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Human Metapneumovirus Induces a Profile of Lung Cytokines Distinct from That of Respiratory Syncytial Virus

Antonieta Guerrero-Plata,[1] Antonella Casola,[1,2,3] and Roberto P. Garofalo[1,2,3]*

Departments of Pediatrics,[1] Microbiology and Immunology,[2] and Sealy Center for Vaccine Development,[3] University of Texas Medical Branch, Galveston, Texas 77555

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Lung cytokine and chemokine production by BALB/c mice infected with human metapneumovirus (hMPV) was compared to respiratory syncytial virus (RSV)-infected mice. hMPV infection induced lower levels of the inflammatory cytokines interleukin-1 (IL-1), IL-6 and tumor necrosis factor alpha but was a more potent inducer of granulocyte-macrophage colony-stimulating factor and triggered a more sustained production of the CXC chemokine KC compared to RSV. hMPV was a stronger inducer of both alpha interferon (IFN-α) and IFN-γ responses than RSV. In regard to immunomodulatory cytokines, hMPV failed to induce detectable IL-10 or IL-12p70 but was a potent inducer of IL-12 p40 subunit. The implications for hMPV pathogenesis are discussed.

Human metapneumovirus (hMPV) was recently identified as a new paramyxovirus pathogen associated with upper and lower respiratory tract infections (27). Based on electron microscopy studies and viral genome sequence, hMPV has been assigned to the *Metapneumovirus* genus of the *Paramyxoviridae* family (26). hMPV contains a nonsegmented, negative-sense RNA with a genomic organization similar but not identical to that of respiratory syncytial virus (RSV) (1, 26). hMPV harbors open reading frames for at least eight viral proteins (3′-N-P-M-F-M2-SH-G-L-5′) but lacks the nonstructural proteins NS1 and NS2 and differs significantly in its gene order. Epidemiological studies indicate that, like RSV, hMPV is a significant human respiratory pathogen with worldwide distribution (6, 14, 22). Indeed, hMPV appears to affect many of the same subpopulations (15) and to cause similar clinical manifestations, including upper respiratory tract infections, bronchiolitis, and pneumonia (2, 5, 17, 28), although of lower severity compared to RSV (31). The profile, relative abundance and kinetics of cytokines that are produced in the airways in the course of viral respiratory infections are all factors that contribute to shaping host immunity, inflammatory response, and ultimately viral shedding (7, 13). Little is currently known about the airway inflammatory and immune responses in hMPV infection and whether a distinct and/or overlapping pattern of cytokines characterizes the host response to hMPV compared to RSV. Therefore, using an experimental mouse model we conducted a comprehensive analysis of the profile and kinetics of proinflammatory and immunoregulatory cytokines, as well as inducible chemokines that are produced in the course of hMPV or RSV infection.

To that purpose, we measured the concentrations of cytokine proteins in the lung of 8- to 10-week-old female BALB/c mice (Harlan Sprague-Dawley Laboratories, Indianapolis, IN) after infection with hMPV (CAN97-83) or RSV (A2), both of which were propagated and sucrose purified as previously described (25). hMPV was obtained from the Centers for Disease Control, Atlanta, GA, with permission from Guy Boivin at the Research Center in Infectious Diseases, Regional Virology Laboratory, Laval University, Quebec, Canada. Mice were infected intranasally with 10^7 PFU of hMPV or RSV diluted in Dulbecco phosphate-buffered saline (D-PBS) (Gibco, Grand Island, NY). As mock treatment, mice were inoculated with an equivalent volume of sucrose diluted in D-PBS. At 0.5, 1, 2, 3, 5, 8, and 10 days postinfection, bronchoalveolar lavage (BAL) fluid was collected by washing the lungs two times with 1 ml of minimal essential medium (Gibco) supplemented with 2% bovine serum (Gibco). BAL fluid was tested for multiple cytokines using the Bio-Plex Mouse Cytokine 18-Plex panel (Bio-Rad Laboratories, Hercules, CA) according to the manufacturer's instructions. The panel included the following cytokines: interleukin-1α (IL-1α), IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-12 p40, IL-12 p70, IL-17, granulocyte-macrophage colony-stimulating factor (GM-CSF), gamma interferon (IFN-γ), tumor necrosis factor alpha (TNF-α), G-CSF, KC, macrophage inflammatory protein 1α (MIP-1α), and RANTES. In addition, IFN-α and IL-18 were measured by enzyme-linked immunosorbent assay (ELISA; PBL Biomedical Laboratories, Piscataway, NJ, and MBL, Nagoya Japan, respectively). The IFN-α ELISA recognizes the αA, α1, α4, α5, α6, and α9 isoforms. All experiments were performed at least three times using four mice/time point/experiment. Statistical analyses were performed with the InStat 3.05 Biostatistics Package (GraphPad, San Diego, CA) using a one-way analysis of variance to ascertain differences between groups, followed by a Tukey-Kramer test to correct for multiple comparisons. Mean ± the standard error of the mean (SEM) cytokine BAL concentrations are shown in the figures.

As shown in Fig. 1, the proinflammatory cytokines IL-1α, IL-1β, IL-6, and TNF-α were differentially induced by hMPV or RSV infections. Indeed, whereas both viruses showed a similar kinetics of production for IL-1α, IL-1β, and IL-6, mice

* Corresponding author. Mailing address: Department of Pediatrics, University of Texas Medical Branch, 301 University Blvd., Galveston, TX 77555-0369. Phone: (409) 772-2658. Fax: (409) 772-1761. E-mail: rpgarofa@utmb.edu.

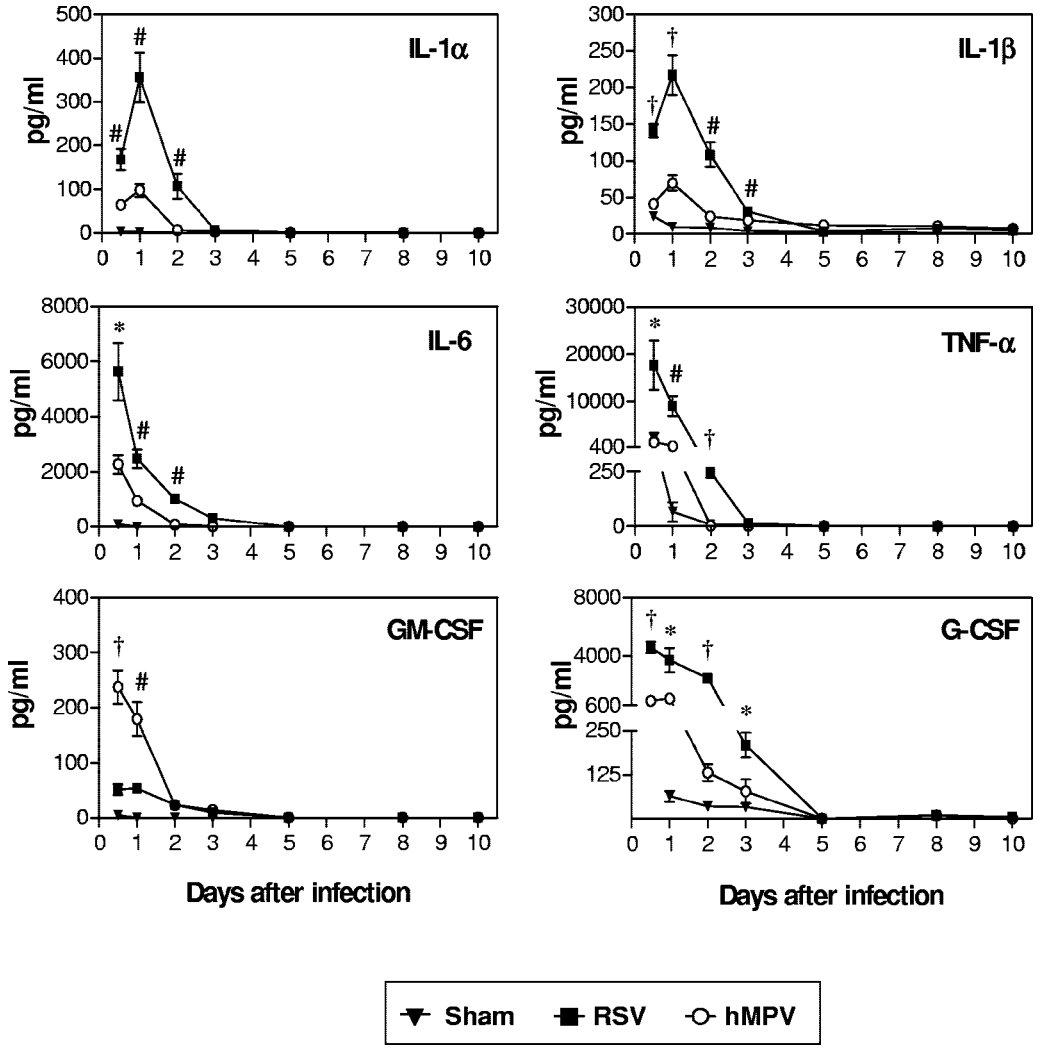

FIG. 1. Production of proinflammatory cytokines in hMPV- or RSV-infected mice. BALB/c mice were infected with 10^7 PFU of sucrose purified hMPV or RSV. BAL fluid was collected at different time points after viral infection and IL-1α, IL-1β, IL-6, TNF-α, GM-CSF, and G-CSF were measured by Bio-Plex Mouse Cytokine 18-Plex panel. Graphs represent mean ± the SEM (n = four mice/group). ✱, $P < 0.05$; #, $P < 0.01$; †, $P < 0.001$ (when hMPV and RSV infections are compared).

infected with RSV had three- to fourfold greater concentrations of IL-1α and IL-1β in BAL samples compared to hMPV (24 h). BAL concentrations of IL-6 were also significantly greater in mice infected with RSV compared to those infected with hMPV. Overall, mice infected with hMPV had concentrations of IL-1α, IL-1β, and IL-6 that were not significantly greater than those measured in sham-inoculated animals ($P > 0.05$ at all time points). Analysis of TNF-α showed that this cytokine was at baseline levels in BAL of hMPV-infected mice (i.e., comparable to sham-inoculated mice), whereas extremely high concentrations were detectable in BAL samples of RSV-infected mice. These data are in agreement with a study performed in nasal samples from naturally infected children showing that those infected by hMPV had lower levels of IL-1β, IL-6, and TNF-α compared to those infected by RSV (17). In addition, our results consistently showed an opposite pattern for virus-induced production of GM-CSF and G-CSF. In this regard, significantly greater concentrations of GM-CSF were observed in hMPV- compared to RSV-infected mice, where the latter had BAL levels of this cytokine that were not significantly higher than those measured in sham-inoculated mice. Production of GM-CSF peaked at 12 h after infection and by day 3 had reached near-constitutive levels (Fig. 1). On the other hand, G-CSF was expressed in significantly greater levels in the BAL samples of RSV-infected mice compared to hMPV-infected mice. In addition to its role as hematopoietic factor, GM-CSF plays a critical role in the recruitment and activation of neutrophils (11, 12), in the process of allergic inflammation (19), and in host immunity. Overexpression of GM-CSF in the lung has been shown to enhance RSV-induced IFN-γ and IL-12 p40 production, to augment proliferation and activation of pulmonary antigen-presenting cells, and to attenuate viral replication (3). Whether the robust expression of GM-CSF, which we found in this studies is linked to the increased production of IFN-γ and IL-12 p40 that was observed in hMPV-infected mice (3 to 5 days after inoculation) com-

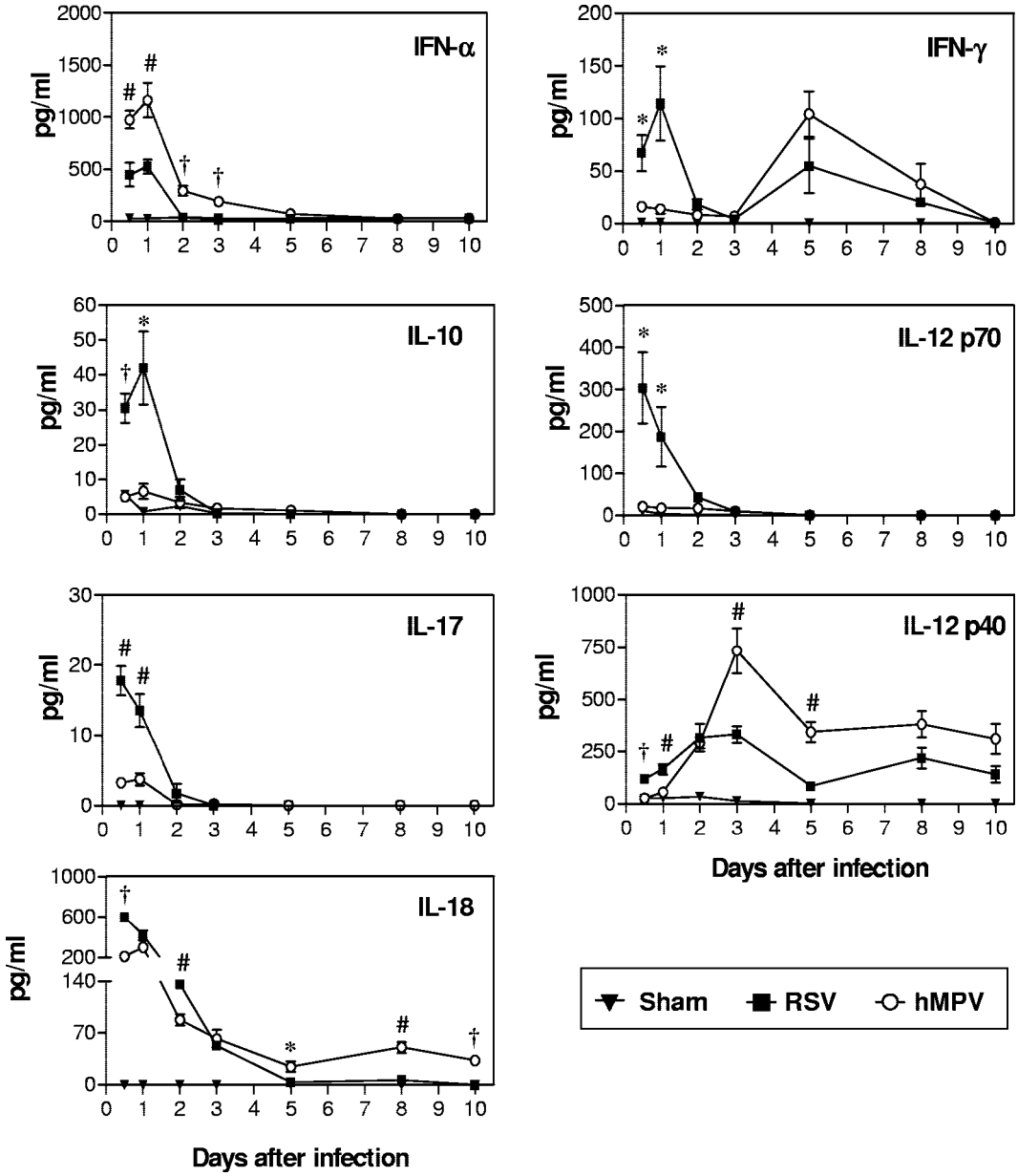

FIG. 2. Production of immunoregulatory cytokines in hMPV- or RSV-infected mice. BALB/c mice were infected as described in Fig. 1, and IFN-γ, IL-10, IL-17, IL-12 p70, and IL-12 p40 levels were measured in BAL fluid by using a Bio-Plex Mouse Cytokine 18-Plex panel. IFN-α and IL-18 were measured by ELISA. Graphs represent mean ± the SEM (n = four mice/group). ✻, $P < 0.05$; #, $P < 0.01$; †, $P < 0.001$ (when hMPV and RSV infections are compared).

pared to RSV-infected ones (see below) remains to be determined. The role of G-CSF, a cytokine that also regulates the production and function of neutrophils, in the context of paramyxovirus infection is currently unknown (4). Overall, our data suggest that hMPV may use different mechanisms than RSV to induce inflammation in the infected hosts.

Immunomodulatory cytokines, including IFN-α, IFN-γ, IL-12 p70, IL-12 p40, IL-10, IL-17, and IL-18, were also differentially induced by hMPV or RSV infections, in terms of profile, amount, and kinetics of release. IFN-α, a critical mediator of innate responses against viruses, was produced by both hMPV and RSV, reaching a peak at 24 h after infection, decreasing by day 2, and returning to undetectable levels by day 5. Confirming recently studies by our group (8), we found that IFN-α was produced in significantly higher amounts by hMPV than RSV at all time points (Fig. 2). These findings further suggest that the lack of NS1 and NS2 proteins in the hMPV genome might enable this virus to induce a more robust IFN-α production compared to RSV, as these viral proteins have been shown to play a mayor role in the suppression of IFN-α/β production (23). Using the same mouse model and identical experimental conditions, we have recently shown that hMPV is significantly more susceptible to the antiviral effect of IFN-α than is RSV (8). Thus, it is possible that the more robust

and sustained production of IFN-α in the airways coupled with increased susceptibility to the antiviral activity of this cytokine and/or other virus-specific factors may all contribute to the overall lower lung virus titers in mice infected with hMPV compared to those infected with RSV (8; A. Casola, unpublished data). Whether these observations may be also pertinent to the poor viral recovery/culture in nasopharyngeal samples of naturally infected human subjects remains to be determined (27). As shown in Fig. 2, during the early phase of infection, hMPV induced small amounts of IFN-γ, barely above the level detected in mock-inoculated mice, whereas RSV infection resulted in a much more robust production of this Th1 cytokine. On the other hand, at later time points (around day 5) we consistently observed a trend for higher production of IFN-γ by hMPV compared to RSV infection, although this difference did not reach statistical significance. By day 10, IFN-γ concentrations in BAL fluid had returned to basal levels in both hMPV- and RSV-infected animals. Of note, hMPV failed to induce production of IL-10, IL-12 p70, and IL-17, all of which were induced after RSV infection (Fig. 2). These results are consistent with other studies by our group, which show that hMPV, different from RSV, does not induce IL-10 and IL-12 p70 production in cultured human dendritic cells (8a). At early stages of the infection (12 and 24 h) IL-12 p40 was also significantly induced by RSV but not hMPV infection compared to sham inoculation. On the other hand, at later time points IL-12 p40 was induced in significantly higher amounts by hMPV compared to RSV, with a peak of production around day 3 of infection and detectable levels still present 10 days after viral inoculation. IL-12 p40 is produced primarily by cells of macrophage and dendritic cell lineage (18), but it is also inducibly expressed in epithelial cells (29). The role of IL-12 p40 in the context of hMPV infection, particularly in view of the relatively high concentrations of the cytokine that we recovered in BAL, is currently unclear. IL-12 p40 is known to antagonize IL-12 p70 activity (16) and to trigger IFN-γ production by NK and T cells (24). Recently, genetic deficiency of IL-12 p40 has been associated with increased lung inflammation, Th2 immune response, and mucus production in an experimental model of acute RSV infection (30). Whether this imbalance in IL-12 p70/p40 production plays a role in shaping the host inflammatory and immune responses during hMPV infection, including the regulation of IFN-γ production, is currently under investigation. IL-18, a cytokine that along with IL-12 is crucial in the development of Th1 responses and has been reported to modulate the inflammatory and immune responses to RSV (30), was induced in greater amounts by RSV than hMPV only at the early time points (up to day 2 after infection). In fact, at later time points the concentrations of IL-18 in BAL were significantly greater in hMPV- than in RSV-infected mice, a pattern similar to that observed for IFN-γ and IL-12 p40. Finally, the Th2 cytokines IL-4 and IL-5 were undetectable or at the lowest limit of detection in BAL samples of either hMPV- or RSV-infected mice, at all time points tested.

Production of three inducible chemokines during infection appeared to follow a virus-specific pattern. As shown in Fig. 3, BAL samples from RSV-infected mice had significantly greater concentrations of the CC chemokines CCL5 (RANTES) at 12 and 24 h, and CCL3 (MIP-1α) at 12, 24, 48 and 72 h

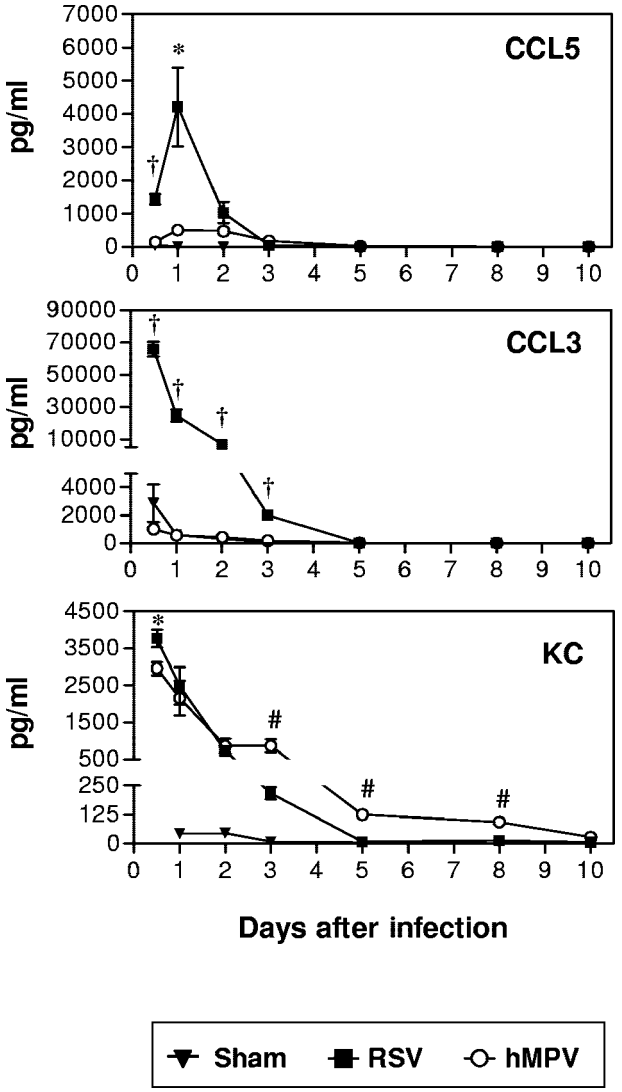

FIG. 3. Production of chemokines in hMPV- or RSV-infected mice. BALB/c mice were infected as described in Fig. 1 and CCL5, CCL3, and KC were measured in BAL fluid by Bio-Plex Mouse Cytokine 18-Plex panel. Graphs represent mean ± the SEM (n = four mice/group). ✱, $P < 0.05$; #, $P < 0.01$; †, $P < 0.001$ (when hMPV and RSV infections are compared).

postinfection compared to those from hMPV-infected mice. The CXC chemokine KC was induced in higher amounts in mice infected with RSV compared to hMPV only at 12 h postinfection, while starting at day 3 and up to day 8 hMPV induced significant amounts of KC compared to the near-baseline levels measured in RSV-infected mice (Fig. 3). Production for CCL3 and CCL5 has been previously reported in RSV-infected mice (9, 21). Indeed, CCL3 plays a critical role in the pathogenesis of RSV disease both in the mouse model and in naturally acquired infections (7, 9). The overall profile and kinetics of chemokines for hMPV are in agreement with those recently published, although in our study levels of CCL5 and CCL3 in BAL fluids were lower (and did not reach statistical significance compared to controls) than those found in lung tissue extracts (10). KC, a mouse IL-8 homologue, is a

potent chemoattractant for neutrophils, a prominent cell type in early airway inflammation both in RSV and hMPV experimental infections (9, 10; A. Casola, unpublished data). Interestingly, hMPV-induced BAL neutrophilia in the murine model appears to be of longer duration compared to that in the RSV infection (10), perhaps due to the more prolonged KC response that we observed with hMPV. Significant levels of IL-8 have also been detected in respiratory secretions of hMPV-infected children (15).

In conclusion, we have demonstrated differential cytokine production patterns in the airways of hMPV-infected mice compared to RSV-infected mice. We have recently reported that under identical experimental conditions, including the use of sucrose-gradient purified viral preparations, volume of viral inoculum, and side-by-side infection of age and gender-matched BALB/c mice, virus titers in the lung were only 1 log lower in hMPV-infected animals than in RSV-infected ones (8). Thus, BALB/c mice appear to be fully permissive to experimental hMPV infection, resulting in pulmonary inflammation and measurable clinical disease (10; A. Casola, unpublished data). Although pathogen-specific factors are likely to affect the replication pattern of hMPV or RSV, comparison of host responses to distinct viral agents is likely to generate meaningful results to understand disease pathogenesis. This is of particular relevance in the case of hMPV and RSV, two genetically distinct pathogens which induce a similar spectrum of clinical disease in humans (5, 20). Overall, hMPV infection induced lower levels of canonical inflammatory cytokines, including IL-1, IL-6, and TNF-α, compared to RSV. On the other hand, hMPV was clearly a more potent inducer of GM-CSF and triggered a more sustained production of a key neutrophil chemoattractant (KC) in the airways. hMPV was also a stronger inducer of both IFN-α and IFN-γ responses compared to RSV. In regard to immunomodulatory cytokines, hMPV failed to induce production of IL-10 or IL-12 p70 but was a potent inducer of IL-12 p40 subunit.

The relevance of these cytokine pathways in shaping the host immune response, modulating pathogenesis of hMPV infection and ultimately causing less severe disease compared to RSV infection (31) will need further investigations. These studies are critical for developing new therapeutic strategies against hMPV infections.

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