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ORIGINAL ARTICLE

Preceding human metapneumovirus infection increases adherence of *Streptococcus pneumoniae* and severity of murine pneumococcal pneumonia

Shen-Hao Lai [a], Sui-Ling Liao [b], Kin-Sun Wong [a], Tzou-Yien Lin [a,*]

[a] *Department of Pediatrics, Chang Gung Memorial Hospital and Chang Gung University, Taoyuan, Taiwan*
[b] *Department of Pediatrics, Chang Gung Memorial Hospital and Chang Gung University, Keelung, Taiwan*

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Background: Coinfection with respiratory virus and *Streptococcus pneumoniae* has been frequently reported in several epidemiologic studies. The aim of this study was to explore the effect of preceding human metapneumovirus (hMPV) inoculation on subsequent pneumococcal infection.
Methods: Hep-2 and A549 cells were infected with hMPV then inoculated with *S. pneumoniae*. Bacterial adhesion was measured using colony forming unit and cytometric-fluorescence assays. *In vivo* bacterial adhesion was examined in hMPV-infected mice after inoculation of fluorescence-conjugated *S. pneumoniae*. Pulmonary inflammation (bacterial titers, cytokine levels, and histopathology) of hMPV-infected mice was investigated after inoculation with *S. pneumoniae*.
Results: In vitro results of bacterial infection with *S. pneumoniae* on A549 and Hep-2 monolayer cells showed that even though cellular adherence was variable among different serotypes, there was significantly enhanced bacterial adherence in A549 cells with preceding hMPV infection. In addition, *in vivo* study of hMPV-infected mice showed increased adhesion of *S. pneumoniae* on the bronchial epithelium with delayed bacterial clearance and exacerbated histopathology. Furthermore, mice with preceding hMPV infection showed repressed recruitment of airway neutrophils with decreased expression of neutrophil chemoattractants during pneumococcal infection.

* Corresponding author. Department of Pediatrics, Chang Gung Memorial Hospital, 5 Fu-Hsing Street, Kwei-Shan, Taoyuan, Taiwan.
E-mail address: pidlin@adm.cgmh.org.tw (T.-Y. Lin).

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Conclusion: These results suggest that hMPV-infected airway cells, especially the lower airway epithelium, express increased adherence with *S. pneumoniae*. Furthermore, hMPV-infected mice showed impaired recruitment of airway neutrophils, possibly leading to delayed bacterial clearance and exacerbated pulmonary inflammation, after secondary infection with pneumococcal isolates.
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Introduction

Streptococcus pneumoniae is a common cause of invasive disease and respiratory tract infections and is among the most common pathogens of community-acquired pneumonia in children.[1–3] Human metapneumovirus (hMPV) is a new member of the genus *Metapneumovirus*. hMPV was first identified in respiratory specimens obtained from young children with various respiratory syndromes,[4] and this virus is genetically and clinically similar to respiratory syncytial virus (RSV). hMPV and RSV are among the most common pathogens causing lower airway infection during infancy.[5]

S. pneumoniae can be easily isolated from the nasopharyngeal airway of healthy children.[6] Colonization of *S. pneumoniae* is common in children, and invasion into the respiratory tract requires bacterial adherence to the epithelial lining of the airway. Reports have shown that preceding or even simultaneous infections with a respiratory virus, including influenza virus, RSV, parainfluenza virus, rhinovirus, and adenovirus, occur during pneumococcal disease.[1–3] The concept of a respiratory virus predisposing to a bacterial infection was first considered during the influenza pandemic in 1918. Decreased pulmonary clearance of *S. pneumoniae* and significant lethal synergism have been observed after influenza infection in animal models,[7,8] which could be mediated by the neuraminidase on the capsid of the influenza virus.[8,9] Enhanced adherence and decreased pulmonary clearance of *S. pneumoniae* were also observed in several *in vitro* and *in vivo* studies of RSV.[10,11] It has been suggested that viral G protein expressed on infected epithelial cells might facilitate the adherence of *S. pneumoniae*.[12] It has also been observed that increasing expression of platelet-activating factor receptor on respiratory epithelial cells after RSV infection can enhance pneumococcal adhesion.[13]

Several recent publications have reported coinfection with hMPV and pneumococcus that resulted in exacerbated pneumococcal pneumonia in mice.[14–17] However, a detailed effect of hMPV infection on subsequent pneumococcal disease has not been studied. The aim of this study was to clarify how hMPV infection causes further exacerbation of pneumococcal pneumonia while focusing on its effect on bacterial adhesion and invasion. To our knowledge, this is the first report to clarify that preceding hMPV infection indeed augments *in vitro* and *in vivo* pneumococcal adhesion, which explains the underlying mechanism of decreased pulmonary clearance of *S. pneumoniae* in hMPV-infected mice.

Materials and methods

Cell cultures, hMPV, and *S. pneumoniae*

Hep-2 (ATCC CCL-23) and A549 cells (ATCC CCL-185) were used for adhesion studies. The hMPV strain CAN97-83 was obtained with kind permission from Dr Roberto P. Garofalo (University of Texas Medical Branch, Galveston, TX, USA). hMPV was propagated in LLC-MK2 cells and viral titer was determined by a cell-based immunoassay.[18] *S. pneumoniae* serotype (ST) 3 and ST14 were attained from clinical samples and resuspended to a concentration of 10^8 colony-forming units (CFU)/mL.[12] To prepare the fluorescein isothiocyanate-labeled *S. pneumoniae* (FITC-SP), the vital bacteria were mixed with FITC and resuspended in a solution at a final concentration of 10^8 CFU/mL.[10]

CFU-based adhesion assay

A549 cells and Hep-2 cells were infected with hMPV at 1.0 multiplicity of infection (MOI) for 1 hour and incubated for 48 hours. The cells were then infected with *S. pneumoniae* at 100 MOI and incubated for 30 minutes. After washing the unbound bacteria, cells were then detached with cell dissociation solution (Sigma-Aldrich, St Louis, MO, USA) and resuspended. CFU of the original bacterial suspension (total CFU) and of the suspension after the adherence assay (final CFU) were estimated by the CFU-counting method.[10] The bacterial concentration (CFU/mL) was calculated as CFU $\times$ dilution $\times$ 10 (10 μL/spot). Adherence percentages were calculated as final CFU/total CFU $\times$ 100.

Cytometric fluorescence adhesion assay and microscopic visualization of adhesion of FITC-SP

A549 cells in plates were infected with hMPV at 1.0 MOI for 1 hour and incubated for 48 hours. FITC-SP ST14 at 25 MOI was then added after removing medium from hMPV-infected or uninfected monolayers cells. The plates were spun to facilitate attachment of bacteria, then incubated for 30 minutes. Cells were detached with cell dissociation solution and fixed with formaldehyde. Cells adhered with FITC-SP were counted and analyzed with a FACSCalibur instrument using CellQuest (BD Bioscience, San Jose, CA, USA) software.

To visualize the adhesion of FITC-SP on cells, A549 cells were inoculated with hMPV and subsequently with FITC-SP ST14 as described above. Cells were then fixed and observed under inverted confocal microscopy (TCS SP2 Leica Microsystems, Buffalo Grove, IL, USA).

Experimental infection protocol in mice

Female 4-week-old BALB/c mice, 14 ± 2 g in weight, were used for the *in vivo* experiments. The Animal Center of Chang Gung Memorial Hospital approved all experiments. Mice were inoculated with hMPV or phosphate-buffered saline (PBS) 5 days before infection with *S. pneumoniae*. For hMPV infection, each mouse was inoculated with 1.5×10^6 plaque-forming units of the virus. For *S. pneumoniae* infection, mice were infected with 1×10^6 CFU of *S. pneumoniae* ST14. Mice were killed 24 hours or 48 hours after *S. pneumoniae* infection, and analytic measurements were then performed.

Localization of FITC-SP in respiratory epithelium

Mice were initially infected with hMPV or PBS as control and inoculated with FITC-SP 5 days later. One hour after FITC-SP inoculation, mice were killed and the lung was lavaged with 1 mL PBS three times before the lungs were collected and fixed with 3.7% buffered formaldehyde. The fixed lungs were embedded in paraffin, sectioned in 5-μm slices, and observed under fluorescence microscopy (DX50 Olympus, Tokyo, Japan) to visualize *in vivo* adherence of *S. pneumoniae* on the respiratory epithelium.

Lung bacterial burden and bronchoalveolar lavage

Infected lungs were removed, weighed, and homogenized. After serial dilutions, 100-mL aliquots of homogenate were quantified for bacterial counts. Data are expressed as CFU/g of lung.

Cellular counts and differential cell counts were determined in bronchoalveolar lavage (BAL) fluid after being stained with Liu's stain. Supernatants were stored for subsequent analysis of chemokines and cytokines.

Histopathology

For histopathologic analysis, lungs were collected on Day 1 or 2 after pneumococcal infection. Multiple 4-μm sections were stained with hematoxylin and eosin. Slides were analyzed and scored for tissue damage in a blinded fashion under light microscopy as previously described.[18] Inflammatory infiltrates were scored by enumerating the layers of inflammatory cells surrounding the vessels and bronchioles. The number of abnormal perivascular and peribronchial spaces was divided by the total number of perivascular and peribronchial spaces and reported as a percentage to provide a pathology score.

Cytokine and chemokine measurement

Chemokines and cytokines in BAL fluids were measured by enzyme-linked immunosorbent assay according to the manufacturer's instructions. Reagents for interleukin (IL)-1α, IL-10, interferon-γ, and cytokine-induced neutrophil chemoattractant (KC) measurements were obtained from R&D Systems (Minneapolis, MN, USA), and tumor necrosis factor-α (TNF-α) and granulocyte–macrophage

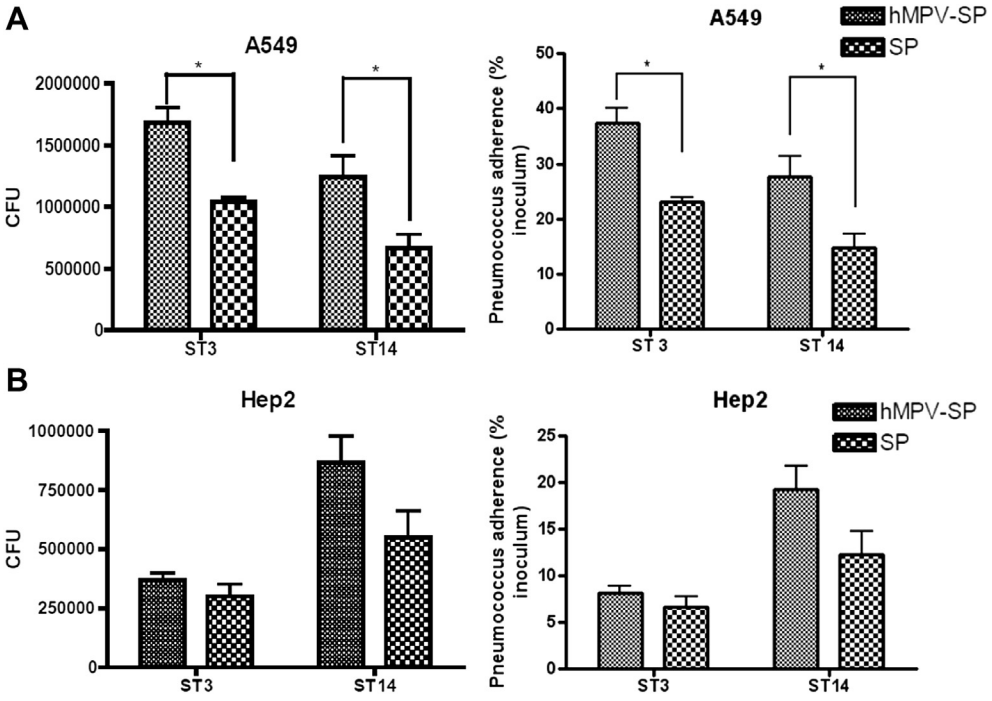

Figure 1. Adherence of *Streptococcus pneumoniae* on human metapneumovirus (hMPV)-infected (hMPV-SP) or non–hMPV-infected (SP). (A) A549; (B) Hep-2 cells. The figure shows the mean and standard deviation (positive value) of absolute colony-forming units (CFU) and inoculum percentage of adhering *S. pneumoniae*/10^7 CFU of inoculum. *$p < 0.05$ between two groups ($n = 6$).

colony-stimulating factor reagents were obtained from BD Biosciences (San Jose, CA, USA).

Statistical analysis

Statistical analyses were performed using the InStat3.05 biostatistics package (GraphPad, San Diego, CA, USA) and a two-tailed, unpaired, Mann—Whitney *U* test. Unless otherwise indicated, the mean value ± standard deviation is shown.

Results

hMPV infection increased *in vitro* and *in vivo* cellular adhesion of *S. pneumoniae*

In A549 cells, adhesion of *S. pneumoniae* was significantly increased in hMPV-infected cells (Fig. 1A). The mean percentage of adhering *S. pneumoniae* ST3 on hMPV-infected and non—hMPV-infected A549 cells was 37.4% and 23.1%, respectively ($p < 0.05$, Fig. 1A) and of *S. pneumoniae* ST14 was 33.6% and 13.5%, respectively ($p < 0.05$). In Hep-2 cells, although there was an increase in pneumococcal adhesion in hMPV-infected cells as well (Fig. 1B), the increment was not as striking as that of A549 *cells*.

S. pneumoniae was conjugated with FITC to enable visualization of the amount of bacterial adherence on respiratory epithelial cells. Results from flow cytometry showed that the positive FITC-signal ratio of hMPV-infected A549 cells was higher than that of noninfected cells (42.9 vs. 19.5%; Fig. 2A). Similar results were observed from confocal microscopy, showing an intense and condensed fluorescence pattern in hMPV-infected cells. In contrast, noninfected cells presented with a more discrete and sparse pattern (Fig. 2B).

To analyze the adherence of *S. pneumoniae in vivo*, we infected mice with FITC-labeled *S. pneumoniae* ST14 five days after hMPV infection. Under fluorescence microscopy, intense fluorescence signals were shown in the bronchial epithelial cells of hMPV-infected mice, but only a few signals were found in the non—hMPV-infected mice (Fig. 3). However, lower in the distal airway, the bronchiolar epithelium showed little fluorescence in both hMPV-infected and noninfected mice.

Decreased recruitment of airway neutrophils after *S. pneumoniae* infection in mice with preceding hMPV infection

Mice were initially inoculated with hMPV or PBS as controls and subsequently infected with *S. pneumoniae* ST14 at Day 5 postinoculation. The total cell count in BAL fluid was higher at 24 hours than at 48 hours postinfection ($p < 0.05$, Fig. 4A). However, the total number of cells in the BAL fluid

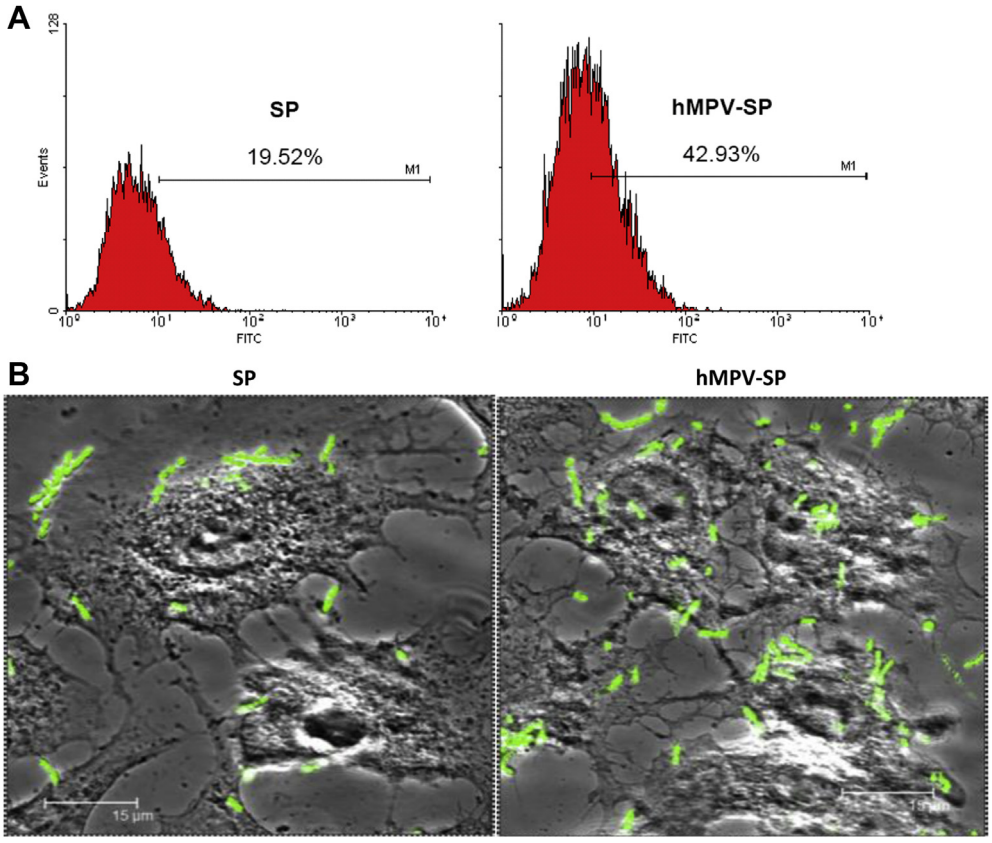

Figure 2. Adherence of fluorescein isothiocyanate-labeled (FITC)-conjugated *Streptococcus pneumoniae* ST14 on human metapneumovirus (hMPV)-infected (hMPV-SP) or non-hMPV-infected (SP) A549 cells. (A) Using flow cytometry, the percentage of A549 cells with adhered FITC-conjugated *S. pneumoniae* is shown. (B) The adhered FITC-conjugated *S. pneumoniae* over the surface of A549 cells were visualized using confocal microscopy.

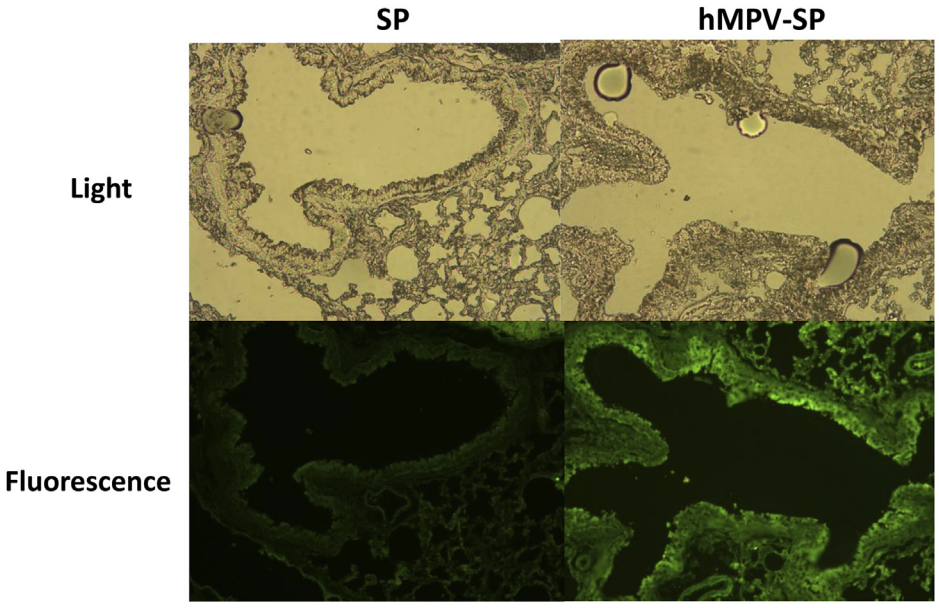

Figure 3. Adherence of fluorescein isothiocyanate-labeled (FITC)-conjugated *Streptococcus pneumoniae* on murine bronchial epithelium. Mice were infected with human metapneumovirus (hMPV-SP) or phosphate buffered saline (SP) and 5 days later, were inoculated with FITC-conjugated *S. pneumoniae* ST14. Under 20× magnification of light and fluorescence microscopy, enhanced adherence of FITC-conjugated *S. pneumoniae* was visualized on bronchial epithelium of hMPV-SP mice.

of hMPV-infected mice (both from 24 hours and 48 hours post infection) was significantly higher than that of mice without hMPV infection ($p < 0.05$, Fig. 4A). Further analysis of the cellular differentials showed that the amount of neutrophils recruited from hMPV-infected mice was significantly lower than that of PBS-inoculated mice ($p < 0.05$, Fig. 4B).

Decreased bacterial clearance and deteriorated histopathology in lungs of mice with previous hMPV infection

Mice with preceding hMPV infection showed significantly decreased clearance of *S. pneumoniae* in the lungs at 24 hours postinfection ($p < 0.001$). Although clearance of the bacteria showed much improvement by 48 hours, the total bacterial counts in the lungs of pre-hMPV-infected mice were still higher than the counts of those that received PBS alone (Fig. 5A).

Pulmonary histopathologic showed that the lungs with preceding hMPV infection exhibited profound inflammation in the peribronchial and perivascular area (Fig. 5B). The histopathologic scores of the peribronchial and perivascular area in mice with hMPV infection followed by *S. pneumoniae* infection were also significantly higher (Fig. 5C). The severity of inflammation was greater at 48 hours after *S. pneumoniae* infection (hMPV-SP vs. SP: peribronchial area, 87% vs. 75%; perivascular area, 72% vs. 57%).

Decreased levels of airway proinflammatory cytokines after *S. pneumoniae* infection in mice with previous hMPV infection

In general, levels of airway cytokine/chemokine significantly waned at 48 hours after pneumococcal inoculation (Fig. 6). At either 24 hours or 48 hours after pneumococcal infection, IL-1α, KC, and granulocyte—macrophage colony-stimulating factor expression of mice with preceding hMPV infection was significantly lower than that of mice without hMPV infection. Airway IL-10 level of mice with previous hMPV infection was also lower than that of mice without hMPV infection at 24 hours after *S. pneumoniae* infection (Fig. 6).

Discussion

Using the most pathogenic pneumococcal serotypes (ST3 and ST14), this study showed that hMPV-infected A549 cells expressed increased bacterial adhesion after *S. pneumoniae* inoculation. However, the increase in adherence was not as significant in Hep-2 cells. A similar cell type-dependent manner was previously observed.[13] A549 and Hep-2 cells are transformed cell lines of type II alveolar and nasopharyngeal carcinoma cells, respectively. Thus, A549 cells are more analogous to the epithelial cells of the lower airway in humans. Therefore, the results indicate the possibility that enhanced bacterial adherence caused by preceding hMPV infection is more prominent in the lower airway.

Using flow cytometry in a fluid phase, increased adherence of *S. pneumoniae* was found in A549 cells inoculated with hMPV. Furthermore, to create a more physiologic condition, a cytometric fluorescence assay was used to explore the efficacy of adherence of *S. pneumoniae* in live monolayer A549 cells. Increased bacterial clumps on hMPV-infected A549 cells were visualized under confocal microscopy. Using live FITC-labeled *S. pneumoniae*, we further showed that the bronchial epithelium of hMPV-infected mice was more susceptible to adherence by

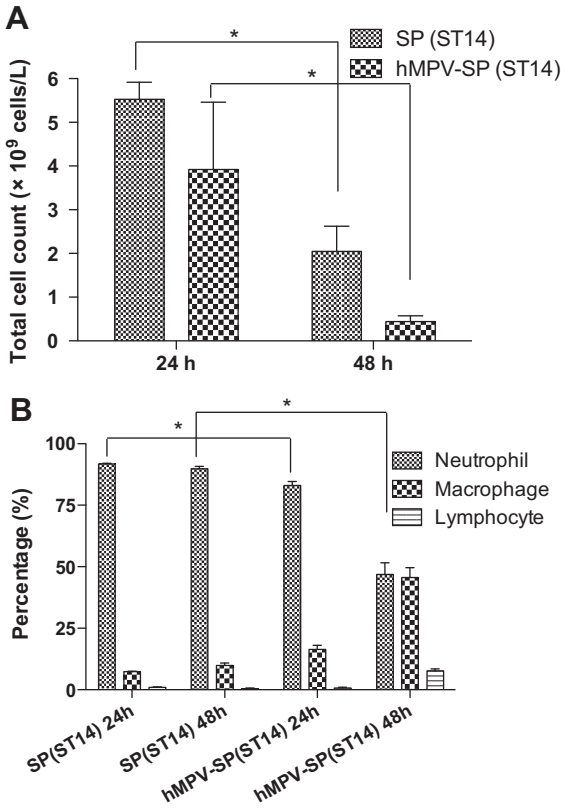

Figure 4. Pulmonary cellular infiltration after *Streptococcus pneumoniae* infection. Mice were infected with human metapneumovirus (hMPV-SP) or phosphate buffered saline (SP) at first and then with *S. pneumoniae* ST14 5 days later. bronchoalveolar lavage was performed at 24 hours and 48 hours after *S. pneumoniae* infection. Mean and standard deviation (positive value) of (A) total and (B) differential cell count in bronchoalveolar lavage fluid are presented. Lower total cell count and lower neutrophil ration were revealed in hMPV-SP mice. $^*p < 0.05$ between 2 groups ($n = 4$—6).

S. pneumoniae. These findings indicated that, with preceding hMPV infection, the adhering bacterial load of *S. pneumoniae* was increased in the host.

Kukavica-Ibrulj et al reported that prior infection with hMPV predisposes mice to exacerbated pneumococcal infection.[17] Their report also showed a profound increase in lung bacterial titer, up to 1000—10,000-fold, 72 hours after *S. pneumoniae* infection. In our report, the increment of bacterial titers was not that high, waned dramatically at 48 hours postinfection, and almost completely vanished at 72 hours postinfection (data not shown). This may be because *S. pneumoniae* ST14, instead of ST3, was used in our experiments in mice. It has been shown that distinct activation of immune responses was elicited by different strains of *S. pneumoniae*.[19,20] *S. pneumoniae* ST3 can cause delayed bacterial clearance and induce stronger host immune reactions in murine lung.[19] Indeed, the inflammatory responses of the host depend critically on the specific strain of *S. pneumoniae*.[20]

According to our unpublished data and previous study,[21] the cytokine/chemokine responses elicited by hMPV declined dramatically at Day 3 after infection. In Fig. 6, comparable levels of cytokine/chemokine were also revealed between mice with hMPV infection alone at Day 6 postinfection (hMPV group at 24 hours) and those at Day 7 postinfection (hMPV group at 48 hours).

Efficient recruitment of neutrophils and macrophages plays an important role in the bacterial clearance of pulmonary pneumococcal infection.[22] Impaired recruitment of neutrophils in the lung can lead to disturbances of the host's immunity to defend against *S. pneumoniae* infection.[23,24] IL-1α plays an important role in the stimulation of neutrophil chemokines after bacterial infection,[25,26] and granulocyte-macrophage colony-stimulating factor is known as a priming factor and chemoattractant of neutrophils.[27] It appears that IL-1α and TNF-α play overlapping roles in the innate response to *S. pneumoniae* infection.[28] In mice with preceding influenza infection, impaired expression of neutrophil chemokines (KC and MIP-2) might contribute to decreased recruitment of neutrophils after secondary challenge with *S. pneumoniae*.[29] Expression of type I interferon, an antiviral response induced by influenza infection, was believed to contribute to the inhibition of neutrophil chemoattractants.[29] In this study, robust reduction of neutrophil chemoattractants (IL-1α, granulocyte-macrophage colony-stimulating factor, and KC) except for TNF-α was found in mice with preceding hMPV infection. These results may contribute to the impaired recruitment of pulmonary neutrophils. However, the role of type I interferon in the mechanism of post-hMPV pneumococcal pneumonia needs to be addressed further.

The exact mechanism of viral infection enhancing subsequent bacterial adherence is still poorly understood. In postinfluenza pneumococcal pneumonia, upregulation of platelet-activating factor receptor and IL-10 was found to be induced by influenza virus.[8,30] Van der Sluijs et al reported that virally infected mice recovering from pneumococcal infection had 50-fold higher levels of IL-10 in the lung than with controls.[30] In their study, the high lethality was markedly reduced by pretreating the animals with anti-IL-10 monoclonal antibody. IL-10 is known to inhibit the expression of major histocompatibility complex class II and costimulatory molecules and reduce the production of pro-inflammatory cytokines.[31] However, on the contrary, our results showed lower levels of pulmonary IL-10 and IL-1α in mice with post-hMPV pneumococcal pneumonia when compared with mice with only pneumococcal infection. Thus, we suggest that in contrast to the role of IL-10 in postinfluenza pneumococcal pneumonia, IL-10 seems to play a less important role in post-hMPV pneumococcal pneumonia.

Some notable limitations of this study should be addressed. First, only two clinical serotype isolates were tested. Future studies will need to test other common clinical isolates to confirm the findings of bacterial adherence. Second, this study showed inhibited recruitment of pulmonary neutrophils after hMPV infection but was unable to confirm whether this effect resulted directly from enhanced bacterial proliferation.

In summary, with preceding hMPV inoculation, an increased adherence of *S. pneumoniae* was seen in *in vitro* airway epithelial cells. A similar finding was noted in the bronchial epithelium of hMPV-infected mice, which resulted in enhanced bacterial proliferation and exacerbated

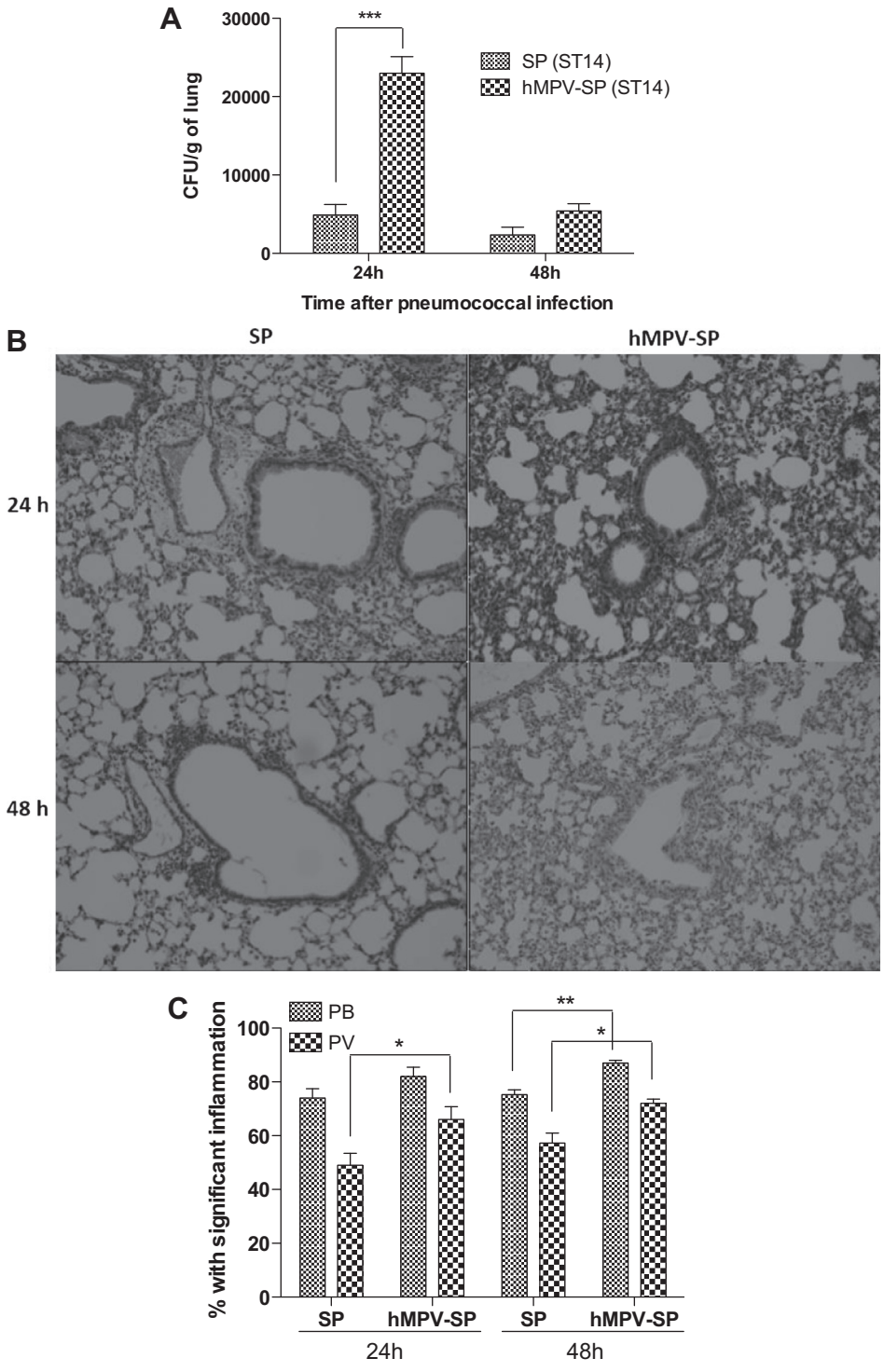

Figure 5. Bacterial loads and histopathologic evaluation of the lungs of mice after *Streptococcus pneumoniae* infection. Mice were infected with human metapneumovirus (hMPV-SP) or phosphate buffered saline (SP) at first and then 5 days later with *S. pneumoniae* ST14. (A) The mean and standard deviation (positive value) bacterial loads in the lung determined at 24 hours and 48 hours after *S. pneumoniae* infection. (B) Histopathologic evaluation of the lung with 20× magnification. CFU = colony-forming units. (C) The severity of lung inflammation is scored for peribronchial (PB) and perivascular (PV) and represented as mean and standard deviation (positive value). Delayed bacterial clearance and more severe histopathology were found in lungs of hMPV-SP mice. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ between 2 groups ($n = 4–6$).

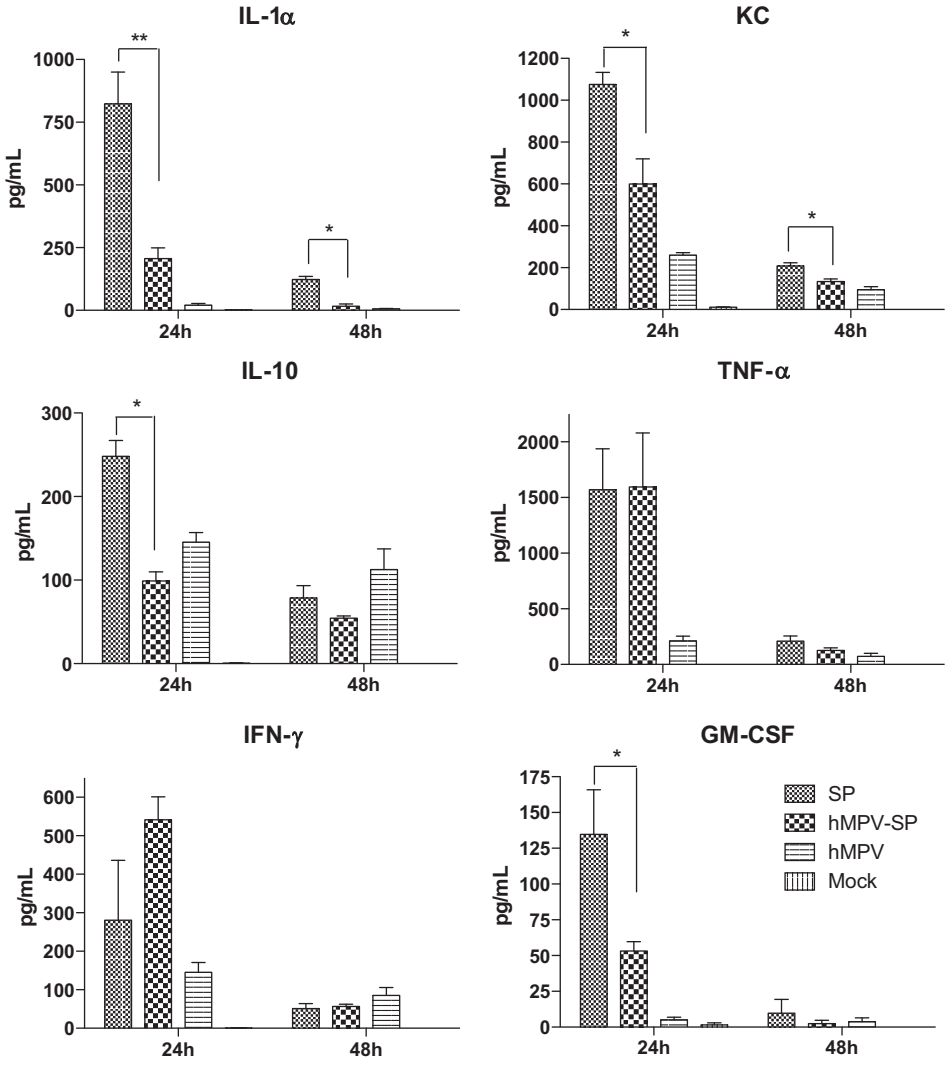

Figure 6. Cytokine and chemokine levels in lungs of mice after *Streptococcus pneumoniae* infection. Mice were inoculated with human metapneumovirus (hMPV) or phosphate buffered saline (PBS) at first and then 5 days later, with *S. pneumoniae* ST14 or PBS. The figure presents the mean and standard deviation (positive value) values of BAL fluid collected at 24 hours and 48 hours after *S. pneumoniae* infection. hMPV-SP and hMPV represent mice with preceding hMPV infection and then *S. pneumoniae* or PBS inoculation, respectively. SP and Mock represent mice with preceding PBS inoculation and then *S. pneumoniae* or PBS inoculation, respectively. *$p < 0.05$, **$p < 0.01$ between 2 groups ($n = 4–5$). GM-CSF = granulocyte-macrophage colony-stimulating factor; IL = interleukin; INF = interferon; KC = cytokine-induced neutrophil chemoattractant; TNF = tumor necrosis factor.

histopathology after pneumococcal infection. In addition, we suggest that deteriorated lung disease in hMPV-infected mice might partially result from failure of recruitment of neutrophils after pneumococcal infection.

Conflicts of interest

All contributing authors declare no conflicts of interest.

Acknowledgments

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