

ORIGINAL ARTICLE

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Effect of Influenza Virus Infection in a Murine Model of Asthma

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ABSTRACT

Respiratory virus infection is a major cause of asthma exacerbation. However, the underlying mechanisms of this exacerbation are unknown. Therefore, to determine the mechanisms, we examined the effect of influenza infection in a murine model of asthma.

Mice were divided into four groups: the phosphate-buffered saline (PBS), house dust mite (HDM), influenza, and HDM/influenza groups. The influenza group and the HDM/influenza group were infected with influenza A virus. We measured airway resistance (Penh value), examined the lung tissue for pathology, and analyzed the cells and cytokines in bronchoalveolar lavage fluid (BALF) by ELISA.

At 50 mg/mL methacholine, the HDM/influenza group showed a significantly higher Penh value than the PBS, HDM, and influenza groups. The number of neutrophils in BALF was higher in the HDM/influenza group than in the HDM group. A significantly greater number of lymphocytes and macrophages were detected in the HDM/influenza group than in the HDM group. IFN- γ and IL-1 β levels were higher in the HDM/influenza group than in the HDM group. IL-5 levels did not vary between the HDM and HDM/influenza groups, IL-10 was significantly lower in the HDM/influenza than in the HDM group. Chemokine (C-X-C motif) ligand 1 (CXCL1) and regulated upon activation, normal T cell expressed and secreted (RANTES) were higher in the HDM/influenza group than in the HDM group.

In a murine model of asthma, influenza-induced airway inflammation appeared to be caused by simultaneous activation of neutrophilic and eosinophilic inflammation.

Keywords: Asthma; Cytokine; Eosinophil; Histology; Influenza; Mouse; Neutrophil

INTRODUCTION

Asthma exacerbations are associated with shortness of breath, coughing, wheezing, and chest tightness, and are accompanied by decreased expiratory airflow manifested by a reduction in peak expiratory flow.

Asthma exacerbations are related to several factors, including allergen exposure, air pollution, and stress; however, the major cause of exacerbation is respiratory virus infection.¹

Much of our knowledge regarding the mechanisms of virus-induced asthma exacerbations is derived from

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of experimental respiratory virus infection in asthmatic volunteers as a model of exacerbation.² Despite the abundant epidemiologic evidence linking respiratory virus infection to asthma exacerbation, the cellular and molecular mechanisms by which these viruses cause exacerbations are not yet known. In a previous experimental study using animal models of asthma, cytokine release by airway epithelial cells after viral infection led to an influx of inflammatory cells. These inflammatory cells release products, such as neutrophil elastase and reactive oxygen species that can cause tissue damage, stimulate mucus production, and further stimulate cytokine production.³

Most of these experimental studies used rhinovirus (RV). However, the pathophysiological changes that occur following infection differ according to virus type. For example, influenza typically causes extensive epithelial necrosis, whereas RV causes only patchy damage of epithelial cells.⁴

In this study, we aimed to determine the effect of influenza infection in a murine model of asthma, and we assessed airway resistance, lung tissue pathology, and the changes in bronchoalveolar lavage fluid (BALF) cells and cytokines (IFN- γ , IL-1 β , IL-5, IL-10, CXCL1, and RANTES) to investigate the mechanism of acute asthma exacerbation induced by influenza virus infection. We compared our results to those in previous studies of influenza and other respiratory viruses.

MATERIALS AND METHODS

Test Animal Groups

For comparison, the test animals were divided into four groups, the phosphate buffered saline (PBS) group, house dust mite (HDM)-exposed group, influenza group, and a group of HDM-exposed mice infected with influenza virus (HDM/influenza). A total of six experiments with 5–8 mice per group were performed.

Sensitization and Allergen Challenge

BALB/c mice aged 6–8 weeks (Orient Bio INC, Gyeonggi-do, Korea) were sensitized and challenged with *Dermatophagoides farinae* crude extract (*D. farinae*; Arthropods of Medical Importance Resource Bank, Seoul, Korea) or PBS. For sensitization, mice in the HDM and HDM/influenza groups were injected intraperitoneally with 200 μ L of a 2-mg/mL solution of

aluminum potassium sulfate and 250 μ g of a crude extract of *D. farinae* or PBS on day 0 and day 14.

For the challenge, mice were administered 50 μ g of a crude extract of *D. farinae* (0.25 mg/mL) intranasally in 50 μ L of PBS on days 14, 15, and 16. The mice were treated with a mixture of aluminum hydroxide (alum) and *D. farinae* crude extract to enhance the immunogenicity of the allergen. We used PBS as a control in all procedures. Successful induction of the mouse asthma model was assessed by determining the total number of cells and eosinophils in BALF and examining lung histopathology. All experimental animal protocols were reviewed by the Committee for Ethics on Animal Experiments of the Catholic University.

Viruses and Infection of Mice

The mouse-adapted influenza A (H1N1) virus was provided by the Korea Bank for Pathogenic viruses (Seoul, Korea) and propagated in monolayer cultures of HeLa cells. On day 17, the influenza A (IA) virus was administered intranasally at a concentration of 3×10^4 fifty percent tissue culture infectivity doses (TCID₅₀)/ μ L; the TCID₅₀ was determined by the Spearman-Kärber method. The mice were euthanized, and samples were collected on day 18 (post-infection day 1) (Figure 1).

Measurement of Airway Hyper-responsiveness

We measured airway responsiveness to methacholine in all four groups of mice. For the HDM/influenza group, we measured airway responsiveness 24 hours after IA virus infection. Airway responsiveness was measured using four different concentrations of methacholine (0 mg/mL, 12.5 mg/mL, 25 mg/mL, and 50 mg/mL) using an OCP3000 instrument (Allmedicus, Anyang, Korea). Methacholine was administered over a period of 3 minutes using an Ultra-Neb ultrasonic nebulizer (3650p; Pulmo-Aide LT Lompressor, Somerset, PA, USA). The enhanced pause (Penh value) variable was measured over a period of 3 minutes.

Bronchoalveolar Lavage (BAL) Procedure

BALF was collected 24 hours after IA virus infection. The BAL was performed by inserting a 22-gauge needle into the trachea. Then, the trachea was ligated and the entire lung was lavaged by instilling 1 mL of PBS. The BALF was harvested once

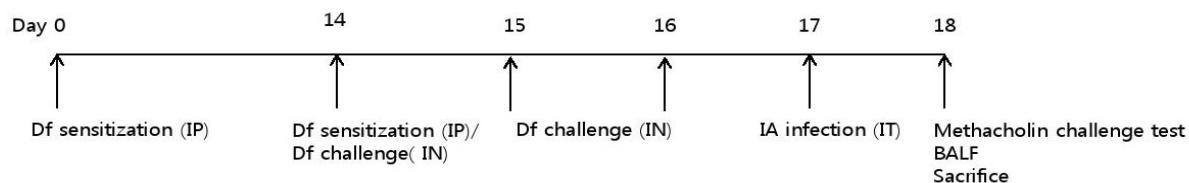


Figure 1. Study design: *Dermatophagoides farinae* (Df)- sensitized mice were immunized with Df and aluminum adjuvant by intraperitoneal injection (IP) once on days 0 and 14. Two weeks later, these mice were inoculated intranasally (IN) once daily with Df for 3 consecutive days (days 14 to 16). Df- sensitized mice were infected with influenza A virus (IA virus) 24 hours after the final Df inoculation. Penh values and cytokine levels in bronchoalveolar lavage (BAL) fluid were measured 24 hours after IA virus infection. IT, intratracheally; IP, intraperitoneal; IT, intratracheal; IN, intranasal.

and centrifuged at $387 \times g$ (2000 rpm) for 10 minutes at 4°C . Supernatants were stored at -70°C .

Assessment of Cells in the BALF

Cell precipitates were suspended in 0.4 mL of PBS, and a 10- μL aliquot was mixed with 10 μL of trypan blue solution (Gibco, Grand Island, NY, USA). The total number of cells in each sample was counted using a hemocytometer. Slides were prepared using a cytocentrifuge (Cytospin 2; Shandon, Runcorn, UK) and stained with Diff-Quick (Sysmex Corporation, Kobe, Japan). Macrophages, eosinophils, lymphocytes, and neutrophils (500 minimum) were counted at $400\times$ magnification.

Measurement of Cytokines and Chemokines in the BALF and Lung Homogenate

Interferon (IFN)- γ , interleukin (IL)-1 β , IL-5, IL-10, chemokine (C-X-C motif) ligand 1 (CXCL1), and regulated upon activation normal T cell expressed and secreted (RANTES) concentrations in the supernatant of the BALF were determined using an enzyme linked immunosorbent assay (ELISA) kit (R&D systems, Inc., Minneapolis, MN, USA), according to the manufacturer's protocol.

Histopathology

We conducted a histopathological analysis of the lung sections of mice in the four groups 24 hours after the final *D. farinae* challenge. Once the washing was complete, the lungs were immediately removed. The removed lung tissues were fixed in 4% paraformaldehyde (Wako pure chemical industries, Ltd., Osaka, Japan) for 24 hours and then embedded in paraffin. The fixed tissues were cut into 5- μm -thick sections and were stained with hematoxylin and eosin (H&E). The sections were observed by light

microscopy to evaluate the degree of airway inflammation. Pathological changes were described using a previously described system with some modification.^{5,6} The degree of peribronchial and perivascular inflammation was evaluated on a subjective scale of 0 to 3. A value of 0 was assigned when no inflammation was detected, a value of 1 was assigned for occasional cuffing with inflammatory cells, a value of 2 was assigned for most bronchi or vessels surrounded by a thin layer (1–5 cells) of inflammatory cells, and a value of 3 was assigned when most bronchi or vessels were surrounded by a thick layer (>5 cells) of inflammatory cells.

Statistical Analysis

All results are shown as mean \pm standard error of the mean (SEM). The data were analyzed with the Kruskal-Wallis test for comparing continuous non-parametric variables in 3 groups or more and the Mann-Whitney test for comparing continuous non-parametric variables in 2 groups. Statistical significance was assessed by using SPSS v. 11.5 (SPSS Inc., Chicago, IL, USA). *P* values less than 0.05 were considered statistically significant.

RESULTS

Increased Airway Resistance

We measured the Penh value using four different concentrations of methacholine. At 50 mg/mL methacholine, the HDM group showed a higher Penh value than the PBS group ($p=0.045$).

At 50 mg/mL methacholine, the HDM/influenza group showed a significantly higher Penh value than the other three groups (PBS, HDM, and influenza, $p < 0.05$; Figure 2).

Analysis of the Inflammatory Cells in BALF

The HDM/influenza group had a significantly higher number of eosinophils than the PBS and influenza groups ($p < 0.001$ and $p < 0.001$). The HDM/influenza group had more neutrophils than the PBS group ($p = 0.003$). The HDM/influenza group had a significantly higher number of lymphocytes than the PBS, HDM, and influenza groups ($p < 0.001$, $p = 0.003$, and $p = 0.001$, respectively). The HDM/influenza group had more macrophages than the control and HDM groups ($p < 0.001$ and $p = 0.003$, respectively; Figure 3).

Cytokine and Chemokine Secretion upon Airway Inflammation

We measured the levels of cytokines and chemokines in the four groups of mice using ELISA. Levels of the cytokines IFN- γ , and IL-1 β were significantly higher in the HDM, influenza, and HDM/influenza groups than in the PBS group ($p < 0.05$). The Th2 inflammation cytokine, IL-5, was higher in the HDM/influenza group than in the PBS group ($p = 0.005$)

and was also higher in the HDM/influenza group than in the influenza group ($p = 0.042$). The regulatory T-cell-related cytokine, IL-10, was lower in the HDM/influenza group than in the HDM group ($p = 0.007$). The neutrophil-related chemokine CXCL1 was higher in the HDM/influenza group than in the HDM group ($p < 0.001$), and the eosinophil-related chemokine RANTES was higher in the HDM/influenza group than in the PBS group ($p = 0.012$, Figure 4).

Histological Analysis of Lungs

Compared to the PBS group, there were histological changes in the bronchial wall, lung parenchyma, interstitium, and the pulmonary vasculature in the HDM, influenza, and HDM/influenza groups (Figure 5).

There were no pathologic changes in the H&E-stained lung sections from the PBS group. Compared to the PBS group, the HDM/influenza group showed greater inflammatory cell infiltration, interstitial inflammation, and thickening of the alveolar wall.

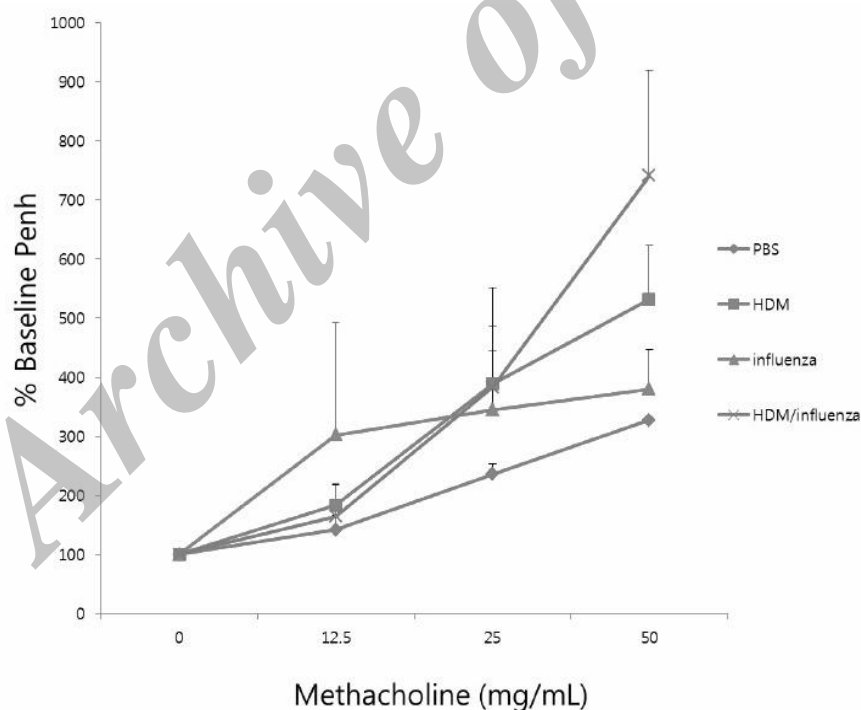


Figure 2. Airway responsiveness determined by measurement of Penh (n=6 mice per group). First bar in each group is at 0 mg/ml concentration of methacholine, second bar in each group is 12.5 mg/ml concentration of methacholine, third bar in each group is 25 mg/ml concentration of methacholine, and fourth bar in each group is 50 mg/ml concentration of methacholine.

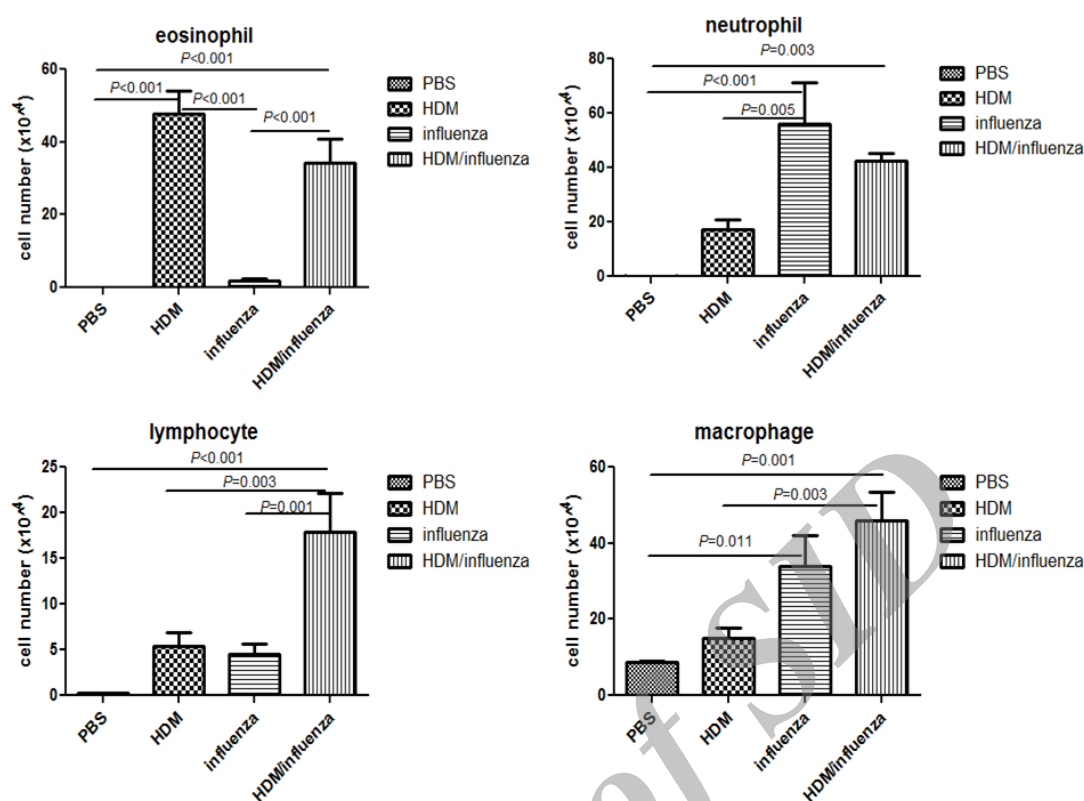


Figure 3. Differential cell counts of neutrophils and eosinophils, lymphocyte, and macrophage in bronchoalveolar lavage fluid (n=6 mice per group).

Compared to the influenza group, the HDM/influenza group showed denudation, dense infiltration of inflammatory cells in the bronchial lining epithelium, and a higher grade of vascular congestion and interstitial widening. Compared to the HDM group, in the HDM/influenza group, a higher grade of multifocal infiltration of various cells, such as lymphocytes, macrophages, plasma cells, and eosinophils was observed in the interstitium. Thus, the HDM/influenza group showed the most severe grade of inflammation.

The HDM/influenza group also had higher eosinophil and neutrophil counts and more prominent neutrophil infiltration than the HDM and influenza groups.

The peribronchial inflammatory scores of the HDM, influenza, and HDM/influenza groups were higher than that of the PBS group. When each group was scored according to the level of eosinophil and neutrophil infiltration, the HDM/influenza group showed proportionally higher neutrophil infiltration than eosinophil infiltration (Table 1).

Table 1. Histological scoring of peribronchial inflammation at day 18 (n = 6 mice per group).

Variables	PBS	HDM	Influenza	HDM/influenza	P value
Cellular Infiltration	0	1.00 ± 0	0.333 ± 0.577	2.667 ± 0.577	0.021
Eosinophil Infiltration	0	1.00 ± 0	0	0.333 ± 0.577	0.041
Neutrophil Infiltration	0	1.00 ± 0	0	2.333 ± 1.155	0.031

Data are expressed as mean ± standard error of the mean.

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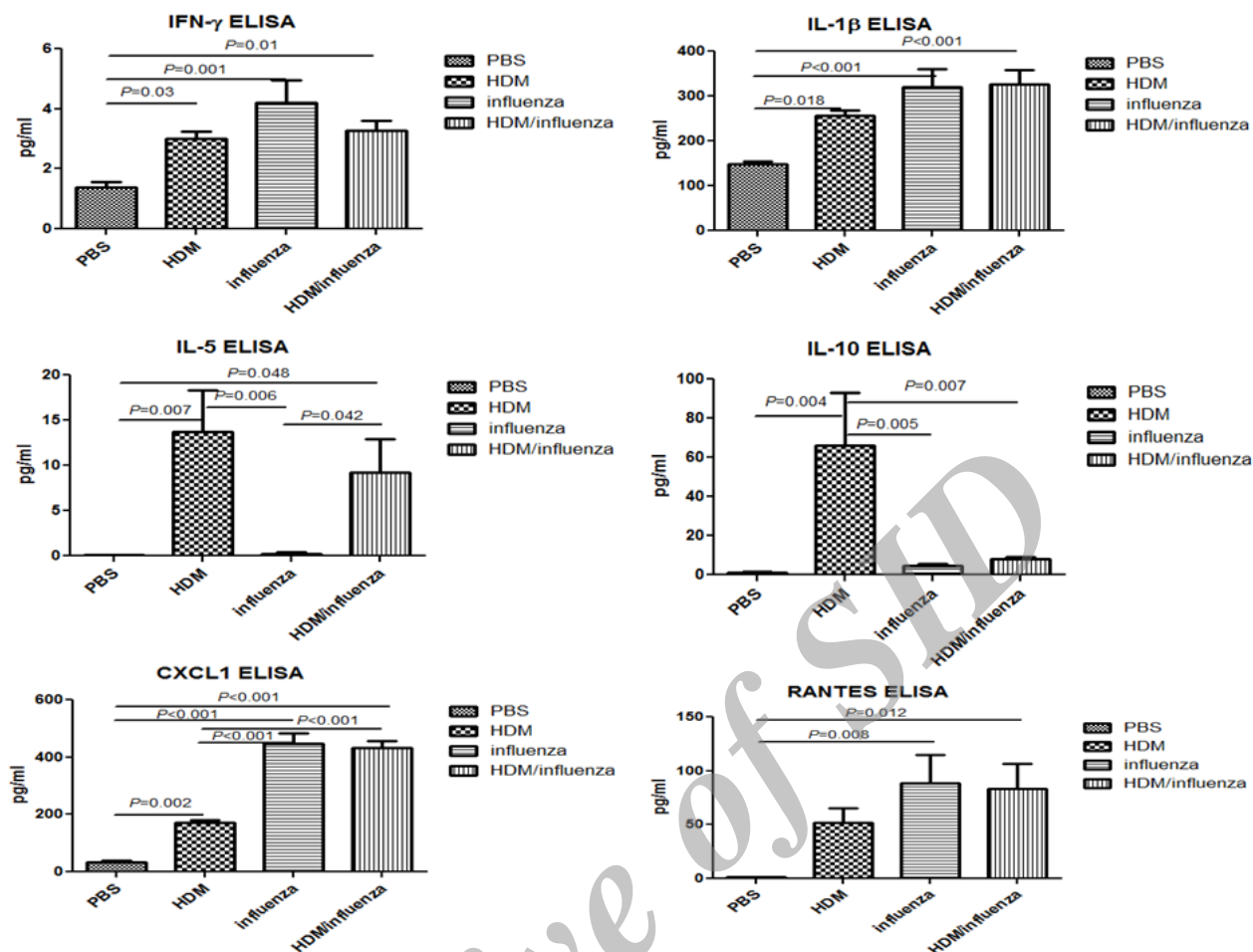


Figure 4. Concentrations of cytokines and chemokines in BALF (n=6 mice per group), IFN: interferon, IL: interleukin, ELISA: enzyme linked immunosorbant assay, CXCL: chemokine ligand, RANTES: regulated upon activation, normal T cell expressed and secreted, BALF: bronchoalveolar lavage fluid.

The perivascular inflammatory scores of the HDM, influenza, and HDM/influenza groups were higher than that of the control (PBS) group. When each group was scored according to the level of eosinophil and

neutrophil infiltration, in the HDM/influenza group, neutrophil infiltration was higher than eosinophil infiltration (Table 2).

Table 2. Histological scoring of perivascular inflammation at day 18 (n = 6 mice per group).

Variables	PBS	HDM	Influenza	HDM/influenza	P value
Cellular infiltration	0	2.667 \pm 0.577	1.000 \pm 0	3.000 \pm 0	0.015
Eosinophil infiltration	0	1.00 \pm 0	0	0	0.012
Neutrophil infiltration	0	2.000 \pm 0	0.333 \pm 0.577	2.000 \pm 0	0.015

Data are expressed as mean \pm standard error of the mean.

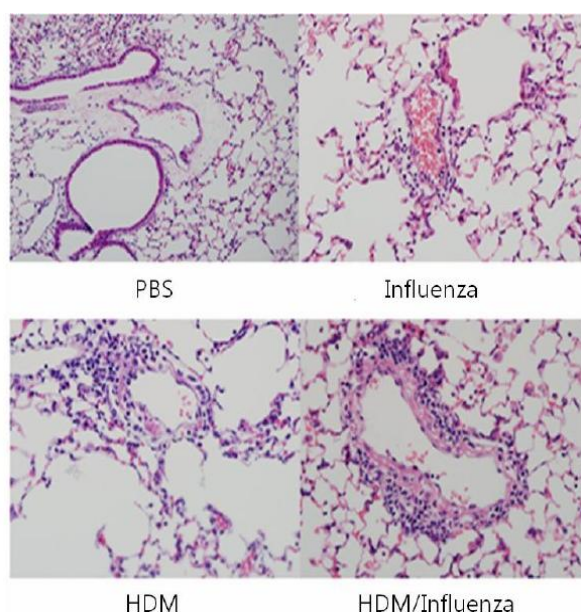


Figure 5. Histopathological changes in lungs of 4 groups. (H&E staining of lung sections; original magnification 400×) HDM/influenza group showed the most severe grade of inflammation compared to the HDM group and the influenza group. The HDM/influenza group also had higher eosinophil and neutrophil counts, together with prominent neutrophil infiltration compared to the HDM group and the influenza group.

DISCUSSION

In this study, we aimed to investigate the effect of influenza infection in a murine model of asthma, and we analyzed airway resistance, lung tissue pathology, and the changes in the cells and cytokines in BALF to determine the mechanism underlying these effects. We found that the mechanism underlying the pathogenic effect of influenza infection in a murine model of asthma includes simultaneous activation of neutrophilic and eosinophilic inflammation.

Several animal models of asthma have been developed to understand the pathogenesis of asthma. The two most commonly used mouse models of asthma are the OVA model of asthma and the HDM model of asthma.⁷ In the mouse model of OVA-induced asthma, active sensitization is achieved either by concurrent administration of Al(OH)₃ as an adjuvant or via repetitive exposure to low doses of ovalbumin.⁷ The use of adjuvant has been reported to induce a mast cell-

independent form of airway inflammation in the mouse model of asthma, led mostly by a strong lymphocyte-driven induced Th2 inflammation and eosinophilia.⁷ Models not using an adjuvant, such as Al(OH)₃, are reportedly dependent on the presence of mast cells for the development of airway inflammation.⁷ However, the shortfall of the OVA mouse model of asthma is the use of a nonantigenic protein, and the development of an artificial immune response to this protein via the use of adjuvant. Therefore, we used the HDM model of asthma, which uses a natural antigenic protein. Latest studies report that administration of HDM extract once a day for 10 days causes an asthmatic response characterized by increased IgE, airway and parenchymal eosinophilia, a Th2 cytokine response, as well as the development of an increased response to methacholine as a measure of airway hyper-responsiveness.⁸ These characteristics indicate that mast cells are likely to play a major role in the development of the asthma-like response in this disease.⁷

Ever since viruses have been implicated as a cause of asthma exacerbation, many studies have attempted to investigate the mechanism of asthma exacerbation caused by viral infection, with conflicting results.

Regarding airway resistance, we found that at 50 mg/mL methacholine, the HDM/influenza group showed a significantly higher Penh value than the PBS, HDM, and influenza groups. This finding shows that influenza infection aggravates airway inflammation in a murine model of asthma. This result was similar to that of a previous study in which mice infected with influenza A and exposed to HDM during infancy exhibited marked functional impairment as measured by significant increases in airway resistance.^{9,10} However, in a recent study, effect of influenza infection on airway resistance differed on age.¹⁰ RV infection has also been shown to increase maximal airway responses to methacholine in normal human subjects.^{11,12}

In our study, we found that the number of inflammatory cells retrieved from BALF was significantly higher in the HDM/influenza group than in the HDM group. Neutrophils were increased in the HDM/influenza group compared with the number in the HDM group. Lymphocytes and macrophages were significantly higher in the HDM/influenza group than in the HDM group. These findings were similar to those of a previous study, which reported a significant increase in the total number of cells retrieved in the

BALF of influenza-treated mice, and early innate immune responses marked by significant increases in neutrophils, macrophages, and other monocytic cells.¹⁰ The adaptive immune response was characterized by marked increases in lymphocytes and eosinophils.¹³

Regarding cytokine and chemokine secretion, we measured IFN- γ , IL-1 β , IL-5, IL-10, CXCL1, and RANTES. The innate immunity cytokines, IFN- γ and IL-1 β , are known to have antiviral activity, IL-5 acts as a proinflammatory cytokine, reflecting Th2 inflammation. IL-10 is a regulatory cytokine with anti-allergic effects, CXCL1 shows neutrophil activation, and RANTES has eosinophil activation activity.

We found that IFN- γ and IL-1 β levels were higher in HDM/influenza group than in the HDM group as a result of influenza infection. IL-5 and IL-10, which are related to Th2 inflammation, showed mixed results. Even though IL-5 showed no significant difference between the HDM group and the HDM/influenza group, IL-10 was significantly lower in the HDM/influenza group than in the HDM group. We postulated that influenza infection can stimulate Th2 inflammation. This also supports findings from a previous study in which IL-4, a Th2 cytokine, was increased in the lung but was not increased in the spleens after influenza infection in a mouse model of asthma.¹⁴ Likewise, CXCL1 and RANTES levels were higher in the HDM/influenza group than in the HDM group. Therefore, we concluded that influenza infection stimulates both neutrophil and eosinophil activation. A previous study of experimental RV infection¹⁵ reported increased levels of IL-8 and IL-1 β in nasal lavage samples from asthmatic patients compared to the levels in samples from control subjects. However, another study¹² reported no differences in IL-6, IL-8, IL-11, and granulocyte-monocyte-colony stimulating factor (GM-CSF) levels in nasal lavage and sputum samples from asthma patients and control subjects. In another study of RV infection, increased production of various pro-inflammatory substances, including IL-1 α , IL-1 β , IL-6, IL-8, IL-11, TNF- α , RANTES, and GM-CSF, was detected in primary cultures of epithelial cells or established cell lines.¹⁶⁻¹⁹ Based on clinical studies, influenza infection induced the NF- κ B-mediated release of cytokines, including IL-1 β , IL-6, and IL-8, from human tracheal epithelial cells.^{20,21} Increases in

cytokines and monokines, including IL-6, IL-8, and RANTES, were also observed in the sera of patients infected with influenza virus.²² Wang et al. demonstrated that influenza virus infection increased the vascular endothelial permeability of mouse lungs by increasing the levels of IL-1 β , IL-6, TNF- α , and trypsin.²³ Our results were quite similar to those in previous studies of both RV and influenza infection.

The peribronchial inflammatory scores of the HDM, influenza, and HDM/influenza groups were higher than that of the PBS group. When each group was scored according to the level of eosinophil and neutrophil infiltration, the HDM/influenza group showed proportionally higher neutrophil infiltration than eosinophil infiltration (Table 1). The perivascular inflammatory scores of the HDM, influenza, and HDM/influenza groups were higher than those of the control group. When each group was scored according to the level of eosinophil and neutrophil infiltration, in the HDM/influenza group, neutrophil infiltration was higher than eosinophil infiltration. To the best of our knowledge, our study is the first to apply a scoring system to assess the effect of viral infection in a murine model of asthma.

We found that influenza virus infection-induced endothelial cell damage may be involved in the mucosal edema associated with airway inflammation due to neutrophil and eosinophil infiltration. This finding is similar to those of previous studies, which have shown that the production of proinflammatory cytokines and mediators and the production of inflammatory substances, such as ECP, in cells other than epithelial cells may also be related to airway hyper-responsiveness in asthma patients infected with respiratory viruses.²⁴

In conclusion, influenza infection may influence asthma via several mechanisms, including airway inflammation, mucus hypersecretion, and bronchial hyper-responsiveness. The pathogenic mechanism of influenza-induced airway inflammation in this murine model of asthma may be a result of simultaneous activation of neutrophilic and eosinophilic inflammation. In addition to the development of vaccines and anti-viral drugs for the treatment of influenza virus infection, the most effective methods for the treatment and prevention of influenza-induced asthma exacerbation should inhibit all of the above-mentioned contributing factors.

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