Cell-type specific recognition of human Metapneumoviruses by RIG-I and TLR7 and viral interference of RIG-I ligand recognition by HMPVB1 Phosphoprotein

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Abstract

Human Metapneumoviruses (HMPV) are recently identified Paramyxoviridae that contribute to respiratory tract infections in children. No effective treatments or vaccines are available. Successful defense against virus infection relies on early detection by germline encoded pattern recognition receptors and activation of cytokine and type I interferon genes. Recently, the RNA helicase Retinoic acid inducible gene (RIG-I) has been shown to sense HMPV. In this study, we investigated the ability of two prototype strains of HMPV (A1 [NL100] and B1 [NL199]) to activate RIG-I and induce type I interferons (IFN). Despite the ability of both HMPV-A1 and B1 to infect and replicate in cell lines and primary cells, only the HMPV-A1 strain triggered RIG-I to induce IFNA/B gene transcription. The failure of the HMPV-B1 strain to elicit type I IFN production was dependent on the B1 phosphoprotein, which specifically prevented RIG-I-mediated sensing of HMPV viral 5′ triphosphate RNA. In contrast to most cell types, plasmacytoid dendritic cells (PDC) displayed a unique ability to sense both the A1 and B1 strains and in this case sensing was via Toll-like receptor (TLR)-7 rather than RIG-I. Collectively, these data reveal differential mechanisms of sensing for two closely related viruses, which operate in cell-type specific manners.

Keywords

Viral; Signal Transduction; Knockout mouse

Introduction

Human metapneumovirus (HMPV) is a newly described virus responsible for lower respiratory tract infections in children (1). The virus was first isolated in the Netherlands in 2001. Compared to its closest human relative, respiratory syncytial virus (RSV), HMPV has a worldwide prevalence and causes a broad spectrum of illness that ranges from asymptomatic infection to severe bronchiolitis. Serological studies have revealed that virtually every child has been exposed to HMPV by the age of 5 (1). Depending on the population analyzed, 5 to

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15% of respiratory infections and 12 to 55% of otitis media may be attributed to HMPV infection (2). Retrospective studies have revealed that HMPV is not a new virus; but rather, one which has been circulating for about 50 years (1). There is currently no effective treatment or vaccine for HMPV. Recent studies in mice have revealed an important role for T cells in anti-viral immunity and pathogenesis (3), however our understanding of the innate immune response to HMPV is limited. HMPV is an enveloped virus containing a single-stranded negative-sense RNA genome, encoding 8 open reading frames. Based on its sequence homology to the avian pneumovirus, HMPV was assigned to the Metapneumovirus genus within the Paramyxoviridae family, which also contains RSV (4). Phylogenetic analysis has revealed two major genetic clusters, designated as group A and B, which have been further subdivided into four main subtypes A1, A2, B1 and B2 (2,5). Unlike most viruses which enter cells by receptor-mediated endocytosis, most paramyxoviruses deliver their genome into the cytoplasm directly by fusion with the plasma membrane (6). The attachment (G, H or HN) and fusion (F) proteins are critical for mediating these events (reviewed in (7)). Most other viruses enter cells by receptor-mediated endocytosis and are delivered to the endosomal compartment where the acidic environment is critical for viral fusion and the release of viral genomes into the cytosol. Several classes of germline encoded pattern recognition receptors have been identified which recognize different components of viruses. In most cases, viruses are sensed via their genomes or their replicative or transcriptional activities (8). The recognition of RNA and DNA viruses has been shown to involve endosomally localized TLRs, including TLR7 and TLR9 which are expressed on plasmacytoid dendritic cells (PDC), the major producers of IFNα in vivo (9). Both Influenza virus and vesicular stomatitis virus (VSV) are sensed by TLR7 in PDC (10), whereby recognition of the genomes of these ssRNA viruses is tightly linked to viral fusion and uncoating (11). DNA viruses are sensed in PDC via TLR9. Induction of IFNα by the TLR7/9 pathway is mediated by the TLR adapter MyD88 and the transcription factor IRF7 (12,13)

Sensing of RNA and DNA viruses also occurs in the cytosol and in the case of RNA viruses is mediated by a second class of immune sensors, the RNA helicases, retinoic acid inducible gene (RIG-I) and melanoma differentiation-associated gene-5 (MDA-5). Genetic evidence has revealed that RIG-I and MDA-5 discriminate between different classes of RNA viruses (9,14). RIG-I is required for triggering anti-viral responses against several Flaviviridae, Paramyxoviridae, Orthomyxoviridae and Rhabdoviridae, whereas MDA-5 is required for the response against picornaviruses like encephalomyocarditis virus (EMCV) (9,15). RIG-I senses the nascent 5’ triphosphate moiety of viral genomes or virus derived transcripts of negative-sense ssRNA viruses, whereas MDA5 is activated by long dsRNA, a typical intermediate of the replication of plus-sense ssRNA viruses. RIG-I and MDA-5 induce type I IFN responses by recruiting a CARD domain containing adapter molecule, mitochondrial antiviral signaling protein (MAVS) (16) (also known as IPS-1, VISA or CARDif (17) (18,19)) and triggering IRF3 activation to regulate type I IFN gene transcription. Some DNA viruses are also sensed by a pathway involving RIG-I, however in this case viral DNA is transcribed by RNA polymerase III into an RNA intermediate which is then recognized by RIG-I (20,21).

A recent study has implicated the RIG-I pathway in sensing of the HMPV strain CAN97-83 (A2 strain). HMPV CAN97-83 induces IFNβ and chemokine gene expression in a RIG-I dependent manner (22). Here, we have examined the role of RIG-I in the detection of HMPV viruses by comparing 2 prototype strains NL\0100 (A1) and NL\1999 (B1) in primary cells and cell lines. Although closely related, only the HMPV-A1 strain activated type I IFN gene transcription in most cell types examined. In both human cell lines as well as highly purified human monocytes the A1 but not the B1 strain induced type I IFN. Induction of IFNα/β by HMPV-A1 was mediated by the recognition of 5’ triphosphate viral RNA through RIG-I and its downstream adaptor MAVS. The failure of the B1 virus to trigger type I IFN relates to its
ability to antagonize IFN production through the phosphoprotein P. In the context of the virus, the HMPV-B1 phosphoprotein prevented RIG-I from sensing the viral genome during infection. In contrast to human cell lines and monocytes, PDC produced type I IFN upon infection with both A1 and B1 strains. In PDC, treatment of cells with lysosomotropic agents that prevented endosomal acidification blocked IFNα induction by both viruses, which was mediated by Toll-like receptor TLR7. Taken together these data emphasize the unique ability of PDC to sense and induce type I IFN in response to viruses that appeared “invisible” to most other cell types. These data also highlight the ability of two closely related viruses to differentially antagonize innate immune sensing mechanisms.

Materials and methods

Cells and mice

Human embryonic kidney (HEK) 293, 293T, alveolar epithelial cells (A549) and Vero cells were from ATCC (Manassas, VA). The human hepatocellular carcinoma cell lines Huh7 and Huh7.5 were from C. Rice (Rockefeller University, New York, NY). B16-FLT3L producing cells were from G. Dranoff (HMS, Boston, MA) (23). HEK293 cells stably expressing the P protein from HMPV-A1 and B1 were generated by transfection with cDNAs encoding P proteins, which were generated by PCR. All of the above cell lines were maintained in DMEM (Mediatech Inc, Herndon VA) supplemented with 5% FBS (Hyclone, Logan UT) and 10μg/ml Ciprofloxacin (Mediatech Inc). BSR-T7/5 cells were from K. Conzelmann (Munich, Germany) and were cultured in DMEM 10%FCS/ciprofloxacin with G418 (0,5μg/ml). C57/Bl6 and C57/Bl6-129 F1 mice were from Jackson Laboratories (Bar Harbor, ME). MAVS−/− mice on a mixed C57/BL6×129 background were from Z.J. Chen (UT Southwestern, Dallas, TX). TLR7−/− and TLR9−/− mice were from S. Akira (Osaka University, Osaka, Japan). Animal studies have been reviewed and approved by the University of Massachusetts Medical School institutional animal care and use committee.

Reagents

The trypsin-independent HMPV viral isolates A1 (NL\1\001) and B1 (NL\1\99) were provided by Medimmune Inc. (Gaithersburg, MD) and were propagated as previously described in IMDM 4% BSA trypsin (24,25). Influenza virus (strain A/PR/8/34) was from Charles River Laboratories (Boston, MA). Newcastle disease virus (NDV, LaSota strain) was from P. Pitha (Johns Hopkins, Baltimore, MD). Herpes Simplex virus-1 (KOS strain) was from D. Knipe (HMS, Boston, MA). HMPV and NDV were inactivated by heating at 56°C for 30 min or by UV cross-linking at a dose of 2 Joules/cm². CpG-A (CpG 2216), CpG 2088 (26) and ISS661 (27) were from IDT (Coralville, IA). Poly (da-dT)•Poly (da-dT) was from GE Healthcare (Piscataway NJ). Chloroquine, and Bafilomycin A1 were from Sigma-Aldrich (St. Louis, MI). The monoclonal antibodies specific for the HMPV F protein were generated and characterized by Medimmune Inc. (Gaithersburg, MD) (24). Mouse monoclonal antibody #338 was used as neutralizing antibody at 10μg/ml and bionylated hamster monoclonal antibody #1017 was used at 1μg/ml for staining of the F protein by immunoblotting and flow cytometry. Anti β-actin was from Sigma-Aldrich.

Plasmids

The IFNβ luciferase, pGL3-PRDII, -PRDIII-I and -PRDIV luciferase reporter genes were from T. Maniatis (Harvard, Cambridge, MA). The IFNα4 luciferase reporter gene was from S. Akira (Osaka, Japan). ISG54-IFN-stimulated regulatory element (ISRE) was from Stratagene (La Jolla, CA). Human RIG-I flag and RIG-IC (helicase domain only) were from T. Fujita (Tokyo, Japan). A dominant negative mutant form of MDA5 (MDA5-T789M) was from D. Conte (C. Mello Lab, UMASS Medical School, Worcester, MA). pME18-NS3/4A-myc and pME18-NS3/4A-S139A-myc were from Z.J. Chen (UT Southwestern, Dallas, Texas). pGL4-TK renilla
luciferase and pGL3-control luciferase were from Promega (Madison, WI). All viral proteins (with the exception of L) from HMPV-B1 were cloned individually into pEF-Bos-Flag by PCR using full-length cDNA as template (28). Clone sequences were verified by sequencing and protein expression in 293T cells by western blotting with anti-Flag M2 (Sigma).

Mouse and human primary cell isolation

Peripheral blood mononuclear cells (PBMC) were freshly isolated by density-gradient centrifugation using Ficoll Hypaque (GE Healthcare). Monocytes and PDC were purified from PBMC using CD14 microbeads and the diamond PDC isolation kit respectively as recommended by the manufacturer (Miltenyi Biotec). Purity of these populations was assessed by staining for CD14 and BDCA2 expression by flow cytometry, and was greater than 95%. Mouse PDC were purified from spleens of mice injected with BL6-FLT3L producing cells. Briefly, mice were injected subcutaneously with $5 \times 10^6$ cells in 300μl PBS. Spleens were harvested within 2 weeks after injection and PDC were isolated using the PDC isolation kit II (Miltenyi Biotec). Purity was assessed by staining for CD11c, Ly6C and mPDCA1 expression by flow cytometry, and was greater than 85%.

Reporter assays

Luciferase reporter assays were conducted as previously described (29). Briefly, 293T cells or A549 cells (2.10⁴ cells/well in 96-well plates) were transfected with the indicated luciferase reporter genes together with thymidine kinase Renilla-luciferase reporter gene and with of the indicated expression plasmids using Genejuice (Novagen, Madison, WI). Luciferase activity was measured as previously described (30). Huh7 and Huh7.5 cells (4.10⁴ cells/well in 96-well plates) were transfected as above except that these cells were also transfected with 40ng of a pGL3 control reporter, which drives constitutive luciferase activity in order to normalize the data between the two cell lines. Data were normalized according to manufacturer recommendations (Promega).

Viral RNA Purification

Viral RNA from HMPV-A1 and B1 was extracted using the QIAamp viral RNA mini kit (Qiagen, Valencia, CA). Viruses were concentrated by ultracentrifugation for 2h at 4 °C at a speed of 27,000 RPM using a SW28 rotor (Beckman, CA). RNA (1.5μg) was treated with or without 10U of calf intestinal alkaline phosphatase (CIAP, Fermentas) for 3h at 37°C or RNase A (Promega) for 1h at 37°C. All samples were then treated for 15min at 85°C to inactivate the enzymes. Viral RNA was transfected into 293T cells using lipofectamine 2000 at a ratio 1:1 (weight/volume) along with the IFNβ luciferase and the TK-renilla reporter genes according to the manufacturer instructions (Invitrogen). Viral RNA from NDV and VSV were from T. Morrison and S. Zhou, respectively (UMASS Medical School, Worcester, MA).

ELISA

For ELISA analysis, human PBMC (2.10⁵/well), monocytes (1.10⁵/well) and PDC (4.10³/well), or mouse PDC (5.10⁴/well) were plated in 96 well plates in 100μl and stimulated for 24h. Poly (dA-dT)•poly (dA-dT) was transfected as previously described (31) at 5μg/ml using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) at a ratio 2:1 (weight/volume). Human IFNα was measured according to the manufacturer's recommendations (Bender Medsystems, Burlingame CA). A murine IFNβ sandwich ELISA was used as previously described (32).

RNA extraction and real time PCR

293T cells (2.10⁶ cells per 10 cm plate) were stimulated with HMPV-A1 or HMPV-B1 (3.2.10⁵pfu/ml) for 5 to 48h and RNA isolated using RNeasy (Qiagen, Valencia CA). cDNA were synthesized as previously described (29) using the SuperScript III enzyme (Invitrogen).
Quantitative real-time PCR analysis was performed using SYBR green reagent (Invitrogen) on a DNA engine Opticon 2 cycler (Bio-Rad, Hercules CA) using the following primers: IFNβ-F CAGCAATTTTCAGTGTCAGAAGC; IFNβ-R CATCCTGTCTTTGAGGCAGT; IFNα-F GTGAGGAAATACCTCAGGAACTCAC; IFNα-R TCTCATGATTTCCTGTCCTGACAA; β-actin-F CCTGGCACCCAGCACAAT; β-actin-R GCGGATCCACACGGACATA; L1-F TTGCATGAGGTACCTTGGATTG; L1-R AGAGTGCATTATCACACATCA. The specificity of amplification was assessed for each sample by melting curve analysis, and the size of the amplicon checked by electrophoresis. PCR efficiency was calculated for both vRNA with tenfold dilutions of the cDNA with the formula $E = 10^{-1/\text{slope}} - 1$ as previously described (33). Relative quantification was performed using standard curve analysis. All gene expression data were normalized with β-actin and are presented as a ratio of gene copy number per 100 copies of β -actin +/- SD.

**Western-Blotting**

Whole cell extracts were prepared using lysis buffer (50 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 10% glycerol, freshly supplemented with protease and phosphatase inhibitors. Cells were placed on ice for 15min and centrifuged for 15min at 14,000rpm. 30μg of protein were analyzed by 10% SDS-PAGE and subjected to immunoblot analysis using anti-F and anti-β-actin antibodies as indicated. The IRF3 dimerization assay was performed as follows. A549 cells (10^10 cells /10ml in 10cm plate) were stimulated with HMPV-A1, B1 (10^5 pfu/ml) or NDV (40 HAU) as indicated. Nuclear proteins were extracted using Nuclear Extraction Kit (Active Motif, CA). 25 μg of protein was analyzed by 8.5% PAGE and subjected to immunoblot analysis using anti-IRF3.

**Generation of chimeric viruses**

Chimeric viruses where the P proteins both HMPV strains were exchanged, were generated by PCR using naturally occurring MluI and PacI enzymes and Esp31 enzyme to generate overlapping PCR fragments. PCR fragments were generated using full length cDNA as previously described (28). For each virus strain, 3 PCR products were generated and cloned in topo cloning vector (Invitrogen). From the MluI site to the ATG of the P protein, the P protein and to the end of P up to PacI site. Ligations of MluI to P and P to PacI from each strain were ligated with the P protein from the opposite virus strain into pCDNA3. HMPV-A1 and B1 full length was generated by partial digestion using MluI and PacI and used to clone the 3 pieces by PCR from pCDNA3. Colonies were screened by digestion and sequenced by PCR.

**Virus recovery and titration**

Recovery of recombinant HMPV was performed as described previously (28). Briefly, BSR-T7 cells were transfected with 5 μg full-length HMPV cDNA plasmid, 2 μg pCITE-N, 2 μg pCITE-P, 1 μg pCITE-L and 1 μg pCITE-M2.1 using Genejuice (Novagen). Two days later, the BSR-T7 cells were scraped and co-cultured with Vero cells in IMDM with 4% BSA for 7 days. Viruses were titered as followed. Twenty-four-well plates containing 95% confluent monolayers of Vero cells were inoculated with 200μl of 10-fold serial virus dilutions. After 2 h at 37 °C, 0.8ml of 0.5% methylcellulose/DMEM with 3% FCS was added. Cells were incubated for an additional 5 days. Methylcellulose overlays were removed and cells were fixed with 80% acetone. Cells were incubated with HMPV-specific 1017 biotinylated antibody (Medimmune) for 1 h at 37 °C, followed by incubation with horseradish peroxidase-labelled streptavidin (BD bioscience, CA). Plaques were quantified after incubation with a freshly prepared solution of 3,3' Diaminobenzidine (DAB) (Vector Laboratories, CA) to determine viral titers.
Statistical analysis

Differences between groups were analyzed for statistical significance by using a t-test in Prism software. P<0.05 was considered statistically significant. ***, ** and * represent p values of <0.001, <0.01 and <0.05, respectively.

Results

Differential induction of type I IFN by HMPV-A1 and B1

In an effort to understand the underlying mechanisms regulating the innate immune response to HMPV viruses, we compared the ability of two closely related strains A1 (NL\1\00) and B1 (NL\1\99) to induce type I IFN by examining IFNβ reporter gene activity in infected cells. 293T cells were transfected with a reporter gene under the control of the IFNβ gene enhancer. The HMPV-A1 strain induced the IFNβ reporter (about 60 fold induction), while the B1 strain failed to drive this reporter (Figure 1a). The HMPV-A1 strain also induced an IFNα4 reporter gene while the HMPV-B1 virus failed to do so (Supplementary Figure 1a). We observed similar results in the human hepatoma cell line, Huh7 (Figure 1b) and the human alveolar epithelial cell line, A549 (Figure 1c). Similar observations were made when the endogenous IFNβ transcript levels were measured. HMPV-A1 induced IFNβ gene transcription while HMPV-B1 failed to do so (Figure 1d).

We also tested these responses in primary cells. Human peripheral blood mononuclear cells (PBMC) were purified from whole blood and infected with HMPV viruses and the production of the type I IFN (IFNα) measured by ELISA. Contrary to previous observations in human cell lines, both the HMPV-A1 and HMPV-B1 strains induced type I IFN in PBMC (Figure 1e). Both IFNα and β mRNA levels were induced as measured by quantitative PCR (Supplementary Figure 1b). Monocytes and PDC are the major producers of type I IFN in PBMC. We therefore purified monocytes and PDC from total PBMC. Monocytes responded to HMPV-A1 but not B1 while PDC responded to both viruses (Figure 1f and g). These results reveal the unique ability of PDC to induce type I IFN in response to HMPV-B1.

To exclude the possibility that the failure of the B1 virus to induce type I IFN induction was due to a failure to infect or replicate in these cells, we compared viral infection and replication in both monocytes and PDC. Upon infection with paramyxoviruses, the viral F protein is first synthesized as an inactive precursor F0, which is subsequently converted into the fusogenic F1 form by cellular proteases (34). A HMPV-specific anti-F antibody has been characterized previously and shown to detect F0 and F1 forms of the F protein from both A1 and B1 strains (24). The level of F-protein expression was monitored in infected cells by flow cytometry. Similar percentages of purified monocytes (about 20% of CD14+ cells) and PDC (about 45% of BDCA-2+ cells) expressing the F protein were observed for both HMPV strains (Figure 2a). This indicated that both strains can infect these cell types. Moreover, in 293T cells, comparable levels of expression of the F protein (both F0 and F1 forms) were detected in both HMPV-A1 and B1 infected 293T cells when examined by western blotting (Figure 2b). Replication of HMPV-A1 and B1 was also examined by quantifying the level of viral transcripts and measuring viral titers in infected cells. To ensure similar PCR efficiency for both strains, the primer-binding sites were designed in a region of the gene encoding the L protein, which was identical in both strains. Equivalent levels of A1 and B1 transcripts were detected in infected cells throughout the course of infection (Figure 2c). Viral titers were also equivalent for both strains (Figure 2d). This was also true in 293T, A549 and Huh7 cells (Figure 2e). Indeed, the levels of B1 virus exceeded that of the A1 strain at later time points (data not shown). We conclude therefore from these studies, that both viruses infect and replicate in all cells tested, however PDC were unique in their ability to induce type I IFN in response to HMPV-B1.
The HMPV-B1 virus fails to activate Interferon Regulatory Factor-3

The transcriptional enhancer of the IFNβ gene contains four positive regulatory domains (PRD1–IV), which bind distinct transcriptional regulators that act cooperatively to activate IFNβ gene expression. The transcription factors that bind to these elements include NFκB, which binds to PRDII; IRF-3 and -7, which bind to adjacent PRDIII and PRDI sites, collectively referred to as PRDIII-I, and the heterodimeric transcription factor ATF-2-c-Jun, which binds to PRDIV. To define the effect of the B1 strain on each of these pathways, we tested the effect of the B1 virus on transcription from individual IFNβ regulatory elements using multimerized reporter assays. Each of these promoter elements when present in multiple copies have been shown to be activated by Sendai virus (35,36). In agreement with the data presented above, only the A1 strain activated the PRDIII-I reporter, which responds to IRF3 and 7 (Figure 3a). In contrast, HMPV-A1 and B1 both induced the PRDII and PRDIV reporter genes, which are activated by NFκB and ATF2/c-jun, respectively (Figure 3b and c). Similar data were obtained when a multimerized NFκB reporter with canonical NFκB binding sites was tested (data not shown). We also monitored activation of a reporter gene containing multimerized ISRE elements from the ISG-54 promoter. Consistent with a failure to induce the PRDIII-I element from the IFNB promoter, HMPV-B1 virus also failed to drive the ISG54-ISRE and IFNα4 reporters (data not shown and Supplementary Figure 1a).

We next monitored endogenous IRF3 activation by examining the formation of IRF3 dimers in virus-infected cells. IRF3 is normally present in the cytoplasm of resting cells as a monomer. Virus infection triggers the phosphorylation and dimerization of IRF3 followed by its nuclear translocation. Infection of 293T cells with HMPV-A1 induced IRF3 dimerization in a manner similar to that observed with NDV, our positive control (Figure 3d). In contrast cells infected with the HMPV-B1 strain failed to lead to dimerization of endogenous IRF3. These observations were confirmed using an in vitro assay for IRF3 and IRF7, which utilizes a hybrid protein consisting of the yeast Gal4 DNA binding domain (DBD) fused to IRF-3 or IRF-7 lacking its own DNA binding domain (37). Reporter gene expression from the Gal4 upstream activation sequence in this assay requires IRF activation (37). HMPV-A1 but not B1 activated both the Gal4-IRF3 and IRF7 reporters (data not shown). Taken together these data provide clear evidence that the B1 strain fails to trigger IRF3/7 activation and as a result fails to trigger IFNβ production.

HMPV-A1 triggers type I IFN gene transcription via RIG-I

Since RIG-I is a sensor of paramyxoviruses and HMPV has been shown to trigger RIG-I signaling (9,22,38,39) we monitored the contribution of the RIG-I pathway to IFNβ gene activation upon HMPV-A1 stimulation. We first examined the role of MAVS, which relays signals from RIG-I to downstream kinases and transcription factors. The involvement of MAVS was assessed using the NS3/4A protease from Hepatitis C Virus. NS3/4A cleaves and inactivates MAVS, thereby disrupting RIG-I signaling (40). NS3/4A can therefore be used as a tool to implicate MAVS signaling in a particular response. The effect of NS3/4A on the HMPV-A1-induced IFN response was tested in 293T cells. Increasing concentrations of wild-type NS3/4A protease dose-dependently blocked the induction of IFNβ by HMPV-A1 as well as that induced by SV (Figure 4a). Importantly, the protease inactive mutant (NS3/4A-S139A) had no effect. We also tested the involvement of RIG-I using the hepatoma cell line (Huh7) and the Huh7.5 sub-line that bears a natural mutation in RIG-I (T55I), which renders it inactive (41). Huh7 and Huh7.5 cells were transfected with the PRDIII-I reporter gene and then infected with HMPV-A1, HMPV-B1 or NDV (Figure 4b). Consistent with our data in 293T cells, only the A1 virus induced this reporter gene in Huh7 cells (Figure 4b). Activation of the PRDIII-I element was completely abrogated in the Huh7.5 cell line in response to HMPV-A1. The IFNβ response following NDV infection (which is known to be RIG-I-dependent) was also inhibited in the Huh7.5 cells, consistent with published results. Moreover, reconstitution of
Huh7.5 cells with wild type RIG-I fully restored the response to both HMPV-A1 and NDV. We also found that a dominant negative version of RIG-I, consisting of the helicase domain only (RIG-IC), dose-dependently inhibited the IFNβ response in 293T cells elicited by HMPV-A1 virus (figure 5d see below). MDA5 did not appear to be involved in the recognition of HMPV-A1, since a dominant negative mutant form of MDA5 did not inhibit the induction of the IFNβ reporter by HMPV-A1, but blocked the IFN response to wild type MDA5 (Figure 4c and d). Interestingly, PRDII (NFκB) and PRDIV (MAP kinase) reporter gene activation by HMPV-A1 and B-1 strains were not inhibited by RIG-IC overexpression in 293T cells or in Huh7.5 cells (Supplementary Figure 2a/2b). Collectively these observations suggest that HMPV viruses can trigger RIG-I to turn on type I IFN production and also trigger RIG-I-independent signaling events leading to NFκB and AP-1 activation. Thus, RIG-I as well as additional as yet unidentified PRR driven pathways contribute to the host response to this virus.

We next examined the requirement for viral replication in the HMPV-A1 induced type I IFN response. We compared live virus to either heat or UV-inactivation HMPV-A1 on IFNβ reporter gene activity in 293T cells. UV- and heat-inactivation of HMPV-A1 and NDV completely blocked their ability to induce IFNβ reporter gene activity (Figure 5a). Similar results were observed in purified human monocytes (Figure 5b). The 5'-triphosphate moiety of the viral RNA is the major ligand recognized by RIG-I (42, 43). To further investigate the nature of the ligand that triggers RIG-I in HMPV viruses, viral RNA was purified from HMPV-A1 and transfected into the cytoplasm of 293T cells via lipofection. Transfection of the viral RNA induced the IFNβ reporter gene and removal of the phosphate groups by calf intestinal alkaline phosphatase (CIAP) or digestion of the viral RNA by RNase A abrogated this response (Figure 5c). Importantly, when we isolated the viral RNA from HMPV-B1 virus and transfected this RNA into 293T cells, IFNβ was induced and this response was also sensitive to CIAP and RNase A treatment. IFN induction by the double stranded DNA analog, poly(dA-dT)-poly(dA-dT), was unaffected by either CIAP or RNase A treatment. Moreover, RIG-IC also dose-dependently inhibited the IFNβ response in 293T cells elicited by either HMPV-A1 virus or by HMPV-A1 or B1 viral RNAs (Figure 5d). These data indicate that 5'-triphosphate RNA is the ligand for RIG-I and also reveal that the naked viral genome of HMPV-B1 virus can be sensed by RIG-I, if delivered to the cytosol. However, the inability of the B1 strain to be sensed suggests that in the context of the virus, the HMPV-B1 viral RNA is not detected.

The HMPV-B1 P protein prevents sensing by RIG-I

To test this hypothesis, we first tested if preincubation with the HMPV-B1 strain could interfere with signaling initiated by the HMPV-A1 strain or other viruses that are sensed by RIG-I. Cells were transfected with the IFNβ reporter gene, pre-incubated for 24 hours with live or UV-inactivated HMPV-B1 strain before infection with the A1 strain or NDV. Luciferase activity was then monitored 24 hours post infection. Preincubation with live HMPV-B1 reduced IFNβ induction upon HMPV-A1 infection (Figure 6a). In contrast, preincubation with UV-inactivated virus did not block the induction of IFNβ. The activation of the IFNβ reporter gene in response to NDV was not affected (Figure 6a). Moreover, IFNβ reporter gene induction induced by overexpression of RIG-I, the downstream adapter MAVS or the IRF3 kinase TBK1 was also unaffected (data not shown). These data suggest that the B1 virus interferes with the induction of IFNβ by A1 virus and that the inhibitory effect was not due to antagonism downstream of RIG-I itself. Therefore, the B1 strain likely blocks sensing of HMPV viral RNA and not RIG-I function per se.

Each individual cDNA from the B1 strain (with the exception of the L polymerase) were cloned into mammalian expression vectors and their effects on type I IFN induction upon infection with the A1 strain examined by reporter assay. Plasmids encoding B1 cDNAs were transfected into 293T cells together with the PRDIII-I reporter gene, whose activity was monitored after...
infection of cells with HMPV-A1 virus. Table 1 shows the percentage identity between A1 and B1 proteins. While the M, SH, M2-1, N, M2-2, F and G proteins had little or no effect, the P protein blocked the induction of the reporter (Figure 6b). Importantly, western blotting revealed equivalent expression levels of all of these proteins in transfected cells (data not shown).

We next generated HEK293 cell lines stably expressing the B1-virus P protein in order to test if the B1 protein could prevent induction of IFNβ upon delivery of viral RNA into the cytoplasm. Viral RNA from both A1 and B1 strains triggered a strong IFNβ response in parental HEK293 cells but the response was largely impaired in HEK293 cells expressing B1-P protein. Importantly, IFNβ induced by vRNA from NDV or VSV was unaffected (Figure 6c). These data are consistent with the possibility that the HMPV-B1 P protein functions in a specific manner as an inhibitor, blocking IFN production in response to HMPV viral RNA.

To further examine the inhibitory effect of the B1 virus P protein in a more physiologically relevant manner and in the context of the virus, we generated chimeric viruses in which the P protein from the A1-strain was replaced with that of the B1 strain. Both wild type and chimeric HMPV-A1 virus were recovered from cDNA and their ability to induce type I IFN examined by reporter assays. Consistent with our data with the A1 clinical isolate, wild type HMPV-A1 virus recovered from cDNA (referred to as A1R) induced IFNβ reporter in HEK293 cells, however when A1R virus expressing the P protein from B1 virus was examined, no reporter gene activity was detected (Figure 6d). Similar data were obtained when A549 cells were examined (Figure 6d). Since both the A1 and B1 strains could induce PRDII and PRDIV-luciferase reporters, we also compared the effect of A1R and APB viruses on induction of these reporters in both HEK293 and A549 cells. In contrast to the IFNβ reporter, both A1R and APB induced both of these reporter genes (Figure 6e). Moreover, analysis of F-protein levels in virus infected cells revealed similar levels in cells infected with all viral strains (Figure 6f). These results provide compelling evidence that the HMPV B1-P protein prevents RIG-I mediated sensing and signaling resulting in IRF3–dependent type I IFN gene transcription.

Detection of HMPV viruses in PDC does not involve RIG-I but occurs via an endosomal sensing pathway

As shown in figure 1, both A1 and B1 strains induce type I IFN production in PDC. The studies outlined above indicate that it is unlikely that RIG-I mediated these events. Quantitative PCR analysis revealed that PDC express RIG-I and MAVS albeit at much lower levels than that found in monocytes (44). To examine the contribution of the RIG-I pathway in PDC responses to HMPV viruses, we monitored PDC from mice lacking MAVS. PDC from MAVS-deficient mice responded normally to both HMPV viruses (as well as to NDV, HSV and Influenza viruses) (Figure 7a). These latter three viruses have been shown to signal in PDC via TLRs (see below), R848 and CpG DNA, ligands for TLR7 and TLR9 respectively, also induced IFNβ normally in MAVS-deficient PDC. Of note MAVS-deficient myeloid DCs or macrophages are severely compromised in NDV or synthetic triphosphate RNA induced IFN responses (data not shown).

In order to define the mechanisms sensing these viruses in human PDC, we investigated the requirement for viral replication in mediating these responses. In contrast to our observations in 293T and monocytes (Figure 5), UV- and heat-inactivation of HMPV-A1, B1 and NDV only partially affected their ability to induce IFNα induction (Figure 7b). Altogether, these studies confirmed that sensing of HMPV viruses in PDC is not mediated via the RIG-I cytosolic pathway.

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Since PDC express high levels of TLR7 and TLR9 and sense viruses via endosomally localized TLRs, we next examined the role of this system in the detection of HMPV-A1 and B1 viruses.
PDC were treated with chloroquine or bafilomycin A1, two lysosomotropic agents that act by raising the intra-endosomal pH or by specific inhibition of the vacuolar ATPase, respectively (11,45). Induction of IFNα by HMPV-A1 and B1 in PDC was totally abrogated when cells were pretreated with either chloroquine or bafilomycin A1 (Figure 7c). Responses to CpG-A were also completely blocked by chloroquine and bafilomycin A1, consistent with published results (45). In contrast to PDC responses, induction of IFNα by HMPV-A1 in monocytes was less affected (Figure 7d). IFNα Induction in response to Influenza is presumable down in chloroquine or bafilomycin A1 treated conditions because of a failure of the virus to infect.

For viruses that enter cells by receptor-mediated endocytosis, viral fusion and uncoating events are tightly coupled to recognition of viral ligands by endosomally localized TLRs. Therefore, inhibition by chloroquine and bafilomycin could implicate TLRs in the sensing of these viruses.

To examine the precise role of endosomal TLRs in the recognition of A1 and B1 viruses, PDC were isolated from WT, TLR7 and TLR9-deficient mice and examined IFNβ secretion post-virus infection. As expected, induction of IFNβ by CpG-A and HSV1 was entirely dependent on TLR9 (Figure 7e). In contrast, IFNα induced in response to influenza was unaffected in TLR9-deficient PDC but was completely defective in TLR7-deficient PDC. HMPV-A1 and -B1 induced IFNβ production in PDC was induced normally in TLR9-deficient PDC and was fully impaired in TLR7-deficient PDC. We also confirmed the TLR7 dependency in the human system using ISS661, a previously characterized oligonucleotide-based inhibitors for TLR7 signaling (26). Pre-treatment with ISS661 blocked the induction of IFNα by HMPV-A1 and B1 (Figure 7f). Pretreatment with CpG2088, an inhibitor of TLR9 signaling (27, 46) had no effect. These results reveal that the unique ability of PDC to induce type I IFN in response to HMPV-B1 correlates with the sensing of HMPV viruses through TLR7, and not the cytosolic RNA helicase pathway.

**Discussion**

HMPV and RSV viruses are major contributors to respiratory tract infections in infants and young children. In most infants, these viruses cause symptoms resembling those of the common cold. However, in infants born prematurely, children with chronic lung disease, or children with congenital heart disease, these viruses can result in a severe or even life threatening disease. As many as 125,000 hospitalizations occur annually in children less than one year old due to lower respiratory infection or bronchiolitis (47). Developing new therapeutics to prevent and treat these infections is therefore of considerable importance.

Limiting virus infection requires rapidly mounted defences, which include in large part the release of type I IFN (IFNα/β). Interferon limits viral replication directly and enhances viral clearance by activating adaptive immunity. Understanding how viruses are sensed and how type I IFN is regulated may facilitate the rational design of novel anti-viral therapeutics and/or better vaccine candidates useful in the prevention or treatment of lower respiratory tract infections in children. In this study, we demonstrate that type I IFN production during infection with HMPV viruses involves differential sensing mechanisms, which work in a cell-type specific manner. Sensing of HMPV-A1 virus occurs via the cytosolic RNA helicase RIG-I in most cell types, with the exception of PDC, where TLR7 mediates these responses. A recent study by Casola and colleagues also implicated RIG-I in the sensing of HMPV in airway epithelial cells (22). We have confirmed these observations using mice with targeted deletions in the RIG-I pathway and have extended these studies to include an analysis of additional cell types and a comparison of two closely related clinical viral isolates. We have identified 5′- triphosphate RNA as the HMPV viral ligand triggering the RIG-I-IFNβ response. Importantly, we also identified a RIG-I-independent pathway for sensing HMPV-A1 and B1 viruses in epithelial cell lines. HMPV-A1 and B1 viruses activated NFκB and AP-1 dependent reporter genes in a RIG-I independent manner. These data suggest additional mechanism of HMPV
sensing. The NLR family member, NOD2 was recently shown to act as a cytosolic sensor for RSV infection (48). Further studies should delineate if NOD2 also senses HMPV to regulate NFκB and AP-1 signalling described herein.

A major focus of this study was a comparison of the innate response to two closely related strains. While both strains induced type I IFN responses in PDC, the B1 strain failed to elicit a type I IFN response in monocytes and cell lines, despite its ability to infect and replicate as efficiently as the A1 virus and despite the ability of naked viral RNA to trigger RIG-I if delivered by lipofection to the cytoplasm. The fact that the B1 virus could prevent type I IFN induction by the A1 virus, but not that induced by NDV indicates that RIG-I signalling per se is not blocked by the B1 virus. Like the A1 virus, purified B1 viral RNA could trigger IFN and pretreatment of the B1 viral RNA with RNase or removal of phosphate groups with alkaline phosphatase ablated sensing of the viral RNA by RIG-I. These studies suggest that RNA of both strains are ligands for RIG-I, however, in the context of the virus, the B1 viral RNA is prevented from being sensed by RIG-I. During viral infection, RNA viruses like HMPV fuse with the cell membrane and deliver their ribonucleoprotein (RNP) complex into cells. The RNP complex consists of the viral RNA associated with the viral polymerase L, the nucleoprotein N and the phosphoprotein P. Upon fusion, the viral RNA is protected from free cellular RNases by this protein complex. Proteins within the RNP therefore could prevent the recognition of the vRNA by the RIG-I pathway. In fact, our studies using overexpressed B1 proteins indicated that the B1 virus P protein but not other HMPV proteins could block IFN production by live A1 virus or viral RNA. Although this approach indicated that the B1 P protein was the most likely candidate, we found that if we overexpressed the A1 P protein we could also observe an inhibitory effect. To determine if the B1 P protein was responsible for the inhibitory effect in the context of the virus, we generated recombinant viruses where we replaced the P protein in the A1 virus with that from the B1 virus. This is a more physiologically relevant system to assess its contribution where associations of the B-1 P with other viral proteins could also be accounted for. A recombinant A1 virus encoding the P protein from the B1 strain was generated and its ability to induce RIG-I signaling examined. Unlike the wild type A1 virus recovered from cDNA, the recombinant A1 virus containing the B virus P protein had a substantially reduced ability to induce IFN, suggesting that in the context of the entire virus, B1-P may indeed prevent RIG-I from sensing HMPV virus. Specific inhibition of RIG-I sensing by the B1-P protein could be because of higher levels of expression of the P protein in the B1 virus rather than what is found in the A1 virus. Another possibility is that the B-1 P protein could have higher affinity for the RNA or for other components of the RNP complex (HMPV RNA or N and L), which would preclude the RNA from being sensed. The two P proteins share 86% identity (see table 1) and therefore unique residues in B1-P could account for these effects.

Usurping IFN induction pathways is a common tactic employed by viruses to enable their replication within host cells. Viral IFN antagonists strike at just about every level of the IFN regulatory network, but by far the best-studied strategies relate to the ability of viral proteins to counteract RNA sensing and signaling events. Examples include Influenza virus NS1, which inactivates RIG-I (43), Hepatitis C Virus, NS3/4A, which cleaves and inactivates MAVS (18) and the phosphoprotein of Borna disease virus and Rabies virus, which target the IRF3 kinase, TBK1 (49,50). In the case of RSV, the genome encodes two non-structural (NS)-1 and NS2 proteins known to inactivate the IFN response (51). A recent study from Casola and colleagues implicated the G protein from another strain of HMPV in evasion of the RIG-I signaling pathway (52). A recombinant hMPV lacking the G protein (rhMPV-ΔG) was developed as a potential vaccine candidate and shown to be attenuated in the respiratory tract of a rodent model of infection. Casola and colleagues found that rhMPV-ΔG-infected airway epithelial cells produced higher levels of chemokines and type I interferon compared to cells infected with rhMPV-WT. They showed that RIG-I was the target of G protein inhibitory
activity. Indeed the G protein associated with RIG-I and inhibited RIG-I-dependent gene transcription. Our data with the HMPV-B1 virus however do not support a role for the B1-virus G protein but rather indicate that the B1 phosphoprotein can prevent RIG-I from sensing the viral RNA.

The inhibitory effect of the B1 virus P protein was restricted to the RIG-I pathway, since the B1 virus did not prevent the induction of IFNα/β in PDC. IFN production in PDC was sensitive to bafilomycin A1 and chloroquine and was dependent on TLR7. The current model of antiviral sensing in PDC suggests that TLR-mediated recognition of viruses occurs without direct infection and that the presence of viral genomic nucleic acids within the endosomal/lysosomal compartment is sufficient to trigger TLRs. Iwasaki and colleagues demonstrated recently that RNA viruses such as VSV which do not enter cells via the endosomal compartment but replicate in the cytosolic compartment where cytosolic viral replication intermediates are then delivered into the lysosomal compartment by the process of autophagy to trigger TLRs (53).

Generally cell entry of paramyxoviruses requires two glycoproteins: the attachment (G, H or HN) and fusion (F) proteins. In the case of HMPV viruses, however, analysis of recombinant viruses lacking the G protein has suggested that attachment and fusion is mainly dependent on the F protein (54). The F protein is a type I glycoprotein, synthesized as an inactive precursor, F0 and subsequently converted into its biologically active form, the heterodimer F1/F2. The majority of Paramyxoviridae F proteins are cleaved intracellularly by host cellular proteases, most notably furin. Cleavage of the F-protein from HMPV however, requires secretory proteases, which restrict HMPV viruses to the lumen of the respiratory and enteric tract for replication in vivo. In vitro the addition of trypsin to process the F0 protein into its mature form allows efficient propagation of the virus (25). In contrast to most other Paramyxoviridae F proteins that require neutral pH for membrane fusion, cleavage of the HMPV F protein might therefore require low pH conditions (55). These findings might indicate a requirement for low-pH compartments such as the endosome for entry of HMPV viruses in PDC. Receptor-mediated endocytosis at low pH was indeed recently shown in Vero cells for HMPV-A2 strain (56). Since PDC do not need to be infected to induce IFN, the ability of PDC to respond may be a result of uptake of viral particles to the endosome directly.

Altogether, our data unveil different mechanisms for sensing of HMPV viruses in different cell types. Such cell type specific involvement of the RIG-I versus TLR pathways in induction of antiviral responses is not unique to HMPV viruses, as this differential sensing has previously been reported in the case of sensing of NDV (14). Understanding how viruses are detected and how viruses exploit innate sensing and signalling pathways is essential for the development of vaccines to harness the power of the innate immune system for the benefit of the host.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

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References


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Figure 1. HMPV A1 and B1 strains differentially induce IFNβ gene transcription

(a-c) 293T, Huh7 and A549 cells were transfected with the full length IFNβ promoter. Cells were infected with HMPV-A1 or HMPV-B1 (from $5 \times 10^4$ to $5 \times 10^2$ pfu/ml for 293T, $5 \times 10^4$ pfu/ml for other lines) for an additional 24 h. Data are expressed as fold induction relative to the reporter-only control and are the mean ± SD. (d) 293T cells were stimulated with HMPV-A1 or HMPV-B1 ($5 \times 10^4$ pfu/ml) for the indicated time. Levels of human IFNβ and β-actin were quantified in RNA samples by real-time PCR. Results are presented in arbitrary units as the ratio of IFNβ over 100 copies of β-actin. (e-g) Total PBMC, monocytes or PDC were stimulated with HMPV-A1, HMPV-B1 ($2 \times 10^5$ pfu/ml), CpG-A 2216 (3 μM), NDV (8 HAU/ml), or poly
(dAdT)•poly(dAdT) (5μg/ml) for 24h. Protein levels were measured in the supernatant of culture by ELISA and presented as the mean ± SD.
Figure 2. HMPV-A1 and HMPV-B1 infect and replicate in primary cells and cell lines
(a) Purified monocytes (6×10^5 cells/well) and PDC (2.10^5 cells/well) were cultured for 24h in presence of HMPV-A1 and B1 (3.2×10^5 pfu/ml). Cells were harvested and stained for specific cell surface antigen (CD14 or BDCA2) and F protein expression using an anti-F biotinylated antibody and PE or APC-conjugated steptavidin. Results are presented as histogram overlays of anti-F antibody staining versus isotype control. Displayed percentages were subtracted from the isotype control. (b). Infection of 293T cells by HMPV was assessed by immunoblot analysis. Cells (6×10^5 cells/well) were infected with HMPV-A1 or HMPV-B1 (5×10^4 pfu/ml) for 2 to 48h. A specific anti-F biotinylated antibody and HRP-conjugated steptavidin were used to detect F protein in cell lysates. F0 and F1 forms of the F protein are indicated by an arrow.
Anti-F #338 neutralizing antibody was used to show the specificity. The lower panel shows β-actin levels in the samples. (c) Replication of HMPV in 293T was quantified by real-time PCR. Cells were infected with HMPV-A1 or HMPV-B1 (5×10^4 pfu/ml) for the indicated time points. Levels of HMPV-L gene and human β-actin were quantified in RNA samples using specific primers. Results are presented in arbitrary unit as the ratio of HMPV-L gene over 100 copies of β-actin. (d-e) Virus titers were determined after infection of several cell types (3×10^5/well) with HMVPA1 or B1 strain (10^5 pfu/ml). Virus titers were measured for the indicated time points after infection in Vero cells (d) or after 24h post infection in 293T, A549 and Huh7 cells (e) and presented as pfu/ml.
Figure 3. Differential activation of IRF3 by the HMPV A1 and B1 strains
(a-c) 293T cells were transfected with the IFNB PRDIII-I, PRDII or PRDIV reporter genes as detailed in Material and Methods. Cells were infected with HMPV-A1 or HMPV-B1 (range from $5 \times 10^4$ to $5 \times 10^2$ pfu/ml) for an additional 24h. Data are expressed as fold induction relative to the reporter-only control and are the mean ± SD. (d) A549 cells were stimulated with viruses for the indicated period of time. Nuclear protein were extracted and analyzed by native PAGE. The monomer and dimeric forms of IRF3 are indicated by arrows.
Figure 4. HMPV-A1 induces IFNβ via the RIG-I/MAVS pathway
(a) 293T cells were transfected with the IFNβ reporter gene and increasing concentrations (5-80ng/well) of the WT or S139A inactive NS3/4A protease from HCV. Cells were stimulated for an additional 24h with HMPV-A1, HMPV-B1 (5×10⁴pfu/ml) or SV (400HAU/ml). Data are expressed as fold induction relative to the reporter-only control and are the mean ± SD. (b) Parental human hepatoma cell line Huh7 and Huh7.5 were transfected with the IFNβ reporter gene in the absence or presence of pEFBos-huRIG-I Flag (40ng/well). Cells were stimulated with HMPV-A1, HMPV-B1 (5×10⁴pfu/ml) or NDV (64HAU/ml) for an additional 24h. Results are normalized by renilla luciferase and presented as arbitrary units after correction between the 2 cell lines using the pGL3-control. (c) 293T cells were transfected with the
IFNβ reporter gene and (d) WT or a dominant negative mutant of MDA-5 (80 ng/well). Cells were stimulated with HMPV-A1 (5×10^4 pfu/ml) for additional 24hrs. Data are expressed as fold induction relative to the reporter-only control and are the mean ± SD.
Figure 5. Induction of IFNβ by HMPV-A1 requires viral replication
(a) 293T cells were transfected with the full length IFNβ promoter. Cells were stimulated 24h later with live, UV- or heat-inactivated HMPV-A1, HMPV-B1 (2×10^5 pfu/ml) or NDV (8HAU/ml) for an additional 24h. Data are expressed as fold induction relative to the reporter-only control and are the mean ± SD. (b) Freshly isolated monocytes were stimulated for 24h with live, UV- or heat-inactivated HMPV-A1, HMPV-B1 (2×10^5 pfu/ml) or NDV (8HAU/ml). Human IFNα protein levels were measured in the supernatant of culture by ELISA and presented as the mean ± SD. (c) 293T cells were transfected with viral RNA purified from HMPV-A1 or B1 (80ng), or poly(dAdT)•poly(dAdT) (20ng) along with the IFNβ and TK-renilla reporter genes. vRNAs and poly(dAdT)•poly(dAdT) were treated with calf intestinal alkaline phosphatase (CIAP) or RNAse A prior to stimulation as indicated. (d) 293T cells were transfected with increasing amounts of RIG-IC (0-2-20ng/well) along with the IFNB reporter and vRNAs (80ng), or stimulate with HMPV-A1 (5×10^4 pfu/ml). In all cases, luciferase activity was measured 24h post-transfection and data are expressed as fold induction relative to the reporter-only control and are the mean ± SD.
Figure 6. HMPV-B1 P protein prevents RIG-I from sensing HMPV vRNA
(a) 293T cells were transfected with the IFNβ reporter gene one day prior to stimulation. Cells were pre-incubated with live or UV (2J/cm²) inactivated HMPV-B1 (10⁵ pfu/ml) for 24 hours. Cells were then stimulated with HMPV-A1 (5×10⁴ pfu/ml) or NDV (8HAU) for an additional 24 hours. Results are normalized by renilla luciferase and presented as fold induction relative to the reporter-only control and are the mean ± SD. (b) 293T cells were transfected with the IFNβ PRDIII-I reporter gene and 40 ng of plasmids encoding HMVB1 proteins as indicated. Cells were stimulated for an additional 24 h with HMPV-A1 (5×10⁴ pfu/ml). Data were normalized by renilla luciferase and are expressed as a percentage of activation relative to conditions with the virus in the absence of exogenous proteins and are the mean ± SD. (c)
HEK293 cells and HEK293 cells stably expressing the P protein from HMPV-B1 were transfected with the full length IFNβ reporter gene along with the viral RNA (80ng) purified from HMPV-A1, B1, NDV, or VSV, or with total RNA (80ng) purified from Vero cells as control. Results are normalized by renilla and pGL3 control luciferase, and presented as mean ± SD. (d-f). 293T and A549 cells were transfected with the full length IFNB or the PRDIV reporter along with the TK-renilla reporter gene, one day prior to stimulation. Cells were infected for 24 hours with the following viruses: HMPV-A1, HMPV-B1, HMPV-A1 recovered from cDNA (A1R), or the chimeric HMPV-A1 virus encoding the P protein from the B1 strain (APB) (5×10^4 pfu/ml). Results are normalized by renilla luciferase and are presented as fold induction relative to the reporter-only control and are the mean ± SD. A western blot of F protein levels is shown (f).
Figure 7. Induction of type I IFN in PDC in response to both HMPV-A1 and B1 is TLR7-mediated
(a). PDC from C57Bl/6/129 and MAVS−/− mice were stimulated for 24h with CpG-A (2μM), Heat-inactivated Influenza (MOI=0.1), HSV (MOI=100) HMPV-A1, HMPV-B1 (2×10⁵pfu/ml). Mouse INFβ protein levels were measured in the supernatant of culture by ELISA and presented as the mean ± SD. (b). Freshly isolated PDC were stimulated for 24h with live, UV- or heat-inactivated HMPV-A1, HMPV-B1 (2×10⁵pfu/ml) or NDV (8HAU/ml). Human INFα protein levels were measured in the supernatant by ELISA and presented as the mean ± SD. (c-d) Freshly isolated PDC and monocytes were stimulated with HMPV-A1, HMPV-B1 (2×10⁵pfu/ml), NDV (8HAU/ml), Heat-inactivated Influenza (MOI=0.1), CpG-A (2μM) for 24 hours. Cells were pre-incubated with chloroquine (1μM) or bafilomycin A1 (4μM for PDC,

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10μM for monocytes) for 1 hour were indicated. Human IFNα protein levels were measured in the supernatant by ELISA and presented as the mean ± SD. (e) Mouse PDC from C57Bl6, TLR7−/− and TLR9−/− mice were stimulated for 24h with CpG-A (2μM), Heat-inactivated Influenza (MOI=0.1), HSV (MOI=100) HMPV-A1, HMPV-B1 (2×10^5 pfu/ml). Mouse IFNβ protein levels were measured by ELISA and presented as the mean ± SD. (f) Freshly isolated PDC were stimulated with HMPV-A1, HMPV-B1 (2×10^5 pfu/ml), R848 (10μM), CpG-A (2μM) for 24 hours. Cells were pre-incubated with CpG2088 (0.1μM) or ISS661 (2μM) for 1 hour were indicated. Human IFNα protein levels were measured in the supernatant by ELISA and presented as the mean ± SD.
Table 1

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