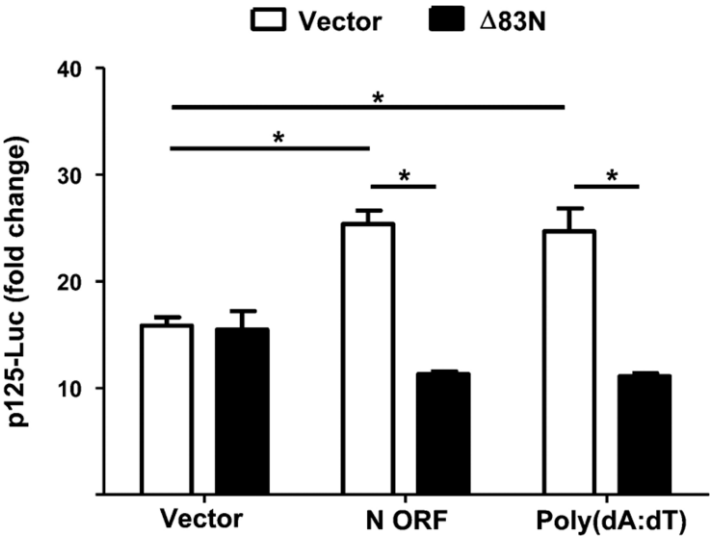

Figure 8. HTNV N RNA protects A549 cells upon VSV challenge. *In vitro*-transcribed HTNV N RNA (0.5 µg) were left untreated or treated with CIAP to remove 5′-triphosphate and transfected into A549 cells. As a positive control A549 cells were treated with poly(dA:dT) (0.5 µg) or type I IFN (2000 U ml^{-1}). After 24 h cells were infected with VSV (m.o.i. of 1). Supernatants from infected cells were collected 24 h p.i. and viral titres were measured by plaque assay. Results are shown as p.f.u. ml^{-1} (mean±sd) derived from two independent experiments (**P*<0.05, ***P*<0.005; Student's *t*-test).