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Cell Type-Specific Recognition of Human Metapneumoviruses (HMPVs) by Retinoic Acid-Inducible Gene I (RIG-I) and TLR7 and Viral Interference of RIG-I Ligand Recognition by HMPV-B1 Phosphoprotein

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Human metapneumoviruses (HMPVs) 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 germ line-encoded pattern recognition receptors and activation of cytokine and type I IFN genes. Recently, the RNA helicase retinoic acid-inducible gene I (RIG-I) has been shown to sense HMPV. In this study, we investigated the abilities of two prototype strains of HMPV (A1 [NL1100] and B1 [NL1199]) to activate RIG-I and induce type I IFNs. Despite the abilities of both HMPV-A1 and HMPV-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 displayed a unique ability to sense both HMPV-A1 and HMPV-B1 and in this case sensing was via TLR7 rather than RIG-I. Collectively, these data reveal differential mechanisms of sensing for two closely related viruses, which operate in cell type-specific manners. *The Journal of Immunology, 2010, 184: 1168–1179.

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 y (1). Depending on the population analyzed, 5–15% of respiratory infections and 12–55% of otitis media may be attributed to HMPV infection (2). Retrospective studies have revealed that HMPV is not a new virus but rather one that has been circulating for ~50 y (1).

There is currently no effective treatment or vaccine for HMPV. Recent studies in mice have revealed an important role for T cells in antiviral 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 eight open reading frames. On the basis of 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 groups A and B, which have been further subdivided into four main subtypes A1, A2, B1, and B2 (2, 5). Unlike most viruses that 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 Ref. 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 germ line-encoded pattern recognition receptors have been identified that 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 (pDCs), the major producers of IFN-α in vivo (9). Both influenza virus and vesicular stomatitis virus (VSV) are sensed by TLR7 in pDCs (10), whereby recognition of the genomes of these ssRNA viruses is tightly linked to viral fusion and uncoupling (11). DNA viruses are sensed in pDCs.
via TLR9. Induction of IFN-α by the TLR7 and TLR9 pathway is mediated by the TLR adapter MyD88 and the transcription factor IFN regulatory factor (IRF)-7 (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 antiviral responses against several Flaviviridae, Paramyxoviridae, Orthomyxoviridae, and Rhabdoviridae, whereas MDA-5 is required for the response against picornaviruses such as echovirus and coxsackievirus (9, 15). RIG-I senses the nascent 5′-triphosphate moiety of viral genomes or virus-derived transcripts of negative-strand ssRNA viruses, whereas MDA-5 is activated by long dsRNA, a typical intermediate of the replication of plus-strand ssRNA viruses. RIG-I and MDA-5 induce type I IFN responses by recruiting a caspase-recruitment domain-containing adapter molecule, mitochondrial antiviral signaling protein (MAVS) (16) [also known as IPS-1, VISA or CARDif (17-19)] and triggering IRF3 activation to regulate type I IFN gene transcription. Some DNA viruses also are sensed by a pathway involving RIG-I; however, in this case viral DNA is transcribed by RNA polymerase III into a RNA intermediate that 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 (HMPV-A2). HMPV-A2 induces IFN-β and chemokine gene expression in a RIG-I-dependent manner (22). In this study, we have examined in detail the role of TLRs and RNA helicases in the detection of HMPVs by comparing two prototype strains NL1/00 (HMPV-A1) and NL1/99 (HMPV-B1) in primary cells and cell lines. Although closely related only, the HMPV-A1 strain activated type I IFN gene transcription in most of the cell types examined. In both cell human lines as well as highly purified human monocytes, HMPV-A1 but not HMPV-B1 induced type I IFN. Induction of IFN-α/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 HMPV-B1 to trigger type I IFN relates to its ability to antagonize IFN production through the phospho-protein 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, pDCs produced type I IFN upon infection with both HMPV-A1 and HMPV-B1. In pDCs, treatment of cells with lysosomotropic agents that prevented endosomal acidification blocked IFN-α induction by both viruses, which was mediated by TLR7. Taken together, these data emphasize the unique ability of pDCs to sense and induce type I IFN in response to viruses that appeared “invisible” to most other cell types. These data also highlight the abilities 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 (A549), and Vero cells were from the American Type Culture Collection (Manassas, VA). The human hepatocellular carcinoma cell lines HuH7 and HuH7.5 were from C. Rice (Rockefeller University, New York, NY). B16-F10 cells producing viral proteins were from G. Dranoff (Harvard Medical School, Boston, MA) (23). HEK293 cells stably expressing the P protein from HMPV-A1 and HMPV-B1 were generated by transfection with CDNA encoding P proteins, which were generated by PCR. All of the above cell lines were maintained in DMEM (Mediatech, Herndon, VA) supplemented with 5% FBS (HyClone, Logan, UT) and 10 µg/ml ciprofloxacin (Mediatech). BSR-T7/5 cells were from K. Conzelmann (Maximilian University of Munich, Munich, Germany) and were cultured in DMEM containing 10% FCS and ciprofloxacin with G418 (0.5 µg/ml). C57/B16 and C57BL6-129 F1 mice were from The Jackson Laboratory (Bar Harbor, ME). MAVS−/− mice on a mixed C57BL6 × 129 background were from Z. J. Chen (University of Texas Southwestern Medical Center, 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 isolates A1 (NL/1/001) and B1 (NL/1/99) were provided by MedImmune (Gaithersburg, MD) and were propagated as described previously in MRC5 cells (14). Influenza virus (strain A/PRI/8/34) was from Charles River Laboratories (Wilmington, MA). Newcastle disease virus (NDV; LaSota strain) was from P. Pitha (Johns Hopkins University, Baltimore, MD). HSV-1 (KOS strain) was from D. Kipnis (Harvard Medical School, 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 J/m². Cpg-A (Cpg 2216), Cpg-2088 (26), and ISG65 (27) were from业绩inbred DNA Test Kit (DNA Technology, Ishikawa, Japan) was from GE Healthcare (Piscataway, NJ). Chloroquine and bafilomycin A1 were from Sigma-Aldrich (St. Louis, MO). The monoclonal antibodies specific for the HMPF P protein were generated and characterized by MedImmune (24). Mouse mAb no. 338 was used as neutralizing Ab at a concentration of 10 µg/ml and biotinylated hamster mAb no. 1017 was used at a concentration of 1 µg/ml for staining of the F protein by immunoblotting and flow cytometry. Anti–β-actin was from Sigma-Aldrich.

Plasmids

The IFN-β luciferase, pGL3-positive regulatory domain (PRD) II, PRDI-II, and -PRDIV luciferase reporter genes were from T. Maniatis (Harvard University, Cambridge, MA). The IFN-α4 luciferase reporter gene was from S. Akira. The ISG54 IFN-stimulated regulatory element (ISRE) was from Stratagene (La Jolla, CA). Human RIG-I flag and RIG-I consisting of the his6 flag domain only (RIG-IC) were from T. Fujita (Tokyo University, Tokyo, Japan). A dominant-negative mutant form of MDA-5 (MDA-5–T78M) was from D. Conte (C. Mello Laboratory, University of Massachusetts Medical School, Worcester, MA). pME18-NS3/4A-myc and pME18-NS3A-S139A-myc were from Z. J. Chen. pGL4-thymidine kinase (TK) Renilla luciferase and pGL3-control luciferase were from Promega (Madison, WI). All of the viral proteins (with the exception of L) from HMPV-B1 were cloned in direct-reading plasmids using the pROK II vector (Promega, Madison, WI). The trypsin-independent HMPV isolates A1 (NL/1/001) and B1 (NL/1/99) were generated by transfection with cDNA as a template (28). Clone sequences were verified by sequencing and protein expression in 293T cells by Western blot analysis with anti-Flag M2 (Sigma-Aldrich).

Mouse and human primary cell isolation

PBMCs were freshly isolated by density-gradient centrifugation using Ficoll Hypaque (GE Healthcare). Monocytes and pDCs were purified from PBMCs using the Flexi MACS microbeads and the Dextran Plasmacytoid Dendritic Cell Isolation Kit, respectively, as recommended by the manufacturer (Milenyi Biotec, Auburn, CA). Purity of these populations was assessed by staining for CD14 and blood dendritic cell antigen 2 (BDCA2) expression by flow cytometry and was >95%. Mouse pDCs were purified from spleens of mice injected with BL6-FLT3L-producing cells. Briefly, mice were injected s.c. with 5 × 106 cells in 300 µl PBS. Spleens were harvested within 2 wk after injection and pDCs were isolated using the Pluronic F68–Dynabeads Cell Isolation Kit II (Milenyi Biotec). Purity was assessed by staining for CD11c, Ly6C, and mPDC1A expression by flow cytometry and was >85%.

Reporter assays

Luciferase reporter assays were conducted as described previously (29). Briefly, 293T or A549 cells (2 × 105 cells per well in 96-well plates) were treated for 16 h with the indicated concentrations of luciferase reporter genes together with Renilla luciferase reporter gene and with the indicated expression plasmids using GeneJuice (Novagen, Madison, WI). Luciferase activity was measured as described previously (30). Huh7 and Huh7.5 cells (4 × 105 cells per well in 96-well plates) were transfected as above except that these cells were also transfected with 40 ng of a pGL3-control reporter, which drives constitutive luciferase activity to normalize the data between the two cell lines. Data were normalized according to the manufacturer’s recommendations (Promega).

Viral RNA Purification

Viral RNA from HMPV-A1 and HMPV-B1 was extracted using the QIAamp Viral RNA Mini kit (Qiagen, Valencia, CA). Viruses were concentrated by ultracentrifugation for 2 h at 4°C at a speed of 27,000 rpm using a SW28 rotor (Beckman Coulter, Fullerton, CA). RNA (1.5 µg) was treated with or without 10 U of Mung bean alkaline phosphatase (CLAP; Fermentas, Glen Burnie, MD) for 3 h at 37°C or RNase A (Promega) for 1 h at 37°C. All of the samples then were treated for 15 min at 85°C to inactivate the enzymes. Viral RNA was transfected into 293T cells using Lipofectamine...
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2000 at a ratio of 1:1 (w/v) along with the IFN-β Incubator and the TK
Renilla reporter genes according to the manufacturer’s instructions (In-
vitrogen, Carlsbad, CA). Viral RNA from NDV and VSV were from T.
Morrison and S. Zhou, respectively (University of Massachusetts Medical
School, Worcester, MA).

ELISA

For ELISA analysis, human PBMCs (2 × 10^5 cells per well), monocytes (1 ×
10^5 cells per well), and pDCs (4 × 10^3 cells per well) or mouse pDCs (5 × 10^6
cells per well) were cultured in 96-well plates in 100 µl and still plated for 24 h.
Poly(dA−dT)poly(dA−dT) was transfected as described previously (31) at a
concentration of 5 µg/ml using Lipofectamine 2000 (Invitrogen) at a ratio of
2:1 (w/v). Human IFN-α was measured according to the manufacturer’s
recommendations (Bender MedSystems, Vienna, Austria). A murine IFN-β
sandwich ELISA was used as described previously (32).

RNA extraction and real time PCR

The 293T cells (2 × 10^6 cells per 10-cm plate) were stimulated with
HMPV-A1 or HMPV-B1 (3.2 × 10^5 PFU/ml) for 5−48 h, and the RNA was
isolated using RNeasy (Qiagen, Valencia, CA). cDNA was synthesized as
described previously (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, Her-
cules, CA) using the following primers: IFN-β F, CAGCAATTTTCAGCTGTCAGAACC; IFN-β R, CATTCCGTCGTCTTGAGGGCTGAT; IFN-α F, GCGGAGAATACATTCCAAGAGTCAAC; IFN-α R, TCTCATGATT TTCTGCTCTGACAA; β-actin F, CAGCAATTTTCAGCTGTCAGAACC; β-actin R, GCCGATCCACACGGAGTA; L1 F, TTGCA TGAGGTACCTTGGATTG; IFN-
βg pCITE-P, 1 µg full-length HMPV cDNA
isolated using RNeasy (Qiagen, Valencia CA). Twenty-five micrograms of protein was analyzed by 8.5% PAGE and
proteins were extracted using a Nuclear Extract Kit (Active Motif, Carlsbad,
CA). Twenty-five micrograms of protein was analyzed by 10% SDS-PAGE and subjected to immunoblot
protein was analyzed by 10% SDS-PAGE and subjected to immunoblot

Western blot analyses

Whole cell extracts were prepared using lysis buffer (50 mM Tris [pH 7.5], 150
mM NaCl, 1 mM EDTA, 1% Nonidet P-40, and 10% glycerol), freshly
supplemented with protease and phosphatase inhibitors. Cells were placed on
ice for 15 min and centrifuged at 15 000 rpm. Thirty micrograms of protein was analyzed by 10% SDS-PAGE and subjected to immunoblot
analysis using anti-F and anti-β-actin Abs as indicated. The IRF3 di-
merization assay was performed as follows. A549 cells (10 × 10^6 cells per 10
ml in a 10-cm plate) were stimulated with HMPV-A1, HMPV-B1 (10^6 PFU/ ml), or NDV (40 hemagglutinating unit [HAU]) as indicated. Nuclear protein
was extracted using a Nuclear Extract Kit (Active Motif, Carlsbad, CA).
Twenty-five micrograms of protein was analyzed by 8.5% PAGE and are presented as a ratio of gene copy
number per 100 copies of β-actin ± SD.

Generation of chimeric viruses

Chimeric viruses, where the P proteins of both HMPV strains were ex-
changed, were generated by PCR using the naturally occurring MluI and
PacI enzymes and Esp31 enzyme to generate overlapping PCR fragments.
PCR fragments were generated using full-length cDNA as described pre-
viously (28). For each virus strain, three PCR products were generated and
cloned into the TOPO cloning vector (Invitrogen): from the MluI site to the
ATG of the P gene, the P protein, and to the end of P up to the 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. Full-length HMPV-
A1 and HMPV-B1 were generated by partial digestion using MluI and PacI
and used to clone the three 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 pcT7E−N, 2 µg pcT7E−P, 1 µg pcT7E−L, and 1 µg pcT7E-
M2.1 using GeneJuice (Novagen). Two days later, the BSR−T7 cells were
scraped and cocultured with Vero cells in IMDM with 4% BSA and 3.75 µg/ml
trypsin (Invitrogen) for 7 d. Viruses were titered as followed. Twenty-four-well plates containing 95% confluent monolayers of Vero cells were inoculated with 200 µl 10-fold serial viral dilutions. After 2 h at 37°C, 0.8 ml 0.5%
methylcellulose/DMEM with 3% FCS was added. Cells were incubated for an additional 5 d. Methylcellulose overlays were removed, and cells were fixed
with 80% acetone. Cells were incubated with HMPV-specific 1017 bio-
tylinated Ab (MedImmune) for 1 h at 37°C followed by incubation with HRP-
labeled streptavidin (BD Biosciences, San Jose, CA). Plaques were quantified
after incubation with a freshly prepared solution of 3,3′-diaminobenzidine
(Vector Laboratories, Burlingame, CA) to determine viral titers.

Statistical analysis

Differences between groups were analyzed for statistical significance by using a t test in GraphPad Prism software (GraphPad, San Diego, CA). p < 0.05 was considered statistically significant. Three, two, and one asterisk represent p values of <0.001, <0.01, and <0.05, respectively.

Results

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

In an effort to understand the underlying mechanisms regulating the
innate immune response to HMPVs, we compared the abilities of
two closely related strains HMPV-A1 (NL1100) and HMPV-B1 (NL1199) to induce type I IFN by examining the IFN-β reporter

gene activity in infected cells. 293T cells were transfected with a reporter gene under the control of the IFN-β gene enhancer. HMPV-A1 induced the IFN-β reporter (~60-fold induction), whereas HMPV-B1 failed to drive this reporter (Fig. 1A). HMPV-A1 also induced an IFN-α reporter gene, whereas HMPV-B1 failed to do so (Supplemental Fig. 1A). We observed similar results in the human hepatoma cell line Huh7 (Fig. 1B) and the human alveolar epithelial cell line A549 (Fig. 1C). Similar ob-

evations were made when the endogenous IFN-β transcript

levels were measured. HMPV-A1 induced IFN-β gene transcription,
whereas HMPV-B1 failed to do so (Fig. 1D).

We also tested these responses in primary cells. Human PBMCs were purified from whole blood and infected with HMPVs, and the production of the type I IFN (IFN-α) was measured by ELISA. Contrary to previous observations in human cell lines, both HMPV-
A1 and HMPV-B1 induced type I IFN in PBMCs (Fig. 1E). Both IFN-α and IFN-β mRNA levels were induced as measured by

quantiative PCR (Supplemental Fig. 1B). Monocytes and pDCs are the major producers of type I IFN in PBMCs. We therefore purified

monocytes and pDCs from total PBMCs. Monocytes responded to
HMPV-A1 but not HMPV-B1, whereas pDCs responded to both

viruses (Fig. 1F, 1G). These results reveal the unique ability of
pDCs to induce type I IFN in response to HMPV-B1.

To exclude the possibility that the failure of HMPV-B1 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 pDCs. 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 Ab has been characterized previously and shown to detect F0 and F1 forms of the F protein from both HMPV-A1 and HMPV-B1 (24). The level of F-protein expression was monitored in infected cells by flow cytometry. Similar percentages of purified monocytes (~20% of CD14+ cells) and pDCs (~45% of BDCA2 positive cells) expressing the F protein were observed for both HMPV strains (Fig. 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 HMPV-

B1−infected 293T cells when examined by Western blot analyses (Fig. 2B). Replication of HMPV-A1 and HMPV-B1 also was ex-
amined by quantifying the levels of viral transcripts and measur-
ing viral titers in infected cells. To ensure similar PCR efficiencies 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 HMPV-A1 and HMPV-B1 transcripts were detected in infected cells throughout the course of infection.
The 293T cells were stimulated with HMPV-A1 or HMPV-B1 (from 5 × 10^5 to 5 × 10^7 PFU/ml for 293T and 5 × 10^5 PFU/ml for other lines) for an additional 24 h. Data are expressed as fold induction relative to the reporter-only control and presented as the mean ± SD. D, The 293T cells were stimulated with HMPV-A1 or HMPV-B1 (5 × 10^7 PFU/ml) for the indicated time. Levels of human IFN-β and β-actin were quantified in RNA samples by real-time PCR. Results are presented in arbitrary units as the ratio of IFN-β and β-actin. E, Total PBMCs, monocytes, or pDCs were stimulated with HMPV-A1, HMPV-B1 (2 × 10^5 PFU/ml), CpG-A (3 μM), NDV (8 HAU/ml), or poly(dA-dT)poly(dA-dT) (5 μg/ml) for 24 h. Protein levels were measured in the supernatant of the culture by ELISA and presented as the mean ± SD.

FIGURE 1. HMPV-A1 and HMPV-B1 differentially induce IFN-β gene transcription (A–C). The 293T, Huh7, and A549 cells were transfected with the full-length IFN-β promoter. Cells were infected with HMPV-A1 or HMPV-B1 (from 5 × 10^5 to 5 × 10^7 PFU/ml for 293T and 5 × 10^5 PFU/ml for other lines) for an additional 24 h. Data are expressed as fold induction relative to the reporter-only control and presented as the mean ± SD. D, The 293T cells were stimulated with HMPV-A1 or HMPV-B1 (5 × 10^7 PFU/ml) for the indicated time. Levels of human IFN-β and β-actin were quantified in RNA samples by real-time PCR. Results are presented in arbitrary units as the ratio of IFN-β and β-actin. E, Total PBMCs, monocytes, or pDCs were stimulated with HMPV-A1, HMPV-B1 (2 × 10^5 PFU/ml), CpG-A (3 μM), NDV (8 HAU/ml), or poly(dA-dT)poly(dA-dT) (5 μg/ml) for 24 h. Protein levels were measured in the supernatant of the culture by ELISA and presented as the mean ± SD.

HMPV-B1 fails to activate IRF3

The transcriptional enhancer of the IFN-β gene contains four PRDs (PRDI–IV) that bind distinct transcriptional regulators that act cooperatively to activate IFN-β gene expression. The transcription factors that bind to these elements include NF-κB, which binds to PRDII; IRF3 and IRF7, which bind to adjacent PRDIII and PRDI sites, collectively referred to as PRDIII-I; and the heterodimeric transcription factor activating transcription factor 2/c-Jun, which binds to PRDIV. To define the effect of HMPV-B1 on each of these pathways, we tested the effect of HMPV-B1 on transcription from individual IFN-β regulatory elements using multimerized reporter assays. Each of these promoter elements when present in multiple copies has been shown to be activated by Sendai virus (35, 36). In agreement with the data presented above, only HMPV-A1 activated the PRDII-I reporter, which responds to IRF3 and IRF7 (Fig. 3A). In contrast, HMPV-A1 and HMPV-B1 both induced the PRDII and PRDIV reporter genes, which are activated by NF-κB and activating transcription factor 2/c-Jun, respectively (Fig. 3B, 3C). Similar data were obtained when a multimerized NF-κB reporter with canonical NF-κB binding sites was tested (data not shown).

We also monitored the activation of a reporter gene containing multimerized ISRE from the ISG54 promoter. Consistent with a failure to induce the PRDIII-I element from the IFN-B promoter, HMPV-B1 also failed to drive the ISG54 ISRE and IFN-α4 reporters (data not shown and Supplemental Fig. 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 (Fig. 3D). In contrast, cells infected with HMPV-B1 failed to lead to the 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 fused to IRF3 or IRF7 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 HMPV-B1 activated both of the Gal4-IRF3 and Gal4-IRF7 reporters (data not shown). Taken together, these data provide clear evidence that HMPV-B1 fails to trigger IRF3 and IRF7 activation and as a result fails to trigger IFN-β production.

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

Because RIG-I is a sensor of paramyxoviruses and HMPV has been shown to trigger RIG-I signaling (9, 16, 22, 38), we monitored the contribution of the RIG-I pathway to IFN-β gene activation upon HMPV-A1 stimulation. We first examined the role of MAVS, which relays signals from RIG-I to downstream kinases and transcription
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 (39). NS3/4A therefore can 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 (WT) NS3/4A protease dose-dependently blocked the induction of IFN-β by HMPV-A1 as well as that induced by Sendai virus (Fig. 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 subline that bears a natural mutation in RIG-I (T55I) that renders it inactive (40). Huh7 and Huh7.5 cells were transfected with the PRDIII-I reporter gene and then infected with HMPV-A1, HMPV-B1, or NDV (Fig. 4B).
Consistent with our data in 293T cells, only HMPV-A1 induced this reporter gene in Huh7 cells (Fig. 4A). Activation of the PRDII-I element was completely abrogated in the Huh7.5 cell line in response to HMPV-A1. The IFN-β response following NDV infection (which is known to be RIG-I–dependent) also was inhibited in the Huh7.5 cells, consistent with published results. Moreover, reconstitution of Huh7.5 cells with WT RIG-I fully restored the response to both HMPV-A1 and NDV. We also found that a dominant-negative version of RIG-I, RIG-IC, dose-dependently inhibited the IFN-β response in 293T cells elicited by HMPV-A1 (Fig. 5D; see below). MDA-5 did not appear to be involved in the recognition of HMPV-A1, because a dominant-negative mutant form of MDA-5 did not inhibit the induction of the IFN-β reporter by HMPV-A1 but blocked the IFN response to WT MDA-5 (Fig. 4C, 4D). Interestingly, PRDII (NF-κB) and PRDIV (MAPK) reporter gene activation by HMPV-A1 and HMPV-B1 were not inhibited by RIG-IC overexpression in 293T or Huh7.5 cells (Supplemental Fig. 2A, 2B). Collectively, these observations suggest that HMPVs can trigger RIG-I to turn on type I IFN production and also trigger RIG-I–independent signaling events leading to NF-κB and AP-1 activation. Thus, RIG-I as well as additional as yet unidentified pattern recognition receptor-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-inactivated HMPV-A1 IFN-β reporter gene activity in 293T cells. Heat and UV inactivation of HMPV-A1 and NDV completely blocked their abilities to induce IFN-β reporter gene activity (Fig. 5A). Similar results were observed in purified human monocytes (Fig. 5B). The 5′ triphosphate moiety of the viral RNA is the major ligand recognized by RIG-I (41, 42). To further investigate the nature of the ligand that triggers RIG-I in HMPVs, viral RNA was purified from HMPV-A1 and transfected into the cytoplasm of 293T cells via lipofection. Transfection of the viral RNA induced the IFN-β reporter gene, and removal of the phosphate groups by CIAP or RNase A treatment. Moreover, RIG-IC also dose-dependently inhibited the IFN-β response in 293T cells elicited by HMPV-A1 or NDV (Fig. 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 can be sensed by RIG-I, if delivered to the cytosol. However, the inability of HMPV-B1 to be sensed suggests that in the context of the virus the HMPV-B1 RNA is not detected.
Preincubation with live HMPV-B1 reduced IFN-β induction upon HMPV-A1 infection (Fig. 6A). In contrast, preincubation with UV-irradiated virus did not block the induction of IFN-β. The activation of the IFN-β reporter gene in response to NDV was not affected (Fig. 6A). Moreover, IFN-β reporter gene induction induced by overexpression of RIG-I, the downstream adapter MAVS, or the IRF3 kinase TANK-binding kinase 1 also was unaffected (data not shown). These data suggest that HMPV-B1 interferes with the induction of the IFN-β reporter by HMPV-A1 and that the inhibitory effect was not due to antagonism downstream of RIG-I itself. Therefore, HMPV-B1 likely blocks sensing of HMPV RNA and not RIG-I function per se.

Each individual cDNA from HMPV-B1 (with the exception of the polymerase L) was cloned into mammalian expression vectors, and their effects on type I IFN induction upon infection with HMPV-A1 were examined by reporter assay. Plasmids encoding HMPV-B1 cDNAs were transfected into 293T cells together with HMPV-A1 were recovered from cDNA, and their abilities to induce type I IFN were examined by reporter assays. 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 RNA.

To further examine the inhibitory effect of the HMPV-B1 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 HMPV-A1 was replaced with that of HMPV-B1. Both WT and chimeric HMPV-A1 were recovered from cDNA, and their abilities to induce type I IFN were examined by reporter assays. Consistent with our data with the HMPV-A1 clinical isolate, WT HMPV-A1 recovered from cDNA (A1R) induced IFN-β reporter in HEK293 cells; however, when A1R expressing the P protein from HMPV-B1 (APB) was examined, no reporter gene activity was detected (Fig. 6D). Similar data were obtained when A549 cells were examined (Fig. 6D). Because both HMPV-A1 and HMPV-B1 could induce PRDII and PRDIV luciferase reporters, we also compared the effects of A1R and APB on induction of these reporters in both HEK293 and A549 cells. In contrast to the IFN-β reporter, both A1R and APB induced both of these reporter genes (Fig. 6E). Moreover, analysis of P-protein levels in virus-infected cells revealed similar levels in cells infected with all of the viral strains (Fig. 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 HMPVs in pDCs does not involve RIG-I but occurs via an endosomal sensing pathway

As shown in Fig. 1, both HMPV-A1 and HMPV-B1 induce type I IFN production in pDCs. The studies outlined above indicate that it is unlikely that RIG-I mediated these events. Quantitative PCR analysis revealed that pDCs express RIG-I and MAVS, albeit at much lower levels than those found in monocytes (43). To
examine the contribution of the RIG-I pathway in pDC responses to HMPVs, we monitored pDCs from mice lacking MAVS. pDCs from MAVS-deficient mice responded normally to both HMPVs (as well as to NDV, HSV, and influenza viruses) (Fig. 7A). These latter three viruses have been shown to signal in pDCs via TLRs (see below). R848 and CpG DNA, ligands for TLR7 and TLR9 respectively, also induced IFN-β normally in MAVS-deficient pDCs. Of note, MAVS-deficient myeloid dendritic cells or macrophages are severely compromised in NDV- or synthetic triphosphate RNA-induced IFN responses (data not shown).

To define the mechanisms sensing these viruses in human pDCs, we investigated the requirement for viral replication in mediating these responses. In contrast to our observations in 293T cells and monocytes (Fig. 5), heat and UV inactivation of HMPV-A1, HMPV-B1, and NDV only partially affected their abilities to induce IFN-α induction (Fig. 7B). Altogether, these studies confirmed that the sensing of HMPVs in pDCs is not mediated via the RIG-I cytosolic pathway.

Because pDCs 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 HMPV-B1. pDCs were treated with chloroquine or bafilomycin A1, two lysosomotropic agents that act by raising the intraendosomal pH or by specific inhibition of the vacuolar adenosine triphosphatase, respectively (11, 44). Induction of IFN-α by HMPV-A1 and HMPV-B1 in pDCs was totally abrogated when cells were pretreated with either chloroquine or bafilomycin A1 (Fig. 7C). Responses to CpG-A were also completely blocked by chloroquine and bafilomycin A1, consistent with published results (44). In contrast to

### Table 1. Percentage of identity between HMPV-A1 and HMPV-B1 proteins

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<th>Protein</th>
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<tr>
<td>P</td>
<td>86.1</td>
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<tr>
<td>N</td>
<td>94.9</td>
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<td>M</td>
<td>97.3</td>
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<td>G</td>
<td>11.3</td>
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pDC responses, induction of IFN-α by HMPV-A1 in monocytes was less affected (Fig. 7D). IFN-α induction in response to influenza is presumably regulated by 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 endosomal TLRs. Therefore, inhibition by chloroquine and bafilomycin A1 could implicate TLRs in the sensing of these viruses. To examine the precise role of endosomal TLRs in the recognition of HMPV-A1 and HMPV-B1, pDCs were isolated from WT and TLR7- and TLR9-deficient mice, and IFN-β secretion was examined postinfection. As expected, the induction of IFN-β by HMPV-A1 and HSV-1 was entirely dependent on TLR7 (Fig. 7E). In contrast, IFN-α induction in response to influenza was unaffected in TLR9-deficient pDCs but was completely defective in TLR7-deficient pDCs. HMPV-A1- and HMPV-B1-induced IFN-β production in pDCs was induced normally in TLR9-deficient pDCs and was fully impaired in TLR7-deficient pDCs. We also confirmed the TLR7 dependency in the human system using ISS661, a previously characterized oligonucleotide-based inhibitor for TLR7 signaling (26). Pretreatment with ISS661 blocked the induction of IFN-α by HMPV-A1 and HMPV-B1 (Fig. 7F). Pretreatment with CpG-2088, an inhibitor of TLR9 signaling (27, 45), had no effect. These results reveal that the unique ability of pDCs to induce type I IFN in response to HMPV-B1 correlates with the sensing of HMPVs through TLR7 and not the cytosolic RNA helicase pathway.

**Discussion**

HMPVs and RSVs 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 <1 y old due to lower respiratory infection or bronchiolitis (46). Developing new therapeutics to prevent and treat these infections is therefore of considerable importance.

Limiting virus infection requires rapidly mounted defenses, which include in large part the release of type I IFN (IFN-α/β). IFN 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 antiviral therapeutics 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 HMPVs involves differential sensing mechanisms that work in a cell type-specific manner. Sensing of HMPV-A1 occurs via the cytosolic RNA helicase RIG-I in most cell types, with the exception of pDCs, where TLR7 mediates these responses. A recent study by Casola and colleagues (22) also implicated RIG-I in the sensing of HMPV in airway epithelial cells. 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 ligand triggering the RIG-I IFN-β response. Importantly, we also identified a RIG-I–independent pathway for sensing HMPV-A1 and HMPV-B1 in epithelial cell lines. HMPV-A1 and HMPV-B1 activated NF-κB– and AP-1–dependent reporter genes in a RIG-I–independent manner. These data suggest an additional mechanism of HMPV sensing. The Nod-like receptor family member nucleotide-binding oligomerization domain containing 2 was recently shown to act as a cytosolic sensor for RSV infection (47). Further studies should delineate whether nucleotide-binding oligomerization domain containing 2 also senses HMPV to regulate NF-κB and AP-1 signaling described herein.

A major focus of this study was a comparison of the innate response to two closely related strains. Although both strains induced type I IFN responses in pDCs, HMPV-B1 failed to elicit a type I IFN response in monocytes and cell lines, despite its ability to infect and replicate as efficiently as HMPV-A1 and despite the ability of naked viral RNA to trigger RIG-I if delivered by lipofection to the cytoplasm. The fact that HMPV-B1 could prevent type I IFN induction by HMPV-A1 but not that induced by NDV indicates that RIG-I signaling per se is not blocked by HMPV-B1. Like HMPV-A1, purified HMPV-B1 RNA could trigger IFN, and pretreatment of the HMPV-B1 RNA with RNase or removal of phosphate groups with alkaline phosphatase ablated sensing of the viral RNA by RIG-I. These studies suggest that RNAs 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 the 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 viral RNA by the RIG-I pathway. In fact, our studies using overexpressed HMPV-B1 proteins indicated that the HMPV-B1 P protein but not other HMPV proteins could block IFN production by live HMPV-A1 or viral RNA. Although this approach indicated that the HMPV-B1 P protein was the most likely candidate, we found that if the HMPV-A1 P protein were overexpressed that an inhibitory effect also could be observed. To determine whether the HMPV-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 HMPV-A1 with that from HMPV-B1. This is a more physiologically relevant system to assess its contribution where associating of the HMPV-B1 P protein with other viral proteins also could be accounted for. Recombinant HMPV-A1 encoding the P protein from HMPV-B1 was generated, and its ability to induce RIG-I signaling was examined. Unlike WT HMPV-A1 recovered from cDNA, the recombinant HMPV-A1 containing the HMPV-B1 P protein had a substantially reduced ability to induce IFN, suggesting that in the context of the entire virus the HMPV-B1 P protein may indeed prevent RIG-I from sensing HMPV. Specific inhibition of RIG-I sensing by the HMPV-B1 P protein could be because of higher levels of expression of the P protein in HMPV-B1 rather than what is found in HMPV-A1. Another possibility is that the HMPV-B1 P protein could have a 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 (Table I), and therefore unique residues in the HMPV-B1 P protein 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 nonstructural (NS) 1, which inactivates RIG-I (42); 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, TANK-binding kinase 1 (48, 49). In the case of RSV, the genome encodes two NS1 and NS2 proteins known to inactivate the IFN response (50). A recent study from Casola and colleagues (51) implicated the G protein from another strain of HMPV in evasion of the RIG-I sensing pathway. 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 IFN compared with those of cells infected with HMPV-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 HMPV-B1, however, do not support a role for the HMPV-B1 G protein but rather indicate that the HMPV-B1 phosphoprotein can prevent RIG-I from sensing the viral RNA.

The inhibitory effect of the HMPV-B1 P protein was restricted to the RIG-I pathway, because HMPV-B1 did not prevent the induction of IFN-α/β in pDCs. IFN production in pDCs was sensitive to chloroquine and bafilomycin A1 and was dependent on TLR7. The current model of antiviral sensing in pDCs 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 (52) demonstrated recently that RNA viruses, such as VSV, which do not enter cells via the endosomal compartment, 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.

Generally cell entry of paramyxoviruses requires two glycoproteins: the attachment (G, H, or HN) and fusion (F) proteins. In the case of HMPVs, however, analysis of recombinant viruses lacking the G protein has suggested that attachment and fusion are mainly dependent on the F protein (53). 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 HMPVs 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 therefore might require low-pH conditions (54). These findings might indicate a requirement for low-pH compartments, such as the...
endosome, for the entry of HMPVs in pDC. Receptor-mediated endocytosis at low pH was indeed recently shown in Vero cells for HMPV-A2 (55). Because pDCs do not need to be infected to induce IFN, the ability of pDCs to respond may be a result of the uptake of viral particles to the endosome directly.

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

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Disclosures

J.T., J.S., N.U., H.J., and A.J.C. are currently employed at MedImmune; P.A.K. is currently employed at Zymegia. K.A.F is a recipient of a MedImmune-funded research development of vaccines to harness the power of the innate immune system for the benefit of the host.

References


Supplementary Figure 1

(a) 293T Cells

(b) PBMC

Graphs showing the comparison of IFNα and IFNβ activity in 293T cells and PBMCs.
Supplementary Figure 2
Supplementary Figure 1. HMPV A1 and B1 strains differentially induce type I IFN gene transcription (a) 293T were transfected with the full length IFNα4 promoter. Cells were infected with HMPV-A1 or HMPV-B1 5x10^4 for an additional 24h. Data are expressed as fold induction relative to the reporter-only control and are the mean ± SD. (b) PBMC cells were stimulated with HMPV-A1 or HMPV-B1 (2.10^5pfu/ml) for 18h. Levels of human IFNα, IFNβ and β-actin were quantified in RNA samples by real-time PCR. Results are presented in arbitrary units as the ratio of IFNα or IFNβ over 100 copies of β-actin.

Supplementary Figure 2. NFκB and MAPkinases pathway activation is RIG-I independent. (a) 293T cells were transfected with or without RIG-Ic (2ng/well) along with the PRDII or PRDIV element reporter and stimulated with HMPV-A1 or -B1 (5x10^4pfu/ml) for an additional 24h. Luciferase 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 PRDIV reporter gene and stimulated with HMPV-A1, HMPV-B1 (5x10^4pfu/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.