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Role of retinoic acid inducible gene-I in human metapneumovirus-induced cellular signalling

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Human metapneumovirus (HMPV) is a recently discovered pathogen that causes a significant proportion of respiratory infections in young infants, the elderly and immunocompromised patients. Very little is known regarding the cellular signalling elicited by this virus in airway epithelial cells, the target of HMPV infection. In this study, we investigated the role of the RNA helicases retinoic acid inducible gene-I (RIG-I) and melanoma differentiation-associated gene-5 (MDA-5) as the main pattern recognition receptors (PRRs) involved in viral detection and subsequent expression of proinflammatory and antiviral genes. HMPV infection readily induced RIG-I and MDA-5 gene and protein expression in A549 cells, a type II-like alveolar epithelial cell line. Expression of dominant-negative (DN) RIG-I or downregulation of RIG-I gene expression using small interfering RNA (siRNA) significantly decreased HMPV-induced beta interferon (IFN-β), interleukin (IL)-8 and RANTES gene transcription, by inhibiting viral-induced activation of nuclear factor (NF)-κB and interferon regulatory factor (IRF), leading to enhanced viral replication. On the other hand, MDA-5 did not seem to play a significant role in HMPV-induced cellular responses. Mitochondrial antiviral signalling protein (MAVS), an adaptor protein linking both RIG-I and MDA-5 to downstream activation of IRF-3 and NF-κB, was also necessary for HMPV-induced cellular signalling. Expression of a DN MAVS significantly reduced IFN-β and chemokine gene transcription, by inhibiting NF-κB- and IRF-dependent gene transcription, in response to HMPV infection. Our results show that HMPV activates the RIG-I–MAVS signalling pathway in airway epithelial cells, leading to the expression of important proinflammatory and antiviral molecules involved in the innate immune response to viruses.

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INTRODUCTION

Human metapneumovirus (HMPV) is a recently identified respiratory RNA virus belonging to the family *Paramyxoviridae*. HMPV is responsible for a significant proportion of upper and lower respiratory tract infections in young children, the elderly and immunocompromised patients, causing bronchiolitis, croup, asthma exacerbation and even pneumonia (Boivin *et al.*, 2002; Esper *et al.*, 2003; Williams *et al.*, 2004), second only to respiratory syncytial virus (RSV) (Kahn, 2006; Principi *et al.*, 2006; Williams *et al.*, 2004), also a paramyxovirus. While there is emerging literature on the clinical and epidemiological features of HMPV infection, very little is known regarding the cellular signalling activated by this pathogen in infected cells. We have recently demonstrated that HMPV is a potent stimulus for cytokine, chemokine and type I interferon (IFN) production in cultured human alveolar epithelial cells, and that HMPV-induced chemokine expression is dependent on viral replication (Bao *et al.*, 2007). We have also shown that HMPV infection induces activation of nuclear factor (NF)-κB and interferon regulatory factor (IRF) transcription factors (Bao *et al.*, 2007), which have been shown to play a fundamental role in controlling the expression of chemokines following paramyxoviral infections (Casola *et al.*, 2001; Garofalo *et al.*, 1996; Groskreutz *et al.*, 2006; Spann *et al.*, 2005). Toll-like receptors (TLRs) and RNA helicases are the pattern recognition receptors (PRRs) most commonly activated by viral infections, leading to signal cascades that regulate the expression of proinflammatory and immune mediators (Akira & Takeda, 2004; Maniatis *et al.*, 1998). The TLRs, located in the endosomal cellular compartment, operate mainly in

plasmacytoid dendritic cells, while two DExD/H box RNA helicases, retinoic acid inducible gene-I (RIG-I) and melanoma differentiation-associated gene-5 (MDA-5) have been identified as being essential for IFN induction by several viruses, including Newcastle disease (NDV), Sendai (SeV) and hepatitis C (HCV) (Andrejeva *et al.*, 2004; Breiman *et al.*, 2005; tenOever *et al.*, 2004). Both RIG-I and MDA-5 share two homologous caspase recruitment domains (CARDs) and a helicase domain that is required for their interaction with viral RNA (Andrejeva *et al.*, 2004; Pichlmair *et al.*, 2006). It has been shown recently that RIG-I is responsible for sensing any viral RNA bearing 5′-triphosphate, while MDA-5 functions as a double-stranded RNA (dsRNA) sensor (Hornung *et al.*, 2006; Pichlmair *et al.*, 2006). The CARD domain of RNA helicases mediates the interaction with the CARD domain of the adaptor molecule IPS-1/MAVS/VISA/Cardif, leading to subsequent activation of important transcription factors, such as IRFs and NF-κB.

In the present study, we showed that HMPV infection readily activated the expression of RIG-I and MDA-5 in human A549 cells, an alveolar type II-like cell line. Overexpression of dominant-negative (DN) mutant RIG-I and gene suppression by small interfering RNA (siRNA) lead to the reduction in expression of type I IFN, as well cytokines and chemokines, indicating a critical role for RIG-I in HMPV-induced cellular signalling. RIG-I gene silencing significantly inhibited HMPV-induced IRF and NF-κB activation, indicating that RIG-I plays a major role in regulating the induction of these important transcription factors in response to HMPV infection. Expression of a MAVS protein lacking the N-terminal CARD domain, which acts as a DN mutant, significantly decreased viral-induced IRF- and NF-κB-dependent gene transcription, including expression of IFN-β and chemokine genes, suggesting a critical role of MAVS in HMPV-induced signalling pathways as well. A detailed understanding of the mechanism by which this viral infection triggers the innate immune response would be helpful for future design of novel therapeutic interventions and effective vaccination strategies.

METHODS

Viral preparation. LLC-MK2 cells (ATCC) were maintained in minimum essential medium (MEM; Invitrogen) supplemented with 10 % fetal bovine serum, penicillin and streptomycin (100 U ml^{-1}). HMPV strain CAN97-83 was obtained from Guy Boivin at the Research Center in Infectious Diseases, Regional Virology Laboratory, Laval University, Quebec, Canada. Virus was propagated in LLC-MK2 cells at 35 °C in the absence of serum and the presence of 1 μg trypsin ml^{-1} (Worthington), and was sucrose purified as described previously (Guerrero-Plata *et al.*, 2005a). Viral titre was determined by immunostaining in LLC-MK2 cells as described previously (Guerrero-Plata *et al.*, 2006). Lipopolysaccharide, assayed using the limulus haemocyanic agglutination assay, was not detected. Virus pools were aliquoted, quick frozen on dry ice/alcohol and stored at −70 °C until used.

Cell culture and infection with HMPV. A549 cells, human alveolar type II-like epithelial cells; 293 cells, a human embryonic kidney epithelial cell line; and Vero cells, an African green monkey kidney cell line (all from ATCC) were maintained in F-12K or MEM, containing 10 % (v/v) fetal bovine serum, 10 mM glutamine, 100 IU penicillin ml^{-1} and 100 μg streptomycin ml^{-1}. Cell monolayers were infected with HMPV at an m.o.i. of 3 (unless otherwise stated) as described previously (Garofalo *et al.*, 1996). An equivalent amount of 30 % sucrose was added to uninfected A549 cells, as a control.

RNA interference. Transfections of siRNA targeting RIG-I, MDA-5, TLR-3 or a scrambled negative control (Dharmacon), into A549 cells were carried out at a final concentration of 100 nM, using A549 transfection reagent (Altogen Biosystems) according to the manufacturer's recommendations. After 48–72 h, A549 cells were mock infected or infected with HMPV for 18 h at an m.o.i. of 3.

RNA extraction and real-time PCR. Total RNA was extracted from control and infected A549 cells by RNeasy kit (Qiagen) according to the manufacturer's instructions. Gene mRNAs were amplified by quantitative real-time PCR (Q-RT-PCR) using Applied Biosystems Assays-On-Demand 20× mix of primers and TaqMan MGB probes (FAM-dye-labelled) for target genes, and 18S rRNA (VIC-dye-labelled probe) TaqMan assay reagent (P/N 4319413E) for control. Separate tubes (singleplex) one-step RT-PCR was performed with 80 ng RNA for both target genes and endogenous control. The cycling parameters for one-step RT-PCR were: reverse transcription, 48 °C for 30 min; AmpliTaq activation, 95 °C for 10 min; denaturation 95 °C for 15 s and annealing/extension 60 °C for 1 min (repeated 30 times) on an ABI 7000. Duplicate threshold cycle (C_t) values were analysed in Microsoft Excel using the comparative C_t (ΔΔCT) method as described by the manufacturer (Applied Biosystems). The amount of target ($2^{-\Delta\Delta CT}$) was obtained by normalizing to an endogenous reference (18S) sample.

Plasmid preparation and transfections. Plasmids containing human IFN-β, RANTES and IL-8 promoters, as well as multimers of the RANTES IFN-stimulated response element (ISRE) site or IL-8 NF-κB site, linked to the luciferase reporter gene have been described previously (Casola *et al.*, 2000, 2001, 2002). DN RIG-I plasmids and full-length MAVS plasmid have been described previously (Foy *et al.*, 2005). Flag-tagged DN MAVS in pEFTaK vector was created by the deletion of the CARD domain, using full-length MAVS as a template. The primers used to generate CARD-deleted MAVS were: forward, 5′-AGCGGCCGCTCCGTTTGCTGAAGACAAGACCTATAAGTATT-GTGAGCTAGTTGATCTCGCGGACGAAGTGGCC-3′ and reverse, 5′-TGTTCGAATGGGTGACCTAGTGCA-3′.

The resulting PCR product was cloned initially into TOPO cloning vector (Invitrogen) and then subcloned into pEFTaK vector using *Not*I and *Bst*BI restriction enzymes. DNA sequencing of the constructs was performed before use. Exponentially growing A549 or 293 cells were transfected in triplicate in 60 mm dishes using FuGene 6 (Roche). A 1 μg sample of the reporter gene plasmid, and 0.2 μg of either empty vector plasmid, or DN expression plasmid (RIG-I or MAVS) were premixed with FuGene 6 in a 1 : 3 ratio (μg μl^{-1}), and added to the cells in 3 ml regular medium. After 30 h transfection, cells were infected with HMPV for 15 or 24 h. Cells were then lysed to measure luciferase and β-galactosidase reporter activity independently as described previously (Casola *et al.*, 2000). Luciferase was normalized to the internal control β-galactosidase activity. All experiments were performed at least two to three times.

Electrophoretic mobility shift assay (EMSA). Nuclear extracts from control and infected A549 cells were prepared using hypotonic/non-ionic detergent lysis, as described previously (Bao *et al.*, 2007). Proteins were normalized by protein assay (Protein Reagent;

Bio-Rad) and used to bind to duplex oligonucleotides corresponding to the RANTES ISRE, IL-8 NF-κB-binding site or an Oct consensus site (Panomics) as described previously (Casola *et al.*, 2000, 2001). Samples (10 μg) of nuclear proteins were incubated with the radiolabelled probe for 15 min at room temperature and then fractionated by 4 % non-denaturing PAGE in 0.5 × TBE buffer (22 mM Tris/HCl, 22 mM boric acid, 0.25 mM EDTA, pH 8) at 120 V. After electrophoretic separation, gels were dried and exposed for autoradiography with Kodak XAR film at −70 °C using intensifying screens. Quantification of the band intensity was done by gel exposure to PhosphoImager and analysis by ImageQuant software (Molecular Dynamics).

Western blot analysis. Total cell lysates were prepared by adding ice-cold lysis buffer (50 mM Tricine-HCl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 0.25 % sodium deoxycholate, 1 mM Na_3VO_4, 1 mM NaF, 1 % Triton X-100 and 1 μg aprotinin, leupeptin and pepstatin ml^{-1}). After incubation on ice for 10 min, the lysates were collected and detergent-insoluble materials were removed by centrifugation at 4 °C at 14 000 ***g***. Proteins (30 μg per sample) were then boiled in 3 × Laemmli buffer (Laemmli, 1970) for 2 min and resolved by SDS-PAGE. Proteins were transferred onto Hybond-enhanced chemiluminescence (ECL) nitrocellulose membrane (Amersham Pharmacia Biotech) and non-specific binding sites were blocked by immersing the membrane in TBST blocking solution [10 mM Tris/HCl, pH 7.6, 150 mM NaCl, 0.05 % Tween-20 (v/v)] containing 5 % skimmed milk powder for 60 min. After a short wash in TBST, the membranes were incubated with the primary antibody (RIG-I from Abgent and MDA-5 from Imgenex) overnight at 4 °C, followed by an anti-rabbit or anti-goat peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) diluted in TBST for 60 min at room temperature. After washing, the proteins were detected using ECL (Amersham Pharmacia Biotech) according to the manufacturer's protocol.

Statistical analysis. Two-tailed Student's *t*-tests using 95 % confidence levels were performed on all experiments. Significance was defined as $P<0.05$.

RESULTS

Role of RNA helicases in HMPV-induced gene expression in airway epithelial cells

The RNA helicase, RIG-I, has been shown to recognize several RNA viruses, including NDV, SeV, vesicular stomatitis virus, Japanese encephalitis virus, HCV and RSV (Chang *et al.*, 2006; Foy *et al.*, 2005; Kato *et al.*, 2005; Liu *et al.*, 2007). On the other hand, MDA-5 has been shown to be essential in detecting picornaviruses (Kato *et al.*, 2006) and to be the target of the IFN-inhibitory activity of paramyxovirus V protein (Andrejeva *et al.*, 2004; Childs *et al.*, 2007; Kawai *et al.*, 2005). The role of RIG-I and MDA-5 in detecting HMPV infection and initiating cellular signalling is currently unknown. To investigate whether HMPV infection of A549 cells induced RIG-I and MDA-5 expression, total RNA was extracted from cells uninfected and infected for various length of time, and used for measurement of the RIG-I and MDA-5 sequences by Q-RT-PCR. As shown in Fig. 1(a and b), HMPV infection caused a marked increase in the expression of both genes, starting around 6 h post-infection (p.i.) and peaking between 15 and 24 h p.i. To determine whether the changes in RIG-I and MDA-5 mRNA levels were paralleled by changes in protein synthesis, we performed Western blot analysis of total cell lysates prepared from A549 cells uninfected or infected for various lengths of time. As shown in Fig. 1(c and d), HMPV-induced RIG-I and MDA-5 protein induction followed a kinetic similar to the one seen for gene expression, with upregulation occurring around 6 h p.i. and progressively increasing up to 24 h p.i.

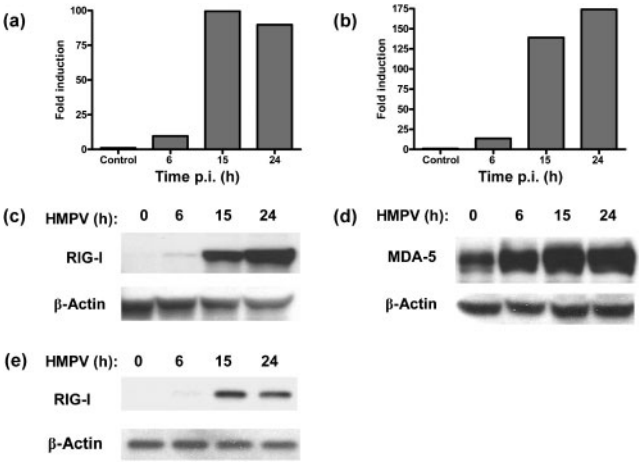

Fig. 1. HMPV-induced RIG-I and MDA-5 gene and protein expression in A549 cells. Total RNA was extracted from uninfected or infected A549 cells at 6, 15 and 24 h p.i. and used for Q-RT-PCR to determine changes in RIG-I (a) and MDA-5 (b) expression at different time points as indicated. Total cell lysates were prepared from uninfected or infected cells at various times p.i. Equivalent amounts of protein were subjected to 8 % SDS-PAGE, followed by Western blot analysis of RIG-I (c and e) and MDA-5 (d) expression. Membranes were stripped and reprobed with anti-β-actin antibody to control for equal loading of the samples. Data are representative of three independent experiments.

Since RIG-I is an IFN-inducible gene (Imaizumi *et al.*, 2004), we asked the question whether its induction following HMPV infection was directly due to the virus or was dependent on the autocrine/paracrine induction of type I IFN. To do so, we investigated RIG-I expression in Vero cells, which lack type I IFN production. Western blot analysis of total cell lysates prepared from Vero cells uninfected or infected for various lengths of time showed that HMPV infection was able to induce RIG-I expression with kinetics similar to those observed in airway epithelial cells, starting at 6 h p.i. However, the magnitude of induction was less in Vero cells and there was a decline in RIG-I expression at 24 h p.i. (Fig. 1e), indicating that HMPV directly induces RIG-I expression and that type I IFN enhances it, probably through an autocrine/paracrine pathway.

We had shown previously that A549 cells secrete a variety of cytokines and chemokines, as well as type I IFNs, upon

HMPV infection (Bao *et al.*, 2007). To investigate the functional significance of RIG-I in initiating cellular signalling, leading to the expression of these important immune mediators, we investigated the effect of expressing a CARD-deleted mutant RIG-I, which acts as a DN mutant by preventing interaction with the adaptor molecule MAVS (Liu *et al.*, 2007), on HMPV-induced IFN-β, RANTES and IL-8 gene transcription. A549 cells were cotransfected with a construct containing either IFN-β, RANTES or IL-8 gene promoter, linked to the luciferase reporter gene, and an expression plasmid containing RIG-I DN or the corresponding empty vector. Expression of the DN mutant RIG-I significantly reduced HMPV-induced luciferase activity of the IFN-β, RANTES and IL-8 gene promoter constructs by approximately 60, 70 and 55 %, respectively (Fig. 2), indicating an important role of RIG-I in viral-induced gene transcription.

To confirm the role of RIG-I in HMPV-induced cellular signalling, we investigated cytokine, chemokine and IFN-β gene expression in A549 cells transfected with either a scrambled siRNA, as a control, or one targeting RIG-I and infected with HMPV. Our results showed that treatment of A549 cells with siRNA targeting RIG-I effectively blocked RIG-I gene expression (approximately 80 %) and protein induction (approximately 40–50 %), as well as IFN-β, RANTES and IL-8 gene expression in response to HMPV infection by approximately 60, 80 and 50 %, respectively (Fig. 3).

To determine the effect of RIG-I inhibition on the production of other immune mediators, supernatants of A549 cells transfected with either the scrambled control or RIG-I siRNA and infected with HMPV were assayed for cytokine and chemokine secretion by Bio-Plex. There was significant reduced secretion of cytokines, such as IL-6, and other chemokines, such as MIP-1α, -β and MCP-1, in cells where RIG-I expression was silenced, compared with control cells, in response to HMPV infection, as shown in Table 1.

The reduction of type I IFN production, and probably IFN-dependent antiviral gene expression, resulted in enhanced viral replication, as shown by increased viral titres in cells infected with HMPV and transfected with RIG-I siRNA, compared with cells transfected with the scrambled siRNA, as shown in Table 2.

To investigate the role of MDA-5 in HMPV-induced cellular signalling, experiments similar to those described above were performed in A549 cells transfected with specific siRNA targeting MDA-5. Our results showed that effective silencing of MDA-5 gene expression (approximately 80 %) did not lead to a significant reduction of HMPV-induced cytokine or chemokine production or changes in viral replication (data not shown), indicating that MDA-5 does not play a major role in initiating cellular responses following HMPV infection.

Since inhibition of RIG-I expression did not completely abolish HMPV-induced cytokine and chemokine production and since we have shown recently that TLR-3 plays a role in cellular signalling in response to another paramyxovirus infection (Liu *et al.*, 2007), we investigated whether it was involved in HMPV-induced signalling too. A549 cells were transfected with specific siRNA targeting TLR-3 or with scrambled siRNA, infected with HMPV for 18 h, and harvested to measure TLR-3 and chemokine gene expres-

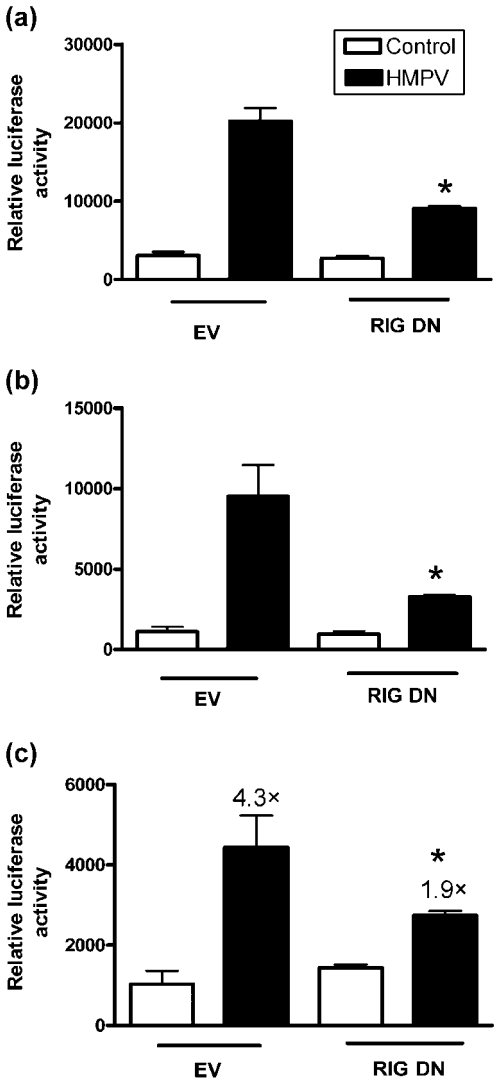

Fig. 2. Inhibition of HMPV-induced IFN-β and chemokines gene transcription by expression of DN RIG-I. A549 cells were cotransfected with a luciferase reporter plasmid containing IFN-β (a), RANTES (b) or IL-8 (c) promoter together with a RIG-I DN mutant expression plasmid (RIG DN), or the corresponding empty vector (EV). Cells were infected with HMPV and harvested at 15 or 24 h p.i. to measure luciferase activity. Uninfected plates served as controls. For each plate, luciferase was normalized to β-galactosidase reporter activity. Data are expressed as mean ± SD of normalized luciferase activity. Bars represent the mean value of triplicate samples of one experiment, n=at least two experiments. Numbers above HMPV columns in (c) are fold change relative to corresponding control values. *, $P<0.05$ relative to HMPV-infected EV-treated cells.

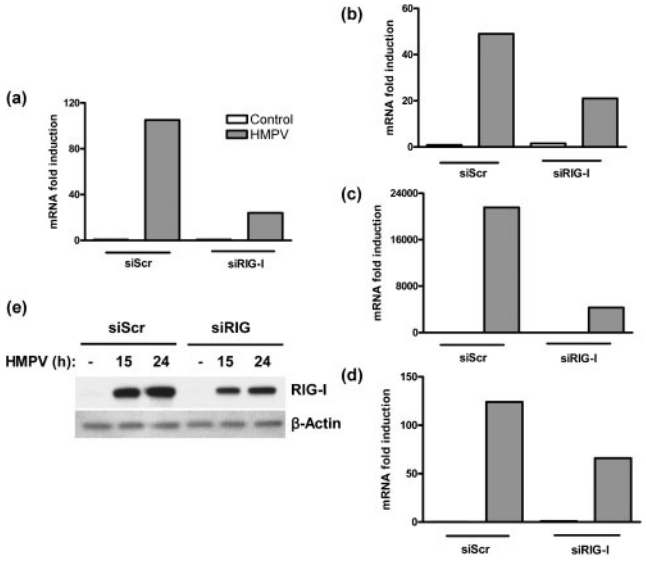

Fig. 3. Effect of RIG-I gene silencing on HMPV-induced IFN-β and chemokine gene expression. A549 cells were transfected with 100 nM siRNA targeting RIG-I (siRIG-I) or a scrambled negative control (siScr). A549 cells were mock infected or infected with HMPV for 18 h at an m.o.i. of 3 and harvested to prepare either total RNA or total cell lysates. Total RNA was harvested for analysis of RIG-I (a), IFN-β (b), RANTES (c) and IL-8 (d) gene expression by Q-RT-PCR. Total cell lysates were subjected to 8 % SDS-PAGE, followed by Western blot analysis of RIG-I (e) expression. Membrane was stripped and reprobed with anti-β-actin antibody to control for equal loading of the samples. Results are representative of three independent experiments.

sion. Our results showed that effective silencing of TLR-3 gene expression (approximately 80–90 %) did not lead to a significant inhibition of HMPV-induced chemokine induction (data not shown), indicating that TLR-3 does not play a major role in initiating cellular responses following HMPV infection.

Table 1. Effect of RIG-I silencing on HMPV-induced chemokine and cytokine secretion

Supernatants from HMPV-infected cells, treated with scrambled (siScr) or RIG-I (siRIG) siRNA, were harvested at the indicated time points of infection. Chemokine and cytokine secretion were measured by Bio-Plex assay. Values are expressed as pg ml^{-1}. Results are representative of three independent experiments.

Gene	siScr			siRIG		
	15 h	24 h	48 h	15 h	24 h	48 h
IP-10	175	574	2885	114*	358*	927*
MCP-1	301	3485	1474	240	2273*	1118*
MIP-1α	1.38	14.45	53	1.51	12	39*
MIP-1β	3	24	70	2	21	47*
IL-6	117	263	2726	127	276	1730*

*$P<0.05$, relative to siScr-treated cells.

Table 2. Effect of RIG-I silencing on HMPV replication

A549 cells were transfected with scrambled (siScr) or RIG-I (siRIG) siRNA, infected with HMPV, at an m.o.i. of 1, and harvested at the indicated time points to determine viral titres. Values are expressed as p.f.u. ml^{-1} and represent the average of two independent experiments.

siRNA	15 h	24 h	48 h
siScr	1.5×10^5	3.6×10^5	8.1×10^5
siRIG	2.7×10^5	1×10^6	2.5×10^6

RIG-I is required for HMPV-induced IRF and NF-κB activation

Transcription factors of the IRF family have been shown to play an essential role in viral-induced expression of type I IFN genes (reviewed by Barnes *et al.*, 2002). They also regulate the induction of several other genes involved in the immune/inflammatory response to viral infections, including the chemokine RANTES (reviewed by Barnes *et al.*, 2002). Similarly, a number of paramyxovirus-inducible inflammatory and immunoregulatory genes require NF-κB for their transcription, as we have shown *in vitro* for IL-8 (Garofalo *et al.*, 1996), RANTES (Casola *et al.*, 2001), and other chemokines and cytokines, secreted proteins and signalling molecules (Tian *et al.*, 2002). To determine whether RIG-I inhibition specifically affected HMPV-induced NF-κB- and IRF-dependent gene transcription, A549 cells were transiently cotransfected with a construct containing multiple copies of either the IL-8 NF-κB site (Casola *et al.*, 2000) or the RANTES ISRE site (Casola *et al.*, 2002) binding IRF proteins (Casola *et al.*, 2001), linked to the luciferase reporter gene, and the RIG-I DN expression plasmid or the empty vector. Expression of the DN mutant RIG-I significantly reduced both HMPV-induced IRF- and NF-κB-dependent gene transcription by approximately 55–60 %, as shown in Fig. 4(a and b), respectively, confirming the role of RIG-I as a central molecule initiating cellular signalling in response to HMPV infection.

NF-κB is a superfamily of ubiquitous transcription factors composed of NF-κB1 or p50, NF-κB2 or p52, Rel A or p65, Rel B and c-Rel proteins, which can form homo- and heterodimers and produce complexes with various transcriptional activities. NF-κB-inducing stimuli cause phosphorylation of inhibitory proteins called IκBs, through activation of the IκB kinase (IKK) complex (Karin & Delhase, 2000), with subsequent IκB proteolytic degradation (Henkel *et al.*, 1993); this allows NF-κB to enter the nucleus and bind to target gene promoters. We have shown recently that HMPV infection of airway epithelial cells induces a time-dependent increase in p50 and p65 nuclear translocation with parallel changes in DNA-binding

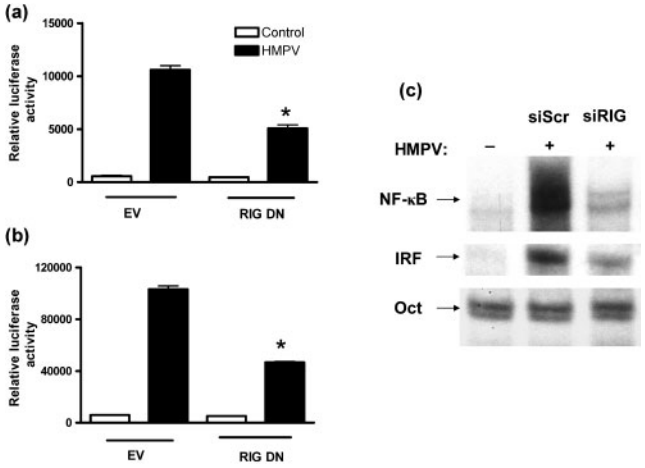

Fig. 4. Effect of RIG-I inhibition on HMPV-induced NF-κB and IRF activation. A549 or 293 cells were cotransfected with a luciferase reporter plasmid containing multimers of the RANTES ISRE site (a) or IL-8 NF-κB site (b) together with a RIG-I DN expression plasmid (RIG DN) or an empty vector (EV). Cells were infected with HMPV and harvested at 15 h p.i. to measure luciferase activity. Uninfected plates served as controls. For each plate, luciferase was normalized to the β-galactosidase reporter activity. Data are expressed as mean ± SD of normalized luciferase activity. Bars represent the mean value of triplicate samples of one experiment, n=at least two experiments. *, $P<0.05$ relative to HMPV-infected EV-treated cells. (c) A549 cells were transfected with scrambled siRNA (siScr) or specific siRNA against RIG-I (siRIG) and mock infected or infected with HMPV for 15 h. Nuclear extracts were harvested and subjected to EMSA with ^{32}P-labelled oligonucleotide probes corresponding to the IL-8 promoter NF-κB site, the RANTES promoter ISRE site or an Oct synthetic probe. Results are representative of two independent experiments.

activity (Bao *et al.*, 2007). To investigate the effect of RIG-I inhibition on NF-κB activation, A549 cells were transfected with either the scrambled or RIG-I siRNA, infected with HMPV and harvested at 15 h p.i. to prepare nuclear extracts. Gel mobility shift assay was used to detect changes in abundance of DNA-binding proteins that recognized the NF-κB-binding site of the IL-8 gene promoter as described previously (Bao *et al.*, 2007). A consensus sequence for Oct proteins, whose DNA binding does not change in response to viral infections, was used as an internal control. As shown in Fig. 4(c), HMPV infection markedly increased binding to the IL-8 promoter NF-κB site, compared with uninfected cells, and RIG-I silencing significantly reduced the inducible binding by approximately 65 % (quantified by PhosphoImager analysis). There was no change in Oct DNA-binding activity, demonstrating specificity of the RIG-I siRNA for NF-κB activation.

Among the different members of the IRF family, IRF-1, -3, -5 and -7 have been identified as direct transducers of viral-induced signalling, with IRF-3 being necessary for IFN-β and RANTES gene expression in response to paramyxovirus infections (Casola *et al.*, 2001). We have shown recently that HMPV infection leads to a time- and replication-dependent activation of IRF-1, -3 and -7 in airway epithelial cells, resulting in increased nuclear translocation and binding to the RANTES promoter ISRE site (Bao *et al.*, 2007). Downregulation of RIG-I expression by siRNA resulted in approximately 50 % reduction of HMPV-induced IRF binding to the RANTES ISRE site (quantified by PhosphoImager analysis), as shown in Fig. 4(c). Together, these data confirm that RIG-I plays an important role in NF-κB and IRF activation, and subsequent gene expression, in response to HMPV infection.

MAVS is necessary for HMPV-induced cellular signalling

MAVS is a mitochondrial adaptor protein linking RIG-I and MDA-5 to downstream kinases responsible for NF-κB and IRF activation, leading to proinflammatory and antiviral gene transcription (Johnson & Gale, 2006; Seth *et al.*, 2006; Sun *et al.*, 2006; Werts *et al.*, 2006). To determine whether MAVS was necessary for HMPV-induced signalling downstream of RIG-I, we investigated the effect of expressing a CARD-deleted mutant MAVS, which acts as a DN mutant by preventing interaction with RIG-I, on HMPV-induced IFN-β, RANTES and IL-8 gene transcription. A549 cells were cotransfected with the IFN-β, RANTES or IL-8 promoter reporter constructs and the MAVS DN expression plasmid or the empty vector. Expression of the DN mutant MAVS increased the basal activity of the IL-8 promoter; however, it significantly reduced HMPV-induced activity of the IFN-β, RANTES and IL-8 gene promoter constructs by approximately 60, 66 and 70 %, respectively (Fig. 5a–c).

Similarly, cotransfection of the MAVS DN plasmid with either the IL-8 NF-κB or the RANTES ISRE reporter constructs resulted in enhanced basal activation of the NF-κB-driven promoter, but also in a significant reduction of both HMPV-induced NF-κB- and IRF-dependent luciferase activity by approximately 70 and 85 %, respectively (Fig. 5d and e). Overall, these results confirm the important role of the RIG-I–MAVS pathway in initiating cellular signalling in response to HMPV infection.

DISCUSSION

Respiratory tract infections are a leading cause of morbidity and mortality worldwide. HMPV is a recently identified RNA virus, belonging to the family *Paramyxoviridae*, and a major cause of lower respiratory tract infections in children, elderly and immunocompromised patients and therefore it is currently considered to be a substantial public health problem (Kahn, 2006; Principi *et al.*, 2006; Williams *et al.*, 2004). The innate immune response represents a critical component of host defence against viruses and is coordinated at the cellular level by

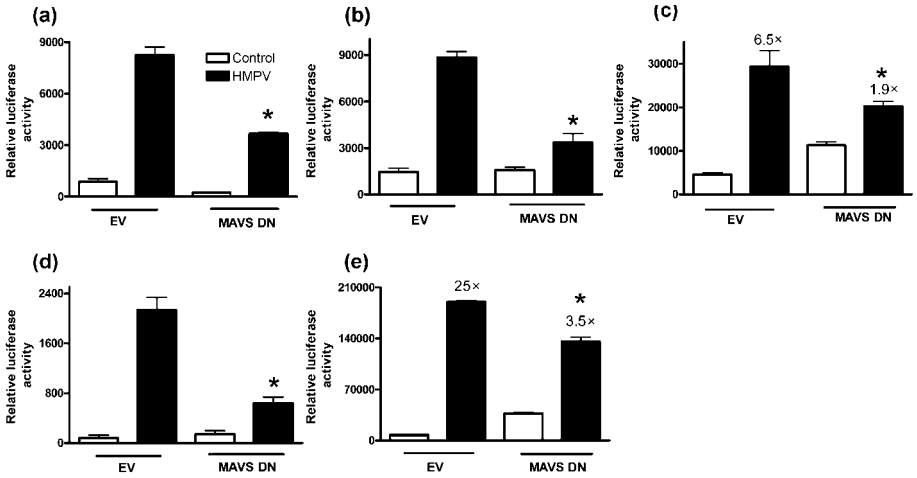

Fig. 5. Inhibition of HMPV-induced IFN-β and chemokines gene transcription by expression of DN MAVS. A549 or 293 cells were cotransfected with a luciferase reporter plasmid containing either the IFN-β (a), RANTES (b), IL-8 (c) promoter or multimers of the RANTES ISRE (d) or IL-8 NF-κB site (e) together with a MAVS DN mutant expression plasmid, or the corresponding empty vector (EV). Cells were infected with HMPV and harvested at 15 or 24 h p.i. to measure luciferase activity. Uninfected plates served as controls. For each plate, luciferase was normalized to the β-galactosidase reporter activity. Data are expressed as mean ± SD of normalized luciferase activity. Bars represent the mean value of triplicate samples of one experiment, n=at least two experiments. Numbers above HMPV columns in (c) and (e) are fold change relative to corresponding control values. *, $P<0.05$ relative to HMPV-infected EV-treated cells.

activation of transcription factors that regulate the expression of inducible gene products with antiviral and/or inflammatory activity. Viruses contain conserved structural moieties, known as pathogen-associated molecular patterns, which are recognized by several families of PRRs, in particular TLRs and RNA helicases. Their relative contribution in virus-triggered cellular signalling is stimulus- and cell-type-dependent (reviewed by Seth *et al.*, 2006).

In this study, we show for the first time, to our knowledge, that HMPV infection of airway epithelial cells induces the expression of the RNA helicases RIG-I and MDA-5 and that RIG-I, but not MDA-5, plays a fundamental role in HMPV-induced cellular signalling, as inhibition of RIG-I expression significantly decreases activation of IRF and NF-κB transcription factors and production of type I IFN and proinflammatory cytokines and chemokines. RIG-I-dependent signalling was necessary to induce a cellular antiviral state, as reduction of RIG-I expression resulted in enhanced HMPV replication. We have recently reported similar findings in airway epithelial cells infected with RSV, also a paramyxovirus and the most common cause of lower respiratory tract infections in children (Liu *et al.*, 2007). A549 cells infected with RSV showed a rapid induction of RIG-I expression and RIG-I binding of viral RNA, and siRNA-mediated RIG-I silencing inhibited both NF-κB and IRF-3 activation, as well as IFN-β and chemokine gene expression (Liu *et al.*, 2007). Although we do not know the exact moiety that mediates RIG-I activation in the course of HMPV infection, we found that RIG-I binds HMPV RNA *in vitro* (data not shown). Together with RIG-I, HMPV also induced MDA-5 expression; however, we did not find a significant role of this helicase in HMPV-induced cellular signalling. Our findings are in agreement with recent studies, indicating that RIG-I is the necessary trigger of innate immune defences in response to a variety of RNA viruses, including other paramyxoviruses, such as NDV and SeV, as well as the orthomyxovirus influenza A/B (Loo *et al.*, 2008). It has been shown that RSV, NDV and SeV infection of MDA-5 −/− mouse embryonic fibroblasts (MEFs) was able to induce IFN and IFN-stimulated gene (ISG) expression, which was almost completely abolished in RIG-I −/− MEFs (Loo *et al.*, 2008). Interestingly, the presence of either RIG-I or MDA-5 was not required for ISG expression in response to dengue virus or reovirus, although double-knockout cells showed significant impairment of ISG expression following infection with both viruses (Loo *et al.*, 2008), indicating variable requirements for RIG-I and MDA-5 in initiating cellular signalling in response to different RNA viruses.

The incomplete abrogation of cellular responses by RIG-I gene silencing suggests the possible involvement of other pathways in HMPV-induced cellular signalling. While the role of TLRs remains speculative in non-immune cells, several studies have shown that TLR-3 is inducible in human epithelial cells and can play a role in regulating proinflammatory response against several viruses, including RSV (Groskreutz *et al.*, 2006; Le Goffic *et al.*, 2007; Liu *et al.*, 2007; Rudd *et al.*, 2006). We have also shown recently that HMPV infection of airway epithelial cells induces

TLR-3 expression, as well the expression of other signalling molecules related to TLR signalling, including TRIF (Bao *et al.*, 2008). However, inhibition of TLR-3 expression did not affect HMPV-induced chemokine gene expression, indicating that it does not play a significant role in HMPV-induced signalling in airway epithelial cells. Whether other TLRs play a role in HMPV-dependent cellular activation needs further investigation.

MAVS is a recently identified adaptor protein that recruits RIG-I and MDA-5 to the outer membrane of mitochondria as part of a signalling complex that activates NF-κB and IRF, leading to IFN and ISG expression (Kawai *et al.*, 2005; Seth *et al.*, 2005). MAVS signalling is initiated by ligand-induced interaction of its N-terminal CARD domain with the CARD domain of either RIG-I or MDA-5. In this study, overexpression of CARD-deleted MAVS greatly diminished HMPV-induced IFN and chemokine promoter activation and more specifically IRF- and NF-κB-dependent gene transcription, indicating a critical role for MAVS in HMPV-induced signalling in airway epithelial cells. Although the roles of RIG-I and MDA-5 in viral recognition and innate immune signalling appear to be different, MAVS seems to be an essential feature of host immunity to RNA virus infection (Sun *et al.*, 2006). In MEFs lacking MAVS expression, infection with SeV, influenza, reovirus and dengue did not induce IRF-3 nuclear translocation, IFN promoter activation and ISG expression (Loo *et al.*, 2008), indicating that MAVS is necessary for innate immune responses to RNA viruses. Recent studies have emphasized the importance of mitochondrial location for MAVS function, implying that other mitochondrial proteins play a fundamental role in the regulation of innate immune responses to RNA viruses (Chen *et al.*, 2007; Li *et al.*, 2005; Seth *et al.*, 2005). Interestingly, both HMPV and RSV share RIG-I as the PRR recognizing their infection in airway epithelial cells. However, we and others have reported distinct differences in abilities to induce cytokines and chemokines, as well as in IFN production between RSV and HMPV, both *in vitro* and *in vivo* (Bao *et al.*, 2008; Guerrero-Plata *et al.*, 2005a, b, 2006). Whether these differences could be attributed to differential recruitment of signalling molecules, such as TRAF-3 (Saha & Cheng, 2006), to the RIG-I/MAVS signalling complex, or are due to the activation of additional distinct antiviral signalling pathways remains to be investigated.

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