AIRWAY EPITHELIAL CELL RESPONSE TO HUMAN METAPNEUMOVIRUS INFECTION

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Abstract

Human metapneumovirus (hMPV) is a major cause of lower respiratory tract infections (LRTIs) in infants, elderly and immunocompromised patients. In this study, we show that hMPV can infect in a similar manner epithelial cells representative of different tracts of the airways. hMPV-induced expression of chemokines IL-8 and RANTES in primary small alveolar epithelial cells (SAE) and in a human alveolar type II-like epithelial cell line (A549) was similar, suggesting that A549 cells can be used as a model to study lower airway epithelial cell responses to hMPV infection. A549 secreted a variety of CXC and CC chemokines, cytokines and type I interferons, following hMPV infection. hMPV was also a strong inducer of transcription factors belonging to nuclear factor (NF)-κB, interferon regulatory factors (IRFs) and signal transducers and activators of transcription (STATs) families, which are known to orchestrate the expression of inflammatory and immuno-modulatory mediators.

Keywords

hMPV; airway epithelial cells; chemokines; inflammation; transcription factors

Introduction

Human metapneumovirus (hMPV), first identified from children with respiratory symptoms in 2001, is a significant respiratory pathogen worldwide (van den Hoogen, de Jong et al., 2001; Falsey, Erdman et al., 2003;Kahn, 2006). hMPV is a member of the genus Metapneumovirus of the family Paramyxoviridae, based on genomic sequence analysis and organization (van den Hoogen, Bestebroer et al., 2002). Studies have shown that hMPV infects ~ 46% of children <1 year, ~70% of children by the time of age 5 and virtually all children by the ages of 5–10 (Principi, Bosis et al., 2006; Williams, Harris et al., 2004). hMPV accounts for not only 10–15% of pediatrics hospitalization but also for 10% of all hospitalizations of elderly patients with respiratory tract infections (Falsey, Erdman, Anderson, & Walsh, 2003). Furthermore, hMPV is a significant cause of morbidity and mortality in immunocompromised

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patients and patients with chronic obstructive pulmonary disease (van den Hoogen, van Doornum et al., 2003; Debiaggi, Canducci et al., 2006; Dare, Sanghavi et al., 2007).

hMPV-induced clinical illnesses range from mild upper respiratory tract infections to severe bronchiolitis and pneumonia (van den Hoogen, van Doornum et al., 2003). Investigations done in animal models of infection have shown that hMPV infection induced important pulmonary inflammation, characterized by alveolitis, interstitial inflammation and increased peribronchiolitis (Alvarez, Harrod et al., 2004; Hamelin, Yim et al., 2005; Wyde, Chetty et al., 2005; Williams, Tollefson et al., 2005). Although hMPV shares similar epidemiological and clinical features with RSV (Principi, Bosis, & Esposito, 2006; Williams, Wang et al., 2006), we and others have shown that they induce a different spectrum of cytokines and chemokines both in vitro, using dendritic cells, and in vivo, either in a mouse model of infection, or in children, suggesting a different ability of these two viruses to induce cellular responses (Guerrero-Plata, Casola et al., 2006; Guerrero-Plata, Casola et al., 2005; Jartti, van den Hoogen et al., 2002; Laham, Israele et al., 2004).

Similar to RSV, airway epithelial cells are the primary target of hMPV infection (Biacchesi, Pham et al., 2006; Alvarez, Harrod et al., 2004). However, little is known of their response to hMPV infection. In this study, we show that hMPV could infect and replicate similarly in epithelial cells from various tracts of the airways. hMPV infection of primary small alveolar epithelial cells (SAE) or of an alveolar type II-like epithelial cell line (A549) induced similar expression of the chemokines RANTES and IL-8, suggesting that A549 cell line can be used as a model to study the interaction of hMPV with lower airway epithelial cells, similar to what we have previously shown for RSV infection (Zhang, Luxon et al., 2001). We then determined whether hMPV induced the production of other inflammatory/immunomodulatory mediators. We found that A549 cells secreted a variety of cytokines and chemokines upon hMPV stimulation. Since activation of transcription factors belonging to the NF-κB and IRF families are critical for the expression of many cytokines and chemokines (Siebenlist, Franzoso et al., 1994; Taniguchi, Ogasawara et al., 2001; Barnes, Lubyova et al., 2002), we also investigated the ability of hMPV to induce their nuclear translocation and DNA-binding to IL-8 and RANTES promoter binding sites. Our results show that hMPV was a strong inducer of p50 and p65, which are members of the NF-κB families, as well as of IRF-1, -3 and -7. Finally, we investigated the ability of hMPV to induce type I interferons and to activate IFN-dependent signaling molecules. HMPV-infected A549 cells secreted significant amounts of both IFN-α and IFN-β. In addition, STAT1, STA2 and IRF-9, which form the intracellular ISGF3 complex necessary to induce antiviral genes (Martinez-Moczygemba, Gutch et al., 1997), were strongly activated following infection with hMPV.

Results

hMPV replication in epithelial cells

hMPV is a recently identified respiratory virus and there is limited knowledge of the response of airway epithelial cells, the target of infection, to this virus. Here, we used normal bronchial epithelial cells (NHBE), SAE and A549 cells, which represent cells from bronchial, terminal bronchioli and alveolar type II-like epithelial cells, respectively, to determine whether hMPV replicates similarly in cells from different tract of the airways. Cells were infected with hMPV at MOI of 3 and viral presence was analyzed by immunofluorescence, using an anti-hMPV polyclonal antibody, at 48 h post-infection (p.i.). Infected cells showed typical intracytoplasmic fluorescent granular inclusions when stained with the specific antibody, but not with isotope control (Fig. 1). Similarly, uninfected cells did not show granular staining with either antibody (data not shown). For each cell type, several pictures were taken and the percentage of cells which showed hMPV staining was counted over the total number of cells examined. All three

cell type showed a similar percentage of infection, which range from 80 to 90% (data not shown).

We then investigated hMPV ability to replicate in NHBE, SAE and A549 cells. Cells were infected at MOI of 0.1, viral inoculum was removed after one hour of incubation, and cells were harvested at 72 h p.i. to determine viral titer. LLC-MK2 cells, the cell line used to propagate hMPV, were used for comparison. We could not investigate viral replication at later time points of infections, above 72h p.i., because there was significant cell death in NHBE and SAE, compared to A549 cells, likely due to the absence of serum required for the infection. We found that, although hMPV replication in NHBE, SAE and A549 was a log less than in LLC-MK2 cells, viral titers among the different airway epithelial cells were very similar (Table 1), indicating that they are equally permissive to hMPV infection. On the other hand, there was no detectable replication of hMPV following inactivation with UV light (data not shown).

A549 and SAE cells secrete similar mediators following hMPV infection

IL-8 and RANTES are prototypes of CXC and CC chemokines, respectively (Miller & Krangel, 1992). To determine whether A549 and SAE cells secreted similar mediators following hMPV infection, supernatants from control or hMPV-infected cells were harvested at different time point of infection to measure IL-8 and RANTES protein by ELISA. As shown in Fig. 2A, hMPV infection induced a similar pattern of IL-8 secretion in both A549 and SAE cells, with induction starting from 6 h p.i. For RANTES, we found that there was a slight delayed kinetic of RANTES production in SAE cells (Fig. 2B), compared to A549 cells, similar to what we have previously shown for RSV infection (Zhang Y, Luxon et al., 2001).

To determine whether chemokine induction in epithelial cells in response to hMPV infection was dependent on viral replication, A549 cells were infected with either live or UV-inactivated virus. At 24 h p.i. cell supernatants were harvested to measure RANTES and IL-8 as described above. Only A549 cells infected with live hMPV released significant amounts of both chemokines, while UV-inactivated hMPV failed to do so (data not shown), indicating that chemokine secretion by hMPV requires viral replication.

A549 cells secret multiple pro-inflammatory mediators upon hMPV infection

Cytokines and chemokines secreted by airway epithelial cells play a critical role in initiating and modulating pulmonary inflammation upon viral infections. Chemokines are small proteins that control cellular migration at the site of infection and their family in mammals contains nearly 50 members. Depending on the arrangement of the first two N-terminal cysteines, chemokines are divided into four subfamilies: CXC, CC, C and CX_3C (Baggiolini, Dewald et al., 1997). The CXC subfamily is further divided in two groups, based on the absence or presence of three specific amino acids (Glu-Leu-Arg), which form the ELR motif (Baggiolini, Dewald et al., 1997). To further investigate the pattern of inflammatory/immune mediators secreted by airway epithelial cells in response to hMPV infection, we used multibead array system, which detects a panel of 24 different cytokines and chemokines. A549 cells were infected with hMPV, MOI of 3, for 6, 12, 24 and 48 h and then harvested to collect cell culture supernatants. We did not detect secretion of IL-1β, IL-2, IL-4, L-5, IL-7, IL-10, IL-12 (p70), IL-13, IL-15, IL-17, TNF-α and IFN-γ by A549 cells following hMPV infection, while we confirmed increased secretion of IL-8 and RANTES, as well as other CXC and CC chemokines (Fig. 3). IL-6 was the only cytokine significantly induced by hMPV infection (Fig. 3D). Among the CXC chemokines, hMPV induced secretion of IP-10 (Fig. 3A), an interferon-inducible protein, which has chemotactic activity for monocytes and T-lymphocytes and has been shown to play a role in limiting viral replication in other infection models (Dufour, Dziejman et al., 2002). IP-10 was secreted in significant amounts from infected epithelial cells starting at 12 h p.i., peaking at 24 h p.i. and declining at 48 h p.i. Among the CC chemokines, hMPV

significantly induced the secretion of MCP-1 (Fig. 3B), a monocyte chemoattractant protein (Rollins, Yoshimura et al., 1990). The secretion pattern and intensity of MCP-1 from infected cells were similar to those of IP-10, although the basal level of MCP-1 was higher than that of IP-10. Regarding macrophage inflammatory protein 1α (MIP-1α), the secretion of control cells was under the detection limit (4 pg/ml) at all time points. Unlike the secretion of the other chemokines, MIP-1α secretion from hMPV-infected cells was not striking, only around 35 pg/ml at 24 h p.i and 40 pg/ml at 48 h p.i. respectively (Fig. 3C), which is consistent with our previous observation of low MIP-1α secretion in bronchoalveolar lavage of hMPV-infected mice (Guerrero-Plata, Casola, & Garofalo, 2005). The secretion patterns and intensity of MIP-1β was very similar to those of MIP-1α (data not shown). Among the tested cytokines, hMPV infection resulted in significant induction of IL-6 in A549 cells (Fig. 3D). Kinetics and intensity of IL-6 secretion were similar to that of IL-8 and RANTES (Fig. 2).

hMPV infection activates transcription factors of the NF-kB and IRF families

In previous studies we have characterized the mechanisms involved in RSV-induced expression of the chemokines IL-8 and RANTES. Both chemokines are dependent on an intact NF-κB signaling pathway for their transcription, as we and others have previously shown (Garofalo, Sabry et al., 1996; Casola, Garofalo et al., 2001; Tian B, Zhang Y et al., 2002). RSV-induced RANTES expression is also dependent on the activation of IRF proteins which are absolutely necessary for RANTES promoter induction (Garofalo, Sabry, Jamaluddin, Yu, Casola, Ogra, & Brasier, 1996;Casola, Garofalo, Haeberle, Elliott, Lin, Jamaluddin, & Brasier A.R., 2001). NF-κB is a superfamily of ubiquitous transcription factors composed of NF-kB1 or p50, NF-kB2 or p52, Rel A or p65, RelB and c-Rel proteins, which can form homo- and hetero-dimers and produce complexes with various transcriptional activities. Their activation is partly controlled by accessory inhibitory proteins called IκBs (Beg & Baldwin, 1993). NF-κB inducing stimuli cause IκB phosphorylation, through activation of the multicomponent IκB kinase (IKK) complex (Karin & Delhase, 2000), with subsequent IκB proteolytic degradation (Henkel, Machleidt et al., 1993), event that allows NF-κB to enter the nucleus and activate target genes. To determine whether hMPV infection produced changes in abundance of DNA-binding proteins that recognized the NF-κB binding site of the IL-8 gene promoter, we initially used gel mobility shift assays (EMSA). As shown in Fig. 4A, a single nucleoprotein complex (C3) was formed from nuclear extracts of control cells on the NF-κB probe, while two other complexes, C1 and C2, were faintly detected. HMPV infection markedly increased the binding of C1 and C2 starting at 6 h p.i., with maximal binding intensity at 24 h p.i. We have previously shown, in the contest of RSV infection, that C2 is constituted by a p50-p65 heterodimer and C1 represents a p65 homodimer (Garofalo, Sabry, Jamaluddin, Yu, Casola, Ogra, & Brasier, 1996). To determine the mechanism for enhanced NF-κB binding activity, we performed Western blot analysis of p50 and p65 using nuclear extracts from control and hMPV-infected A549 cells (Fig. 4B). Both proteins were detected in the nuclear compartment of uninfected cells. However, upon hMPV infection, a time-dependent increase in p50 and p65 was observed, starting between 3 and 6 h p.i., with maximal nuclear translocation detected at 24 h p.i. The time-dependent increase in nuclear p50 and p65 was parallel to the changes in DNA binding activity observed in EMSA.

IRF transcription factors have been shown to play a fundamental role in the induction of several genes involved in the immune/inflammatory response to viral infections, including type I IFNs, chemokines and cytokines, like IL-15. Activation of IRFs is stimulus- and cell type-dependent. Among all members of the IRF family, IRF-1, IRF-3 and IRF-7 are the ones best characterized. IRF-1 and IRF-7 are inducible proteins, while IRF-3 is constitutively expressed. IRF-3 and -7 are phosphorylated on several serine/threonine residues, upon viral infection. This event allows dimerization, nuclear localization and DNA-binding (Taniguchi, Ogasawara, Takaoka, & Tanaka, 2001). To determine whether hMPV infection produced changes in the abundance of

DNA binding proteins recognizing the ISRE (interferon-stimulated response element) of the RANTES gene promoter, we performed EMSA similar to the one described for NF-κB. As shown in Fig. 5A, one inducible nucleoprotein complex was formed on the ISRE probe, starting at 6 h p.i. We have previously shown that in response to RSV infection, IRF-1, -3 and -7 all bind to the RANTES ISRE (Casola, Garofalo, Haeberle, Elliott, Lin, Jamaluddin, & Brasier A.R., 2001). To determine whether hMPV infection induced a similar activation of the IRF proteins, we performed Western blot analysis of IRF-1, -3 and -7 using nuclear extracts from control and hMPV-infected A549 cells (Fig. 5B). We found increased IRF-3 nuclear translocation starting around 6 h p.i., while IRF-1 and -7 appeared later, around 12 h p.i. Altogether, these data suggest that both NF-κB and IRF proteins are activated in airway epithelial cells in response to hMPV infection, and they are likely to play a key role in regulating pro-inflammatory gene expression.

hMPV induces type I interferon production and activates interferon-dependent signaling pathways in airway epithelial cells

Interferons (IFNs) are a superfamily of cytokines with antiviral, as well as anti-proliferative and immuno-modulatory functions (Smith, Lombardi et al., 2005). Type I IFNs, which include IFN-α,-β and –λ, are the first line of defense against viral infections. As IFNs play an essential role in limiting virus replication, we determined whether hMPV was able to induce type I IFN production in airway epithelial cells. A549 cells were infected with hMPV, MOI of 3, and cell supernatants were harvested at different time points of infection to measure IFN-α and-β by ELISA. We found that both IFN-α and -β were induced by hMPV infection of A549 cells in a time-dependent manner (Fig. 6), with a more significant induction of IFN-β than IFN-α.

IFN-β gene expression is a primary cellular response to viral infection and depends on activation of the transcription factors NF-κB and IRF-3 (Hiscott, Pitha et al., 1999), constitutively expressed in infected cells. Binding of IFN-β to its receptor activates STAT1 and STAT2, which, together with IRF-9, form the ISGF3 complex, responsible for the expression of a variety of interferon stimulated genes (ISGs), including IRF-7, which is required for IFN-α gene expression (Sato, Suemori et al., 2000). STAT proteins are constitutively expressed and located in the cytoplasm of unstimulated cells. Upon activation, they are phosphorylated on specific tyrosine residues, post-translational modifications necessary for dimerization, nuclear translocation and DNA binding [Reviewed in (Imada & Leonard, 2000)]. To determine whether hMPV infection of A549 cells induced activation of STAT1, STAT2 and IRF-9, we performed Western blot analysis of nuclear proteins extracted from A549 cells uninfected or infected for various lengths of time. As shown in Fig. 7, hMPV infection induced a time-dependent increase in tyrosine phosphorylation and nuclear translocation of STAT1, starting around 6 h p.i. It also induced increased nuclear translocation of STAT2 and IRF-9 between 6 and 12 h p.i., indicating that hMPV can activate the ISGF3 complex necessary to mediate interferon-dependent cellular responses, in particular antiviral gene expression.

Discussion

Human metapneumovirus has been identified as major viral pathogen, causing both upper and lower respiratory tract infections. Many children with a LRTI have a clinical syndrome consistent with bronchiolitis, with fever, cough, tachypnea, wheezing and hypoxia (Boivin, Abed et al., 2002; Esper, Boucher et al., 2003;Falsey, Erdman, Anderson, & Walsh, 2003). Chest radiographs demonstrate focal infiltrates and peribronchial cuffing (Esper, Boucher, Weibel, Martinello, & Kahn, 2003). Although the mechanisms mediating hMPV-induced lung disease are largely unknown, airway inflammation is likely to play a major role, as suggested by the recently developed small animal models of infection (Hamelin, Prince et al.,

2006b;Williams, Tollefson, Johnson, & Crowe, Jr., 2005;Guerrero-Plata, Casola, & Garofalo, 2005). Both in mice and cotton rats, hMPV infection induces significant lung inflammation and treatment with a combination of steroids and ribavirin, which affects hMPV replication, induce a significant reduction in lung pathology and disease (Hamelin, Prince et al., 2006a). Airway epithelial cells represent the major target of respiratory viruses and they are able to synthesize and secrete soluble mediators, upon injury or infection, which are important for the recruitment and activation of immune/inflammatory cells [Reviewed in (Lemanske, Jr., 1992)]. In this study, we show that hMPV can infect and replicate similarly both in normal airway epithelial cells, isolated from different tracts of the airways, as well as in cell lines. Replication of hMPV induces a variety of cytokines and chemokines, whose expression is dependent on viral replication. The finding that hMPV can induces IL-8 and RANTES secretion in SAE and A549 cells in a similar manner suggests that A549 cells can be used as an in vitro model of hMPV lower respiratory tract infection. We have previously shown that SAE cells display a similar profile of chemokine/cytokine gene expression, transcription factor and signaling pathway activation compared to A549 cells, following infection with respiratory syncytial virus (RSV), another member of the paramyxovirus family and the number one cause of viral lower respiratory tract infections in children (Garofalo, Sabry, Jamaluddin, Yu, Casola, Ogra, & Brasier, 1996; Olszewska-Pazdrak, Casola et al., 1998; Casola, Burger et al., 2001;Zhang Y, Luxon, Casola, Garofalo, Jamaluddin, & Brasier A.R., 2001; Pazdrak, Olszewska-Pazdrak et al., 2002).

In this study, we found that hMPV is a strong inducer of both CXC and CC chemokines, as well as of proinflammatory cytokines such as IL-6. While IL-8 secretion was detectable at 6 h p.i., induction of other chemokines, including IP-10, MCP-1 and RANTES was significant starting at 15 h p.i. IL-8 is the prototype of ELR-containing CXC chemokines, which recruit and activate neutrophils. We and others have shown that hMPV induces CXC chemokine production in a mouse model of infection, in which neutrophils are the prominent cell type seen in early airway inflammation (Hamelin, Yim, Kuhn, Cragin, Boukhvalova, Blanco, Prince, & Boivin, 2005;Guerrero-Plata, Casola, & Garofalo, 2005). Furthermore, significant levels of IL-8 have been detected in respiratory secretions of hMPV-infected children with bronchiolitis (Jartti, van den Hoogen, Garofalo, Osterhaus, & Ruuskanen, 2002;Laham, Israele, Casellas, Garcia, Prugent, Hoffman, Hauer, Thumar, Name, Pascual, Taratutto, Ishida, Balduzzi, Maccarone, Jackli, Passarino, Gaivironsky, Karron, Polack, & Polack, 2004).

Mononuclear cells, including macrophages/monocytes and lymphocytes, represent also an important component of the lung inflammatory infiltrate present in animal models of hMPV infection (Hamelin, Yim, Kuhn, Cragin, Boukhvalova, Blanco, Prince, & Boivin, 2005). Among the non-ELR-containing CXC chemokines, hMPV induced the expression of IP-10, which is an interferon-inducible protein and has been shown to play an important role in limiting viral replication in other infection models (Dufour, Dziejman, Liu, Leung, Lane, & Luster, 2002; Hamilton, Mahalingam et al., 2004). Among the CC chemokines, hMPV induced the expression of RANTES, MCP-1 and MIP-1α/β. These molecules have been shown to activate a variety of cell types, including monocytes, T- lymphocytes, eosinophils, basophils and dendritic cells, which are likely to play a significant role in the pathogenesis of hMPV-induced inflammatory lung disease, as well as in the modulation of immune responses, as it has been previously shown in RSV and other models of respiratory viral infections (Haeberle, Kuziel et al., 2001; Bonville, Lau et al., 2004; Domachowske, Bonville et al., 2000). In comparison to RSV, hMPV infection displayed a delayed kinetics and lower induction of chemokines in airway epithelial cells (Olszewska-Pazdrak, Casola, Saito, Alam, Crowe, Mei, Ogra, & Garofalo, 1998)data not shown), in particular for MIP-1α and -1β secretion, which could be due to hMPV slower viral replication and lower viral titers achieved in A549 cells (RSV titer in A549 cells at 24 h is similar to the hMPV titer at 72 h p.i. and it is ten fold higher at later time points, data not shown). A lower chemokine and cytokine induction by hMPV,

compared to RSV, was also observed in dendritic cells, as well as in a mouse model of infection and in children (Guerrero-Plata, Casola, Suarez, Yu, Spetch, Peeples, & Garofalo, 2006;Guerrero-Plata, Casola, & Garofalo, 2005;Laham, Israele, Casellas, Garcia, Prugent, Hoffman, Hauer, Thumar, Name, Pascual, Taratutto, Ishida, Balduzzi, Maccarone, Jackli, Passarino, Gaivironsky, Karron, Polack, & Polack, 2004).

Pro-inflammatory cytokines, such as IL-1, IL-6 and TNF, have been implicated in the pathogenesis of paramyxovirus-induced lung disease [Reviewed in (Graham, Johnson et al., 2000;Garofalo & Haeberle, 2000)]. We did not detect significant production of IL-1 and TNF, but there was significant secretion of IL-6 in response to hMPV infection. IL-6 production has been previously observed both in an animal model of hMPV infection and in infected children (Guerrero-Plata, Casola, & Garofalo, 2005;Laham, Israele, Casellas, Garcia, Prugent, Hoffman, Hauer, Thumar, Name, Pascual, Taratutto, Ishida, Balduzzi, Maccarone, Jackli, Passarino, Gaivironsky, Karron, Polack, & Polack, 2004). Whether pro-inflammatory cytokines play a role in hMPV-induced lung inflammation and disease pathogenesis will require further investigations.

Different patterns of inflammatory mediator secretion in response to RSV and hMPV infection may be also caused by different activation of intracellular signaling pathways, and subsequent different induction of transcription factors by the two viruses. To start to address this question, we investigated two of the key transcription factors involved in proinflammatory gene expression, NF-κB and IRF proteins. Our results show that hMPV is a strong inducer of both p50 and p65, belonging to the NF-κB family, as well as of IRF-1, -3 and -7. HMPV infection of A549 cells induced nuclear translocation of these proteins and increased binding to their specific DNA binding sites present in the IL-8 and RANTES gene promoters. We have previously shown that RSV infection of A549 cells is a potent activator of p65/Rel A, which is absolutely required for RSV-inducible IL-8 gene transcription (Garofalo, Sabry, Jamaluddin, Yu, Casola, Ogra, & Brasier, 1996). We have also shown that RSV infection of A549 cells induces IRF-1 synthesis, nuclear translocation and binding to a newly identified responsive element of IL-8 promoter involved in RSV-induced IL-8 transcription (Casola, Garofalo et al., 2000). IRF-7 synthesis is also induced in RSV-infected A549 cells, while IRF-3 is constitutive, and in alveolar epithelial cells IRF-1, -3 and -7 are the IRF proteins necessary for RSV-induced RANTES promoter activation (Casola, Burger, Liu, Jamaluddin, Brasier A.R., & Garofal, 2001). Therefore, it is likely that both NF-κB and IRF play a critical role in cytokine and chemokine gene expression in response also to hMPV infection.

Different from RSV, we found that hMPV induced IFN-α secretion in infected A549 cells. This result is consistent with our previous observation in monocyte-derived dendritic cells, as well as in a mouse model of infection, in which hMPV, but not RSV infection, induces significant production of IFN-α (Guerrero-Plata, Casola, Suarez, Yu, Spetch, Peeples, & Garofalo, 2006;Guerrero-Plata, Casola, & Garofalo, 2005). RSV failure to induce IFN-α production in A549 cells has been previously reported (Spann, Tran et al., 2004). Two RSV non-structural proteins, NS1 and NS2, have been shown to partially inhibit activation of NF-κB and IRF-3 and disrupt STAT-2-mediated signaling pathway, which is important for type I IFN-mediated signaling, including IFN-α production and expression of antiviral genes (Spann, Tran et al., 2005; Ramaswamy, Shi et al., 2006; Wright, Karron et al., 2006). Compared to RSV, hMPV does not have NS1 and NS2, and this could explain hMPV ability to induce higher levels of both IFN-α and -β in epithelial, as well as non-epithelial cells. However, we have also reported that hMPV, similar to RSV, can inhibit Toll-like receptor (TLR)-dependent type I IFN production (Guerrero-Plata, Baron et al., 2005). This observation, together with the finding that the amount of IFN-α secreted by hMPV-infected A549 cells is much less than that secreted by monocyte-derived dendritic cells, would suggest that hMPV also might have evolved

strategies to modulate interferon production and interferon-dependent signaling pathways. Which viral protein(s) is responsible for this activity remains to be investigated.

In summary, in this study we have started to characterize the airway epithelial cell responses to hMPV, an important respiratory pathogen. As the airway epithelium is the primary target of hMPV infection, the identification of genes and pathways activated by this respiratory virus is highly relevant for a better understanding of the pathogenesis of hMPV-induced lung disease.

Material and Methods

Materials

The following reagents were purchased from the indicated suppliers: Eagle's minimal essential medium, F12K medium, L-glutamine, penicillin-streptomycin-amphotericin B, (Gibcol, Carlsbad, CA); LLC-MK2 cells (Rhesus monkey kidney ATCC CCL-7) and human alveolar type II-like epithelial cells (A549 cells) (ATCC; Manassas, VA); small alveolar epithelial cells (SAE) and normal bronchial epithelial cells (NHBE) (Clonetics, San Diego, CA); double antibody ELISA kit (Duo-Set, R&D Systems, Minneapolis, MN); antibodies for IRFs, p50 and lamin B (Santa Cruz, Santa Cruz, CA); antibody for p65 (Upstate, Charlottesville, VA); RiboQuant kit (Pharmingen, San Diego, CA); radioactive probe (Du Pont NEN Research Products, Boston, MA); human interferon-α and -β ELISA kit (PBL Biomedical Laboratories, Piscataway, NJ); Bio-Plex Human Cytokine panel (Bio-Rad Laboratories, Hercules, CA).

Cell culture and viral preparation

LLC-MK2 cells were maintained in MEM supplemented with 10% Fetal Bovine Serum (FBS), Penicillin and Streptomycin (100U/ml). A549 cells were maintained in F12K medium containing 10% (v/v) FBS, 10 mM glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin. Primary cultured SAE and NHBE cells were grown according to the company instructions. For most experiments, confluent cells were infected with hMPV in serum-free media with 1.0 μg trypsin/ml at MOI 3 to 5. Viral stocks were obtained by infecting LLC-MK2 cells at MOI 0.1. Around day 5 post-infection, cells were disrupted using glass beads, and then scraped completely from the flasks. Total medium was collected and sonicated on ice for 5 min. The preparation was then vortexed and centrifuged at 2900g for 15 min on ice. Crude virus was collected and then purified on a sucrose cushion. Viral titers were determined by immunostaining, as previously described (Guerrero-Plata, Casola, Suarez, Yu, Spetch, Peeples, & Garofalo, 2006).

Immunofluorescence

Replication of hMPV in NHBE, SAE and A549 cells were verified by immunochemistry staining using anti-hMPV polyclonal antibody (a gift from MedImmune Inc., Gaithersburg, MD). Cells were plated in a slide chamber and infected with hMPV using MOI of 3 for 24 h. Cells were washed with PBS and fixed with 1% paraformaldehyde at room temperature for 15 min. Cells were then washed with PBS twice, followed by incubation with 0.2% Triton 100-X. Following PBS washes, slides were incubated with a primary guinea pig isotype control Ab or anti-hMPV antibody (1:200) for one hour. Cells were washed again and incubated with a secondary FITC-conjugated anti-guinea pig antibody. Picture was taken using Zeiss LSM Image Browser software v. 3.0 (Zeiss, Thornwood, NY).

Bio-Plex

Chemokines and cytokines (IL-1RA, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 p70, IL-13, IL-17, G-CSF, GM-CSF, IFN-γ, IP-10,EOTAXIN, MIP-1α, MIP-1β, GCSF, FGFB, PDGF, VEGF and TNF-α) were quantified by the Luminex-based Bio-Plex system

(Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. The lower limit of detection for all cytokines measured by Bio-Plex is 3 pg/ml.

ELISA

Human IL-8 and RANTES concentrations in cell culture supernatants were determined using DuoSet ELISA kits (R&D, Systems Minneapolis, MN). IFN-α and -β were determined by Human interferon-α and- β ELISA kit ((lower limit of detection, 12.5 pg/ml, PBL Biomedical Laboratories, Piscataway, NJ) according to manufactory's instructions.

EMSA

Nuclear extracts of uninfected and infected cells were prepared using hypotonic/nonionic detergent lysis, according to Schaffner protocol (Schreiber, Matthias et al., 1989). To prevent contamination with cytoplasmic proteins, isolated nuclei were purified by centrifugation through 1.7 M sucrose buffer A for 30 minutes, at 12,000 rpm, before nuclear protein extraction, as previously described (Brasier, Spratt et al., 2004). After normalization, nuclear proteins were used to bind to duplex oligonucleotides corresponding to either the RANTES ISRE or the IL-8 NF-κB binding sites, as previously described (Casola, Garofalo, Haeberle, Elliott, Lin, Jamaluddin, & Brasier A.R., 2001;Casola, Garofalo, Jamaluddin, Vlahopoulos, & Brasier, 2000). DNA-binding reactions contained 10- 15 μg nuclear proteins, 5% glycerol, 12 mM HEPES, 80 mM NaCl, 5 mM DTT, 5 mM Mg_2Cl, 0.5 mM EDTA, 1 μg of poly dI-dC and 40,000 cpm of ^{32}P- labeled double-stranded oligonucleotide in a total volume of 20 μl. The nuclear proteins were incubated with the probe for 15 min at room temperature and then fractionated by 6% nondenaturing polyacrylamide gels (PAGE) in TBE buffer (22 mM Tris-HCl, 22 mM boric acid, 0.25 mM EDTA, pH 8). After electrophoretic separation, gels were dried and exposed for autoradiography using Kodak XAR film at −70 °C using intensifying screens.

Western blot analysis

Nuclear extracts were prepared as described above. Proteins (10 to 30 μg per sample) were then boiled in 2X Laemmli buffer for 2 min and resolved on SDS-PAGE. Proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad). Nonspecific binding sites were blocked by immersing the membrane in TBST blocking solution [10mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.05% Tween-20 (v/v)] containing 5% skim milk powder or 5% bovine serum albumin for 30 min. After a short wash in TBST, the membranes were incubated with the primary antibody for 1 h, followed by an anti-rabbit peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology), diluted 1:10,000 in TBST for 30 min. After washing, the proteins were detected by autoradiography using ECL regular or plus (Amersham Pharmacia Biotech) according to manufacturer's protocol. Equal loading of proteins was evaluated by stripping and reprobing the membranes with anti-lamin B antibody.

Statistical Analysis

Statistical significance was analyzed by using analysis of variance (ANOVA). *P* value of less than 0.05 was considered significant. Mean ± SEM is shown.

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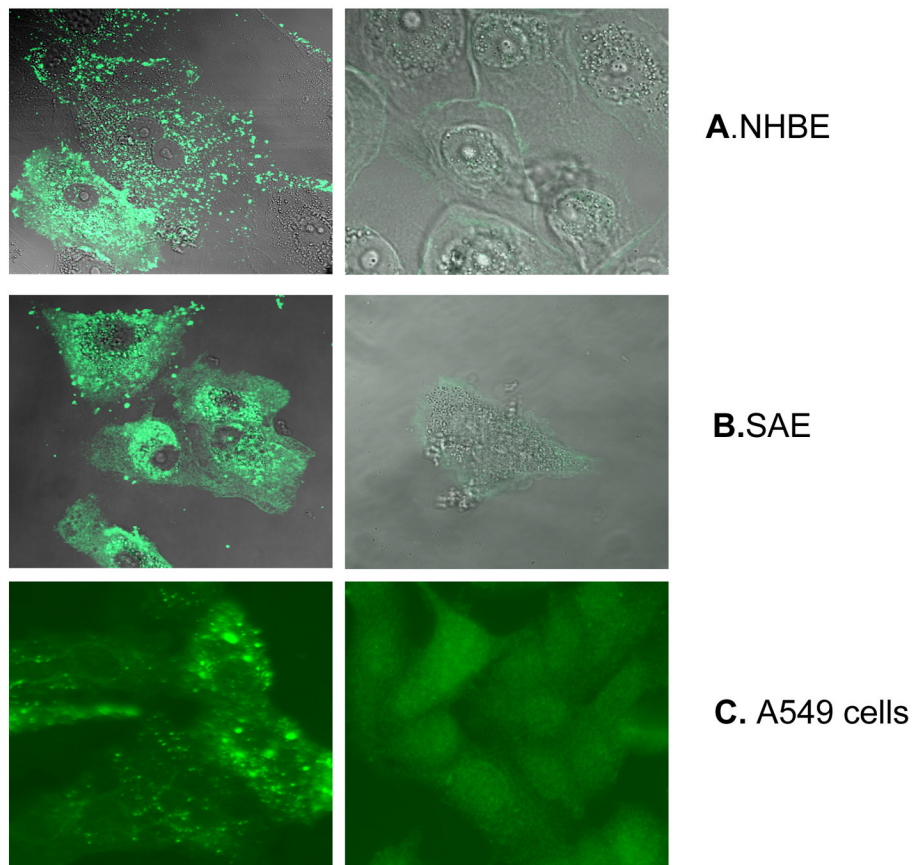
Fig. 1. Immunofluorescence of hMPV-infected human airway epithelial cells
Normal bronchial epithelial cells (NHBE, A), small airway epithelial cell (SAE, B) and human alveolar type II-like epithelial cell line (A549, C) were infected with hMPV at MOI 3 for 48 h and stained with nti-hMPV polyclonal antibody (left panel) or isotype antibody (right panel). Cells were analyzed using a Zeiss LSM 510 meta-ultraviolet laser scan confocal microscope for A and B or fluorescent microcopy for C. No staining was observed in mock-infected cells when similar staining was done (data not shown).

Fig.2A

A549

SAE

Fig.2B

A549

SAE
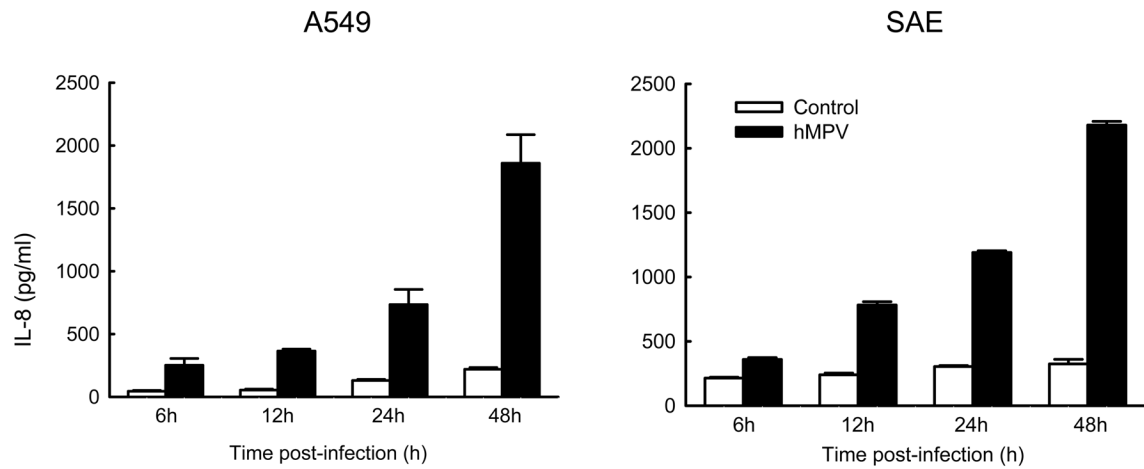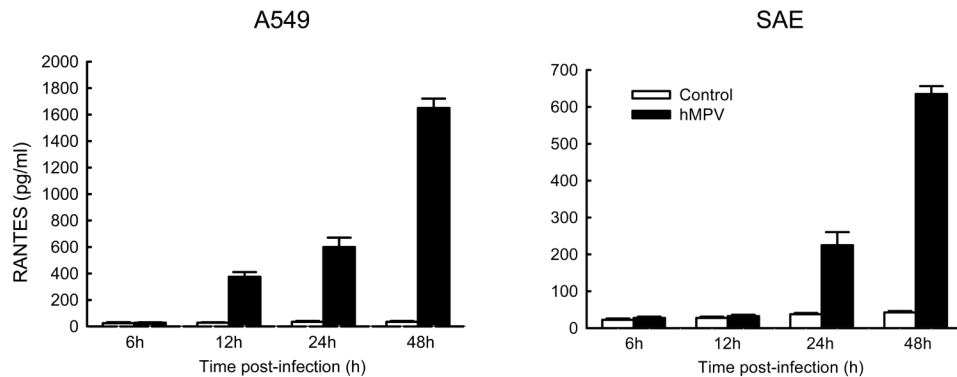
Fig. 2. Chemokines induced by hMPV in A549 and SAE cells
A549 and SAE cells were infected with hMPV at MOI of 3 for various length of time. Secretion of IL-8 (A) and RANTES (B) by hMPV-infected A549 and SAE cells was assayed by ELISA. Data are expressed as mean ± standard error of two independent experiments performed in triplicates.
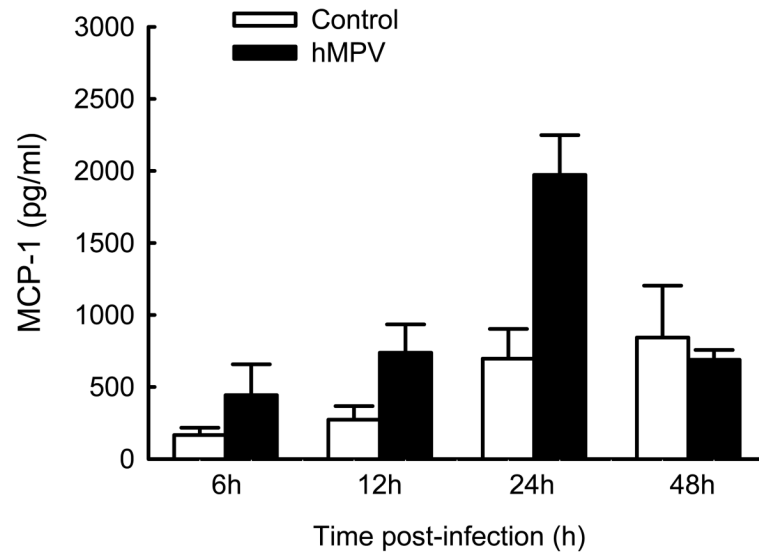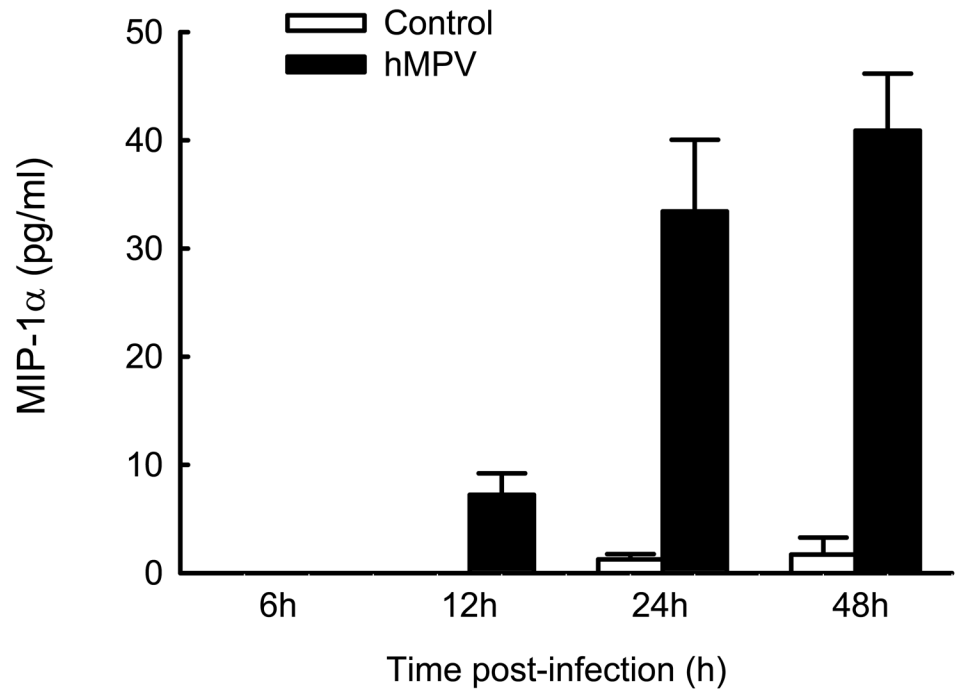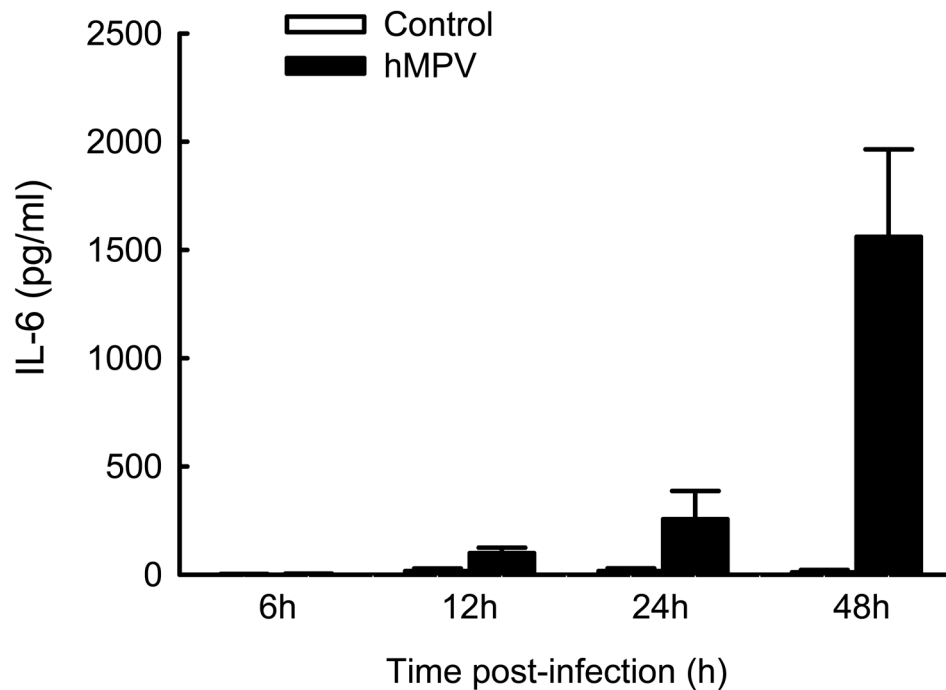
Fig.3A
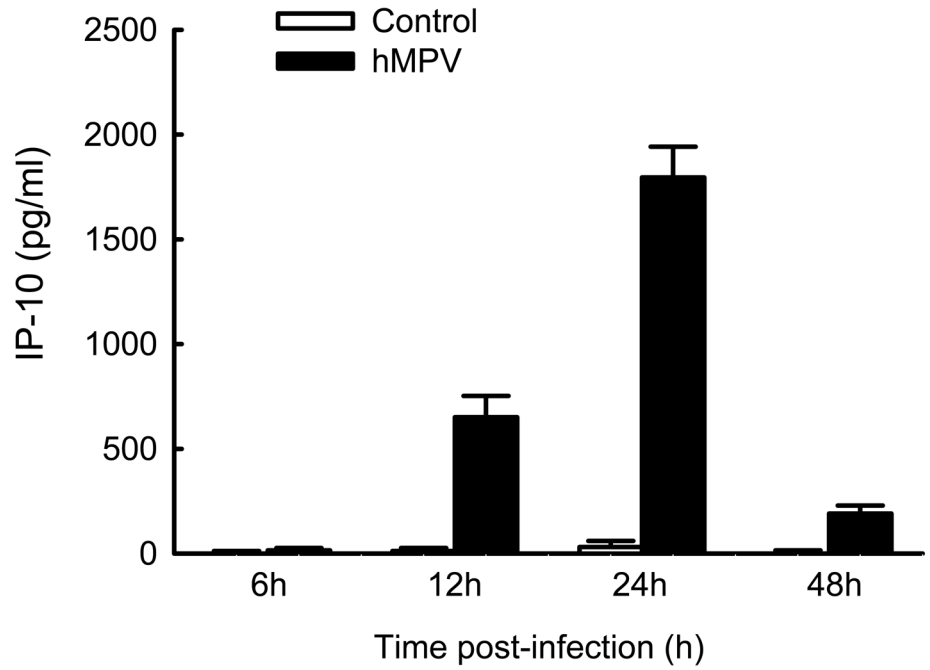
Fig.3B

Fig.3C

Fig.3D

Fig. 3. Cytokine and chemokine production by A549 cells infected with hMPV
A549 cells were infected with hMPV at MOI of 3. Cell supernatants were collected at different time points of infection and tested for cytokines and chemokine secretion by multiplex bead assay. Data are expressed as mean ± standard error of two independent experiments performed in triplicates. Open bars represent uninfected and solid bars hMPV-infected cells.
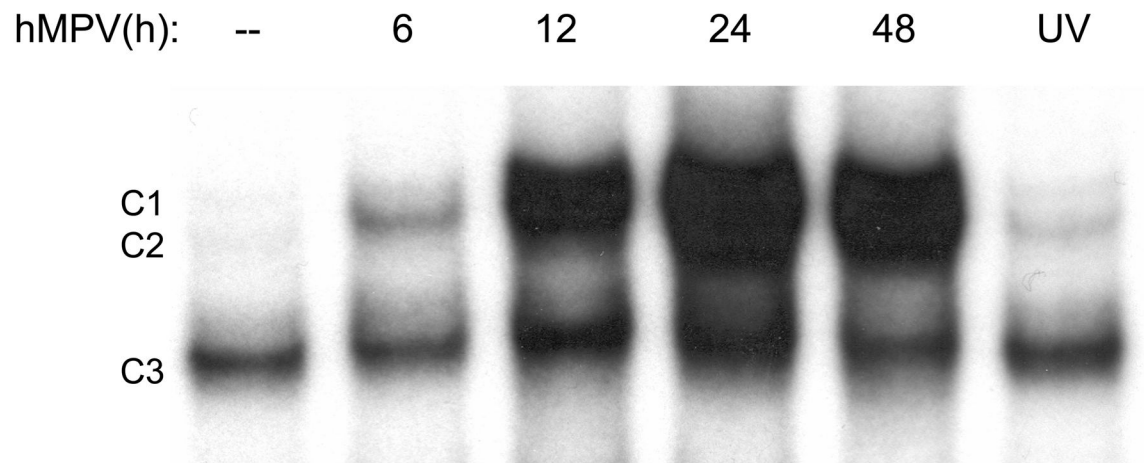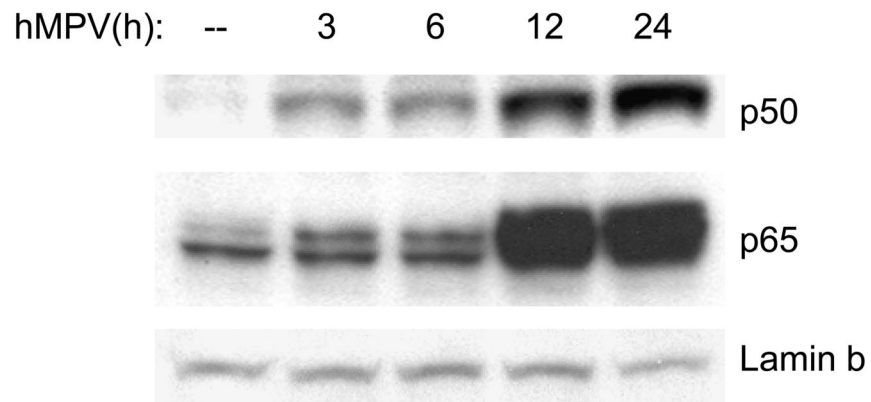
Fig.4A

hMPV(h): -- 6 12 24 48 UV

C1
C2

C3

Fig.4B

hMPV(h): -- 3 6 12 24

p50

p65

Lamin b

Fig. 4. NF-kB activation in response to hMPV infection in A549 cells
(A). Nuclear extracts were prepared from A549 cells control and infected with hMPV, MOI
of 3, for 6, 12, 24 and 48 h and used for binding to the IL-8 NF-kB probe in EMSA. Shown
are the nucleoprotein complexes formed on the probe in control cells and in response to the
infection. C1 and C2 are hMPV-inducible complexes, while C3 is constitutive. UV indicates
UV-inactivated virus. (B) Western blot of p50 and p65 in hMPV-infected cells. Nuclear
proteins were prepared from control and A549 cells infected for various length of time,

fractionated on a 10% SDS-PAGE, transferred to PVDF membranes and probed with the appropriate antibody. Lamin b was used as an internal control to determine equal loading of the samples.
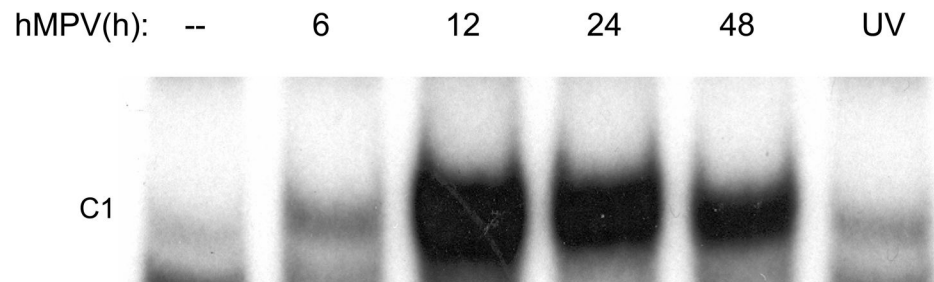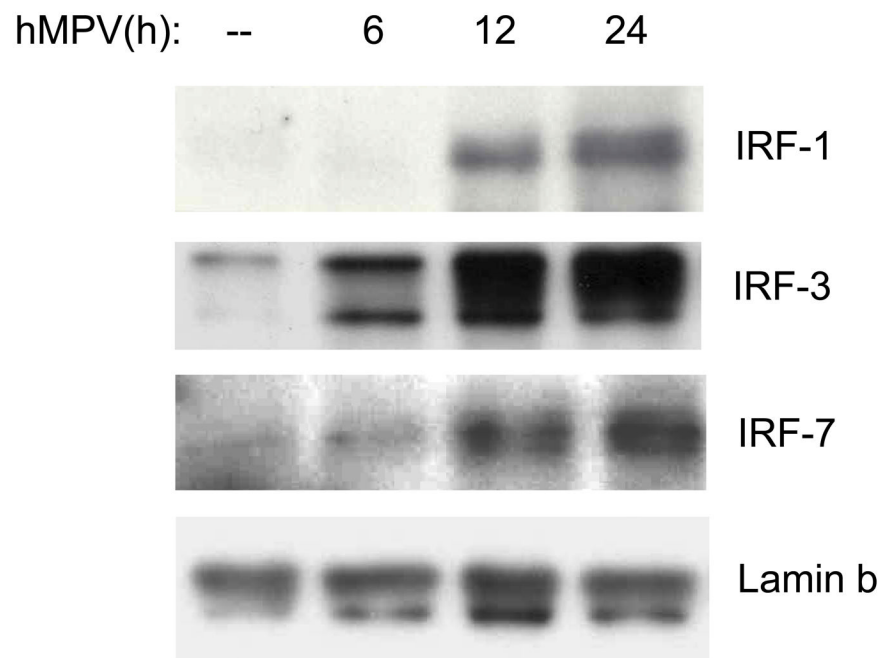
Fig.5A

hMPV(h):   --   6   12   24   48   UV

C1

Fig.5B

hMPV(h):   --   6   12   24

IRF-1

IRF-3

IRF-7

Lamin b

Fig. 5. IRF activation induced by hMPV infection in A549 cells
(A). Nuclear extracts were prepared from A549 cells control and infected with hMPV, MOI
of 3, for 6, 12, 24 and 48 h and used for binding to the RANTES ISRE probe in EMSA. Shown
is the inducible nucleoprotein complex formed on the probe in response to the infection. UV
indicates UV-inactivated virus. (B) Western blot of IRF-1,-3, and -7 in A549 cells infected
with hMPV. Nuclear proteins were prepared from control and A549 cells infected for various
length of time, fractionated on a 10% SDS-PAGE, transferred to PVDF membranes and probed

with the appropriate antibody. Lamin b was used as an internal control to determine equal loading of the samples.
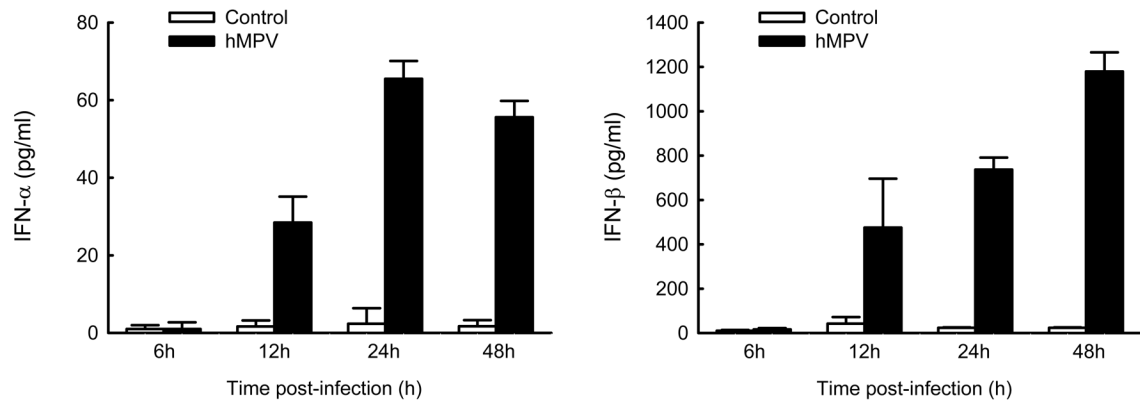
Fig. 6. Type I IFN production by airway epithelial cells infected with hMPV
A549 cells were infected with hMPV at MOI of 3. Cell supernatants were collected at 6, 12, 24 and 48 h p.i. and tested for type I interferon production by ELISA. Data are expressed as mean ± standard error of two independent experiments performed in triplicates. Open bars represent uninfected and solid bars hMPV-infected cells.
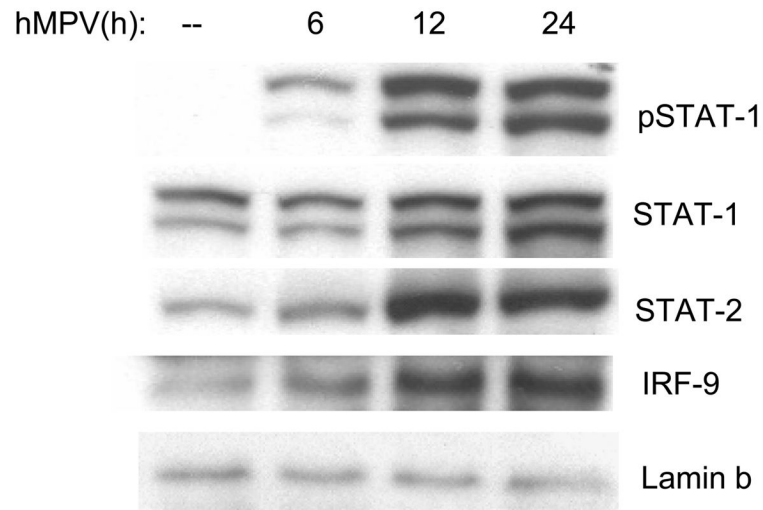
Fig. 7. ISGF3 complex activation in response to hMPV infection
A549 cells were infected with hMPV, MOI of 3, for 6, 12 and 24 h. Cells were harvested to prepare nuclear extracts and equal amounts of protein from control and infected cells were assayed for phosphorylated and total STAT1, total STAT2 and IRF-9 by Western blot. Lamin b was used as internal control to determine equal loading of the samples.

Table 1

hMPV replication in primary airway epithelial cells and cell lines

Cell type	Viral titer (pfu/ml)
NHBE	1.6×10^4
SAE	1.6×10^4
A549	2.15×10^4
Hep2	4.08×10^5
LLC-MK2	8.5×10^5