

RESEARCH ARTICLE

Role of Th2 cytokines on the onset of asthma induced by *meta*-xylene in mice

Seong-Gyeol Hong | Yun-Ho Hwang | Seul-Ki Mun | Su-Jin Kim | Ho-Yeol Jang |
Hangun Kim | Man-Jeong Paik | Sung-Tae Yee 

Department of Pharmacy, Sunchon National University, Suncheon, Republic of Korea

Correspondence

Man-Jeong Paik and Sung-Tae Yee,
Department of Pharmacy, Sunchon National University, 255, Joongang-Ro, Seokhyeon-Dong, Suncheon 57922, Republic of Korea.
Email: paik815@sunchon.ac.kr (M.-J.P.) and
Email: sungtae@sunchon.ac.kr (S.-T.Y.)

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Abstract

meta-Xylene (*m*-xylene) is one of three isomers of xylene, which is widely used as a solvent and detergent in various industries and medical technology. Exposure to volatile organic compounds, such as *m*-xylene, causes pulmonary inflammation and airway inflammation, thereby contributing to the onset of asthma. Exposure to *m*-xylene increases acute wheezing and intensity of asthma symptom. However, the mechanism of the onset of asthma by *m*-xylene has not been studied yet. C57BL/6 mice were sensitized and challenged by *m*-xylene at 100 or 300 mg/kg. The mice were then sacrificed after the last challenge. Exposure to *m*-xylene increased the total number of inflammatory cells and the production of interleukin (IL)-4, IL-5, IL-13, and immunoglobulin E related to the Th2 immune response. In contrast, the production of interferon- γ related to the Th1 immune response was decreased. In addition, the airway resistance increased according to the airway hyper-responsiveness measurements. Finally, a histological analysis revealed infiltration of inflammatory cells, mucus production, and lung fibrosis. These results suggest that *m*-xylene is a potential risk factor for asthma and the onset of asthma is caused by TH2 cytokines.

KEYWORDS

AHR, asthma, *m*-xylene, VOCs

1 | INTRODUCTION

Asthma is a chronic inflammatory disease that affects the lower airways. The features of asthma include variable airflow obstruction and airway hyper-responsiveness (AHR). Respiratory symptoms associated with asthma include wheezing, shortness of breath, chest tightness, and cough of depending on the duration and intensity of disease.¹ According to the World Health Organization, over 3 million people worldwide have asthma. In many countries, the prevalence and impact of asthma are rapidly increasing, and the burden of asthma treatment is growing.² Factors thought to play an important role in the occurrence of respiratory diseases, such as asthma, include passive smoking, indoor air pollution, and outdoor air pollution (eg, vehicle emissions).³ Type 1 and 2 T-helper cells (Th1 and Th2) are known to play an

important role in asthma. An imbalance of Th1 and Th2 cells causes asthma, which is predominant with the Th2 immune response. In addition, Th2 mediated cytokines of interleukin 4 (IL-4), IL-5, and IL-13 are over-produced by Th2 cells, a subset of CD4⁺ T cells. These cytokines increase airway reactivity via the production of immunoglobulin E (IgE), growth and proliferation of granulocytes and mucus secretion.⁴

Indoor exposures to nitrogen dioxide (NO₂), sulfur dioxide (SO₂), volatile organic compounds (VOCs), and formaldehyde (FA) are associated with the onset of asthma.⁵⁻⁷ A wide distribution of outdoor VOCs affects indoor VOC concentrations.⁸ Typical indoor VOCs include benzene, toluene, xylene (BTX), and formaldehyde (FA). The main sources of VOCs indoors are furnishings with synthetic coating furniture, carpets, polyvinylchloride flooring, building materials and are cleaning agents. In addition, sources also include indoor smoking. Previous studies demonstrated

that simultaneous exposure to BTX and FA induced pulmonary inflammation and airway inflammation in Kunming mice.^{9,10}

FA exposure induced airway inflammation, and exposure to FA in asthma model induced by ovalbumin (OVA) aggravated asthma-related symptoms.^{11,12} Toluene exposure led to increased airway inflammation.^{13,14} In other research, benzene inhalation was associated with airway inflammation or pulmonary inflammation. It has been shown to be caused by inflammatory cells different from basophils.¹⁵

Xylene exposure is known to affect various tissues and the central nervous system. In terms of its effects on the lungs, xylene causes not only chest pain and dyspnea but also excessive exposure leads to pulmonary edema.¹⁶ In addition, xylene induced acute wheezing and aggravated intensity of asthma.^{17,18} Especially, *meta*-xylene (*m*-xylene) accounts for the highest proportion in "mix xylene, which is used as an industrial solvent".¹⁹ Moreover, *m*-xylene is known to common antigenic determinant in commercial topical anesthetics (Las) and causes delayed hypersensitivity in allergic reactions.²⁰ However, the mechanism of the onset of asthma by *m*-xylene has not been studied yet. In this study, we investigated whether the effects of exposure to *m*-xylene causes pathological symptoms similar to asthma and examined its mechanism.

2 | MATERIALS AND METHODS

2.1 | Materials

m-Xylene (296325) and ovalbumin (OVA) (A5503) were purchased from Sigma-Aldrich (St. Louis, Missouri). Purified rat anti-mouse IL-4,

purified rat anti mouse IL-5, purified rat anti-mouse interferon (IFN)- γ , purified rat anti mouse IgE (R35-72), biotin rat anti mouse IL 4, biotin rat anti mouse IL-5, biotin rat anti mouse IFN- γ , and biotin rat anti mouse IgE (R35-118) were purchased from BD Biosciences (San Diego, California). Mouse IL-13 antibody and mouse IL-13 biotinylated antibody were purchased from R&D systems (Minneapolis, Minnesota).

2.2 | Animals

Specific pathogen-free female C57BL/6 mice (7-8 weeks old) were purchased from ORIENT BIO (Seongnam, Korea). The animals were housed in cages at a temperature of $22 \pm 2^\circ\text{C}$ and the humidity of $50 \pm 5\%$. This study complied with the guidelines for the care and use of laboratory animals of Sunchon National University Institutional Animal Care and Use Committee (SCNU IACUC). All experimental procedures were approved by SCNU IACUC (approval ID: SCNU IACUC-2018-09).

2.3 | Administration of *m*-xylene to animals

The sensitization and challenge experiments were performed as described previously, with slight modifications.²¹ Schematic representation of *m*-xylene administration to animals is depicted in Figure 1. The C57BL/6 mice were randomly divided into four groups, with five mice in each group: phosphate buffer saline (PBS) group (control group), OVA group, *m*-xylene 100 mg/kg group, and *m*-xylene 300 mg/kg group. The mice were primary sensitized by an intraperitoneal injection

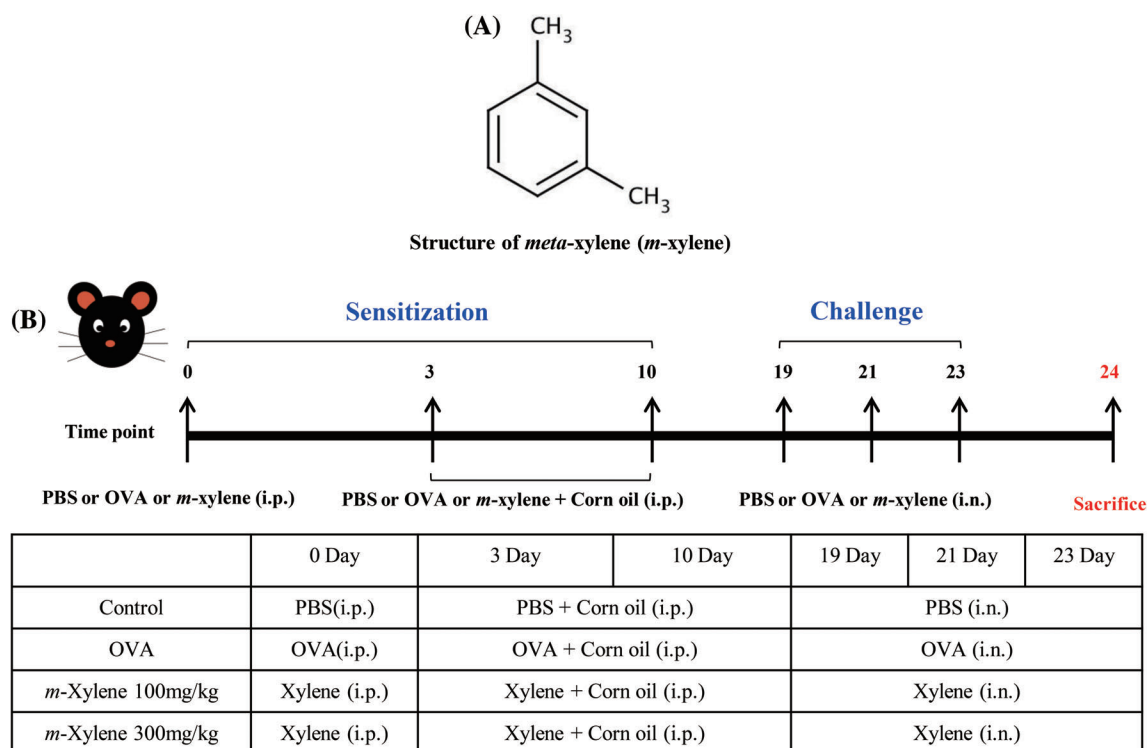


FIGURE 1 A, Molecular structure of *m*-xylene; and B, experimental protocol for the induction of airway inflammation along with the treatment scheme [Color figure can be viewed at wileyonlinelibrary.com]

of 100 µg/mL of OVA or *m*-xylene (100 and 300 mg/kg) in 200 µL of PBS on day 0. The mice were secondary sensitized by an intraperitoneal injection of 100 µg/mL of OVA or *m*-xylene (100 and 300 mg/kg) dissolved in corn oil on days 3 and 10. In the challenge experiment, the mice received 5 mg/mL of OVA or *m*-xylene (100 and 300 mg/kg) dissolved in 50 µL of PBS by intranasal administration under anesthesia with Zoletil/Rumpun on days 19, 21, and 23. The mice in the control group received equal amounts of OVA group and *m*-xylene groups (100 and 300 mg/kg). On day 24, blood was collected from a retro orbital plexus and placed in heparin coated tubes. After centrifugation (5000 rpm, 4°C, 5 minutes), the serum was obtained and stored at -80°C for analysis of IgE using by enzyme-linked immunosorbent assay (ELISA). Bronchoalveolar lavage fluid (BALF) was obtained by lavaging the lungs four times with 0.5 mL of PBS using a 1 mL polyethylene syringe. After centrifugation (1200 rpm, 4°C, 5 minutes), the supernatant of BALF recovered of PBS was stored at -80°C for cytokine analysis using by ELISA. Red blood cells were removed with tris buffered ammonium chloride, and the nucleated cells were counted using the hemacytometer after cell staining with trypan blue dye.

2.4 | Hematological analysis of blood

The blood collected from a retro orbital plexus was placed in EDTA coated tubes. Hematological changes were analyzed using by

automatic analyzer (HEMAVET 850, Drew Scientific, Inc., Dallas, Texas). The numbers of white blood cells (WBCs), lymphocytes, monocytes, eosinophils, neutrophils, and basophils were determined within 24 hours after the last airway challenge.

2.5 | Measurement of the levels of cytokines, and total IgE

Cytokines and immunoglobulin were obtained at the place where they were usually measured.²¹⁻²³ BALF supernatant and serum were stored at -80°C. The levels of IL-4, IL-5, IL-13 and IFN-γ in BALF and the levels of total IgE in serum were determined by ELISA.

2.6 | Measurement of AHR

As described in previous studies, AHR was measured in anesthetized mice within 24 hours of the final challenge.^{21,22} An 18G metal cannula was inserted into the trachea to expose the methacholine (MCH). The mice were placed in a flow-type body plethysmograph and connected to a computer-controlled ventilator (FlexiVent, SCIREQ, Inc., Montreal, Canada). The mice were then exposed directly through the ventilator to increasing concentrations of MCH (0, 3.125, 6.25, 12.5, 25, and 50 mg/mL). The airway resistance (Rn) following exposure to each dose of MCH was determined.

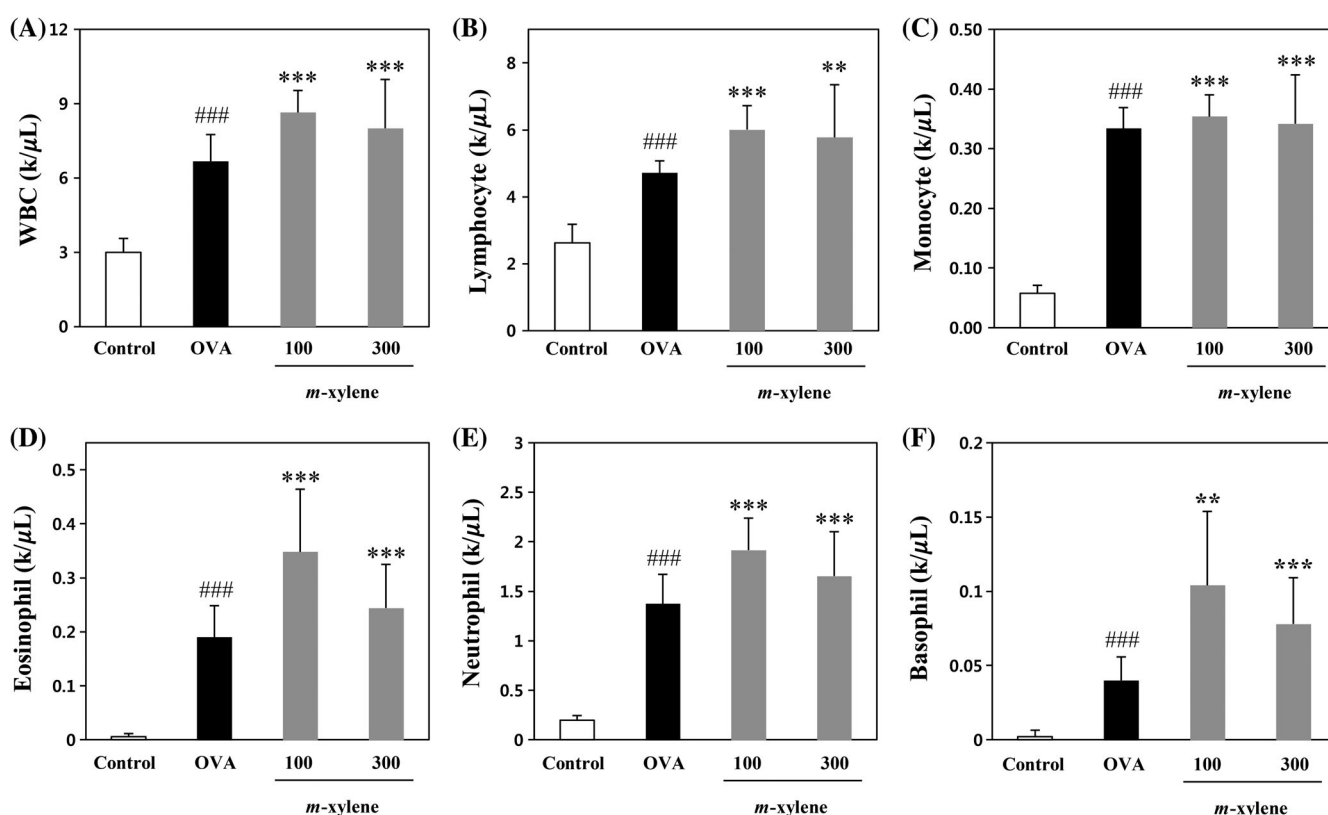


FIGURE 2 Effects of *m*-xylene on hematological changes. Blood was collected 24 hours after the last challenge. An automatic analyzer (HEMAVET 850, Drew Scientific, Inc., Dallas, Texas) was used to measure: A, white blood cells (WBCs); B, lymphocytes; C, monocytes; D, eosinophils; E, neutrophils; and F, basophils. The data represent five mice per group. All data are expressed as mean ± SD. ###*P* < 0.01 and ###*P* < 0.001 control vs OVA. ***P* < 0.01 and ****P* < 0.001 control vs *m*-xylene group. OVA, ovalbumin

2.7 | Histopathological analysis of lung tissue

The left lungs were collected, fixed in 4% formalin, and embedded in paraffin. The lung tissues were sliced of 4 μ m thickness. The lung sections of embedded paraffin were stained with hematoxylin and eosin (H&E), periodic acid-Schiff (PAS), and Picrosirius red. Image of the sections were acquired using a microscope at $\times 200$ magnification.

2.8 | Statistical analysis

All data are given as means $\pm$ SD. SPSS version 22 (Chicago, Illinois) was used for statistical analysis. The differences between the groups were determined using the Student's *t* test. *P*-values of less than 0.05 were considered statistically significant.

3 | RESULTS

3.1 | Change in the number of inflammatory cells in the blood of the *m*-xylene exposed mice

To investigate the effect of *m*-xylene exposure on inflammatory cells in the blood, hematological changes in each group were determined using by automatic analyzer. As compared with the control group, the number of lymphocytes, eosinophils, and neutrophils, all of which are major features

of asthma, significantly increased in OVA group and *m*-xylene groups (100 and 300 mg/kg) (Figure 2B,D,E), as well as a larger the number of WBCs, including monocytes and basophils (Figure 2A,C,F). These results suggest that exposure to *m*-xylene induces inflammatory responses by increasing the number of inflammatory cells, including granulocytes.

3.2 | Change in the total cells and cytokines in the BALF of the *m*-xylene exposed mice

We examined the effect of *m*-xylene on inflammation cell infiltration and the Th1/Th2 mediated cytokine equilibrium in mice. As compared with the control group, the total number of BALF cells and the level of Th2 cytokines (IL-4, IL-5, and IL-13) were significantly increased in the OVA group and *m*-xylene groups (100 and 300 mg/kg) (Figure 3A-D). In contrast, the production of IFN- γ in the BALF was decreased in the OVA group and *m*-xylene groups (100 mg/kg and 300 mg/kg) (Figure 3E). These results suggest that exposure to *m*-xylene increases inflammatory cells in the lung and induces predominant Th2 immune response.

3.3 | Change in IgE production in the serum of the *m*-xylene exposed mice

We additionally measured the effect of *m*-xylene on total IgE, a typical marker of asthma in serum. The total IgE level in the OVA mice group

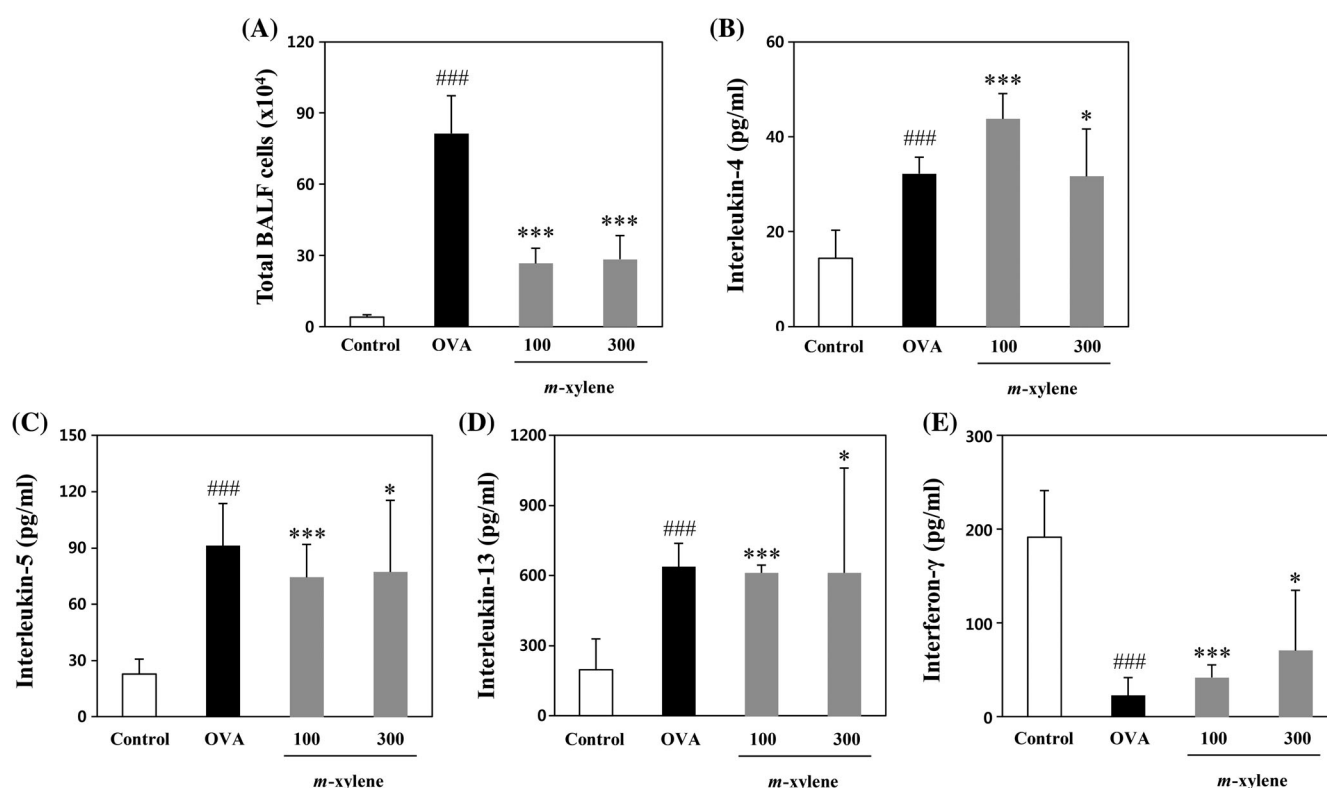


FIGURE 3 Effects of *m*-xylene on the total number of cells and Th2 cytokines in BALF. BALF was collected 24 hours after the last challenge. A, The lavage fluids was centrifuged, and the cell pellets were counted using by hemacytometer after cell staining with trypan blue. The levels of (B) IL-4, (C) IL-5, (D) IL-13, and (E) IFN- γ in the supernatants were measured by ELISA. The data represent five mice per group. All data are expressed as mean $\pm$ SD. ###*P* < 0.01 and ###*P* < 0.001 control vs OVA. **P* < 0.05 and ****P* < 0.001 control vs *m*-xylene group. BALF, bronchoalveolar lavage fluid; ELISA, enzyme-linked immunosorbent assay; OVA, ovalbumin

and *m*-xylene groups (100 and 300 mg/kg) were significantly increased in comparison with that in the control group (Figure 4). These results suggest that exposure to *m*-xylene increase the level of total IgE, which is associated with the Th2 immune response.

3.4 | Change in AHR of the *m*-xylene exposed mice

We examined the effect of *m*-xylene on the development of AHR in mice. AHR was determined by measuring Rn value following exposure to different doses of MCH (Figure 5). There were no significant between-group differences in the Rn values at MCH concentrations of 0 to 6.25 mg/mL. As compared with the control group, the Rn values significantly increased in accordance with the MCH concentration in OVA group and *m*-xylene 300 mg/kg group. In the *m*-xylene 100 mg/kg group, the Rn value increased only at the concentration of

12.5 mg/mL. This results suggest that the induction of AHR at a concentration of 300 mg/kg higher than 100 mg/kg of *m*-xylene.

3.5 | Histological changes in the lung of the *m*-xylene exposed mice

To observe histological changes following exposure to *m*-xylene, sections of lung tissue were stained with H&E, PAS, and Picrosirius red. As compared with the control group, H&E staining revealed infiltration of inflammatory cells and epithelium thickening in OVA group. The *m*-xylene groups (100 and 300 mg/kg) also increased numbers of inflammatory cells in the airway epithelium, although the numbers were lower than those observed in the OVA group (Figure 6A). The secretion of mucus in the airway epithelium was observed by PAS staining. As compared with the control group, the numbers of PAS positive mucous cells increased in the OVA group. The numbers of PAS positive cells in the *m*-xylene groups (100 and 300 mg/kg) also were higher than those in the control group (Figure 6B). The deposition of subepithelial collagen was examined by Picrosirius red staining. The collagen deposition in the OVA group and *m*-xylene groups (100 and 300 mg/kg) were higher than those in the control group (Figure 6C). These results suggest that *m*-xylene induces the infiltrations of inflammatory cells, production of mucus, and deposition of collagen.

4 | DISCUSSION

The several studies showed that exposure to VOCs is associated with asthma. In particular, substances contributing to the onset and promotion of allergies and asthma include BTX and FA, which are often found indoors.^{9,10,24,25} Studies also showed that single exposure to benzene, toluene, and formaldehyde induced airway inflammation, which is directly related to asthma.¹¹⁻¹⁵ Exposure to *m*-xylene is known to not only induce wheezing but also aggravate intensity of asthma. In addition, *m*-xylene causes a delayed hypersensitivity reaction in the allergic response.^{17,18,20} However, there is a lack of research into the mechanism of the onset of asthma by *m*-xylene. In this study, experiments conducted to investigate the alone effect of *m*-xylene and mechanism of onset of asthma.

AHR causes airflow disorders due to hypersensitivity to various airway stimuli, and it is considered a hallmark clinical symptom in the diagnosis of asthma.^{26,27} In the present study, single exposure to *m*-xylene significantly increased AHR at high concentrations of MCH (12.5, 25, and 50 mg/mL). In addition, the 100 mg/kg dose of *m*-xylene resulted in significantly elevated airway resistance (Rn) at an MCH concentration of 12.5 mg/mL, whereas the 300 mg/kg dose of *m*-xylene increased the Rn value at MCH concentrations of 12.5, 25, and 50 mg/mL. Although there was no change in AHR at low concentrations of MCH (0, 3.125, and 6.25 mg/mL), the results provide evidence that exposure to *m*-xylene alone may cause AHR.

Various chemokines are known to play a role in inflammatory cell migration and infiltration. The lung is a unique organ in that it receives

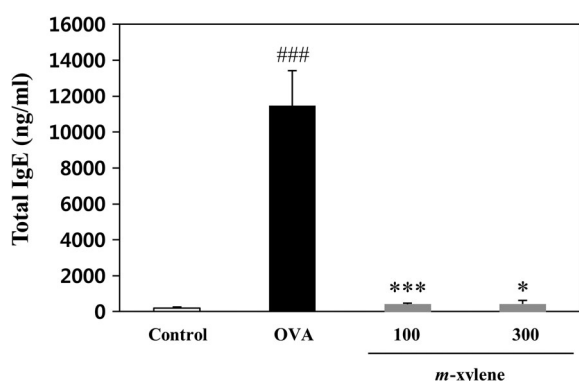


FIGURE 4 Effects of *m*-xylene on IgE production in serum. Blood was collected 24 hours after the last challenge. The levels of total IgE in serum were measured by ELISA. The data represent five mice per group. All data are expressed as mean $\pm$ SD. ### $P < 0.001$ control vs OVA. * $P < 0.05$ and *** $P < 0.001$ control vs *m*-xylene group. ELISA, enzyme-linked immunosorbent assay; IgE, immunoglobulin E; OVA, ovalbumin

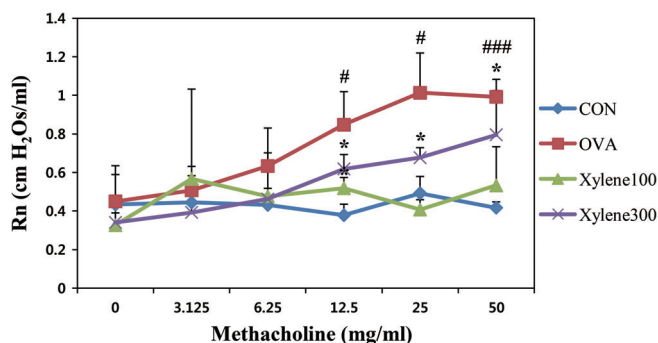


FIGURE 5 Effects of *m*-xylene on AHR in mice. Airway resistance (Rn) was measured 24 hours after the last challenge using the forced oscillation technique. The data represent three mice per group. All data are expressed as mean $\pm$ SD. # $P < 0.05$ and ### $P < 0.001$ control vs OVA. * $P < 0.05$ control vs *m*-xylene group. AHR, airway hyper-responsiveness; CON, control; OVA, ovalbumin [Color figure can be viewed at wileyonlinelibrary.com]

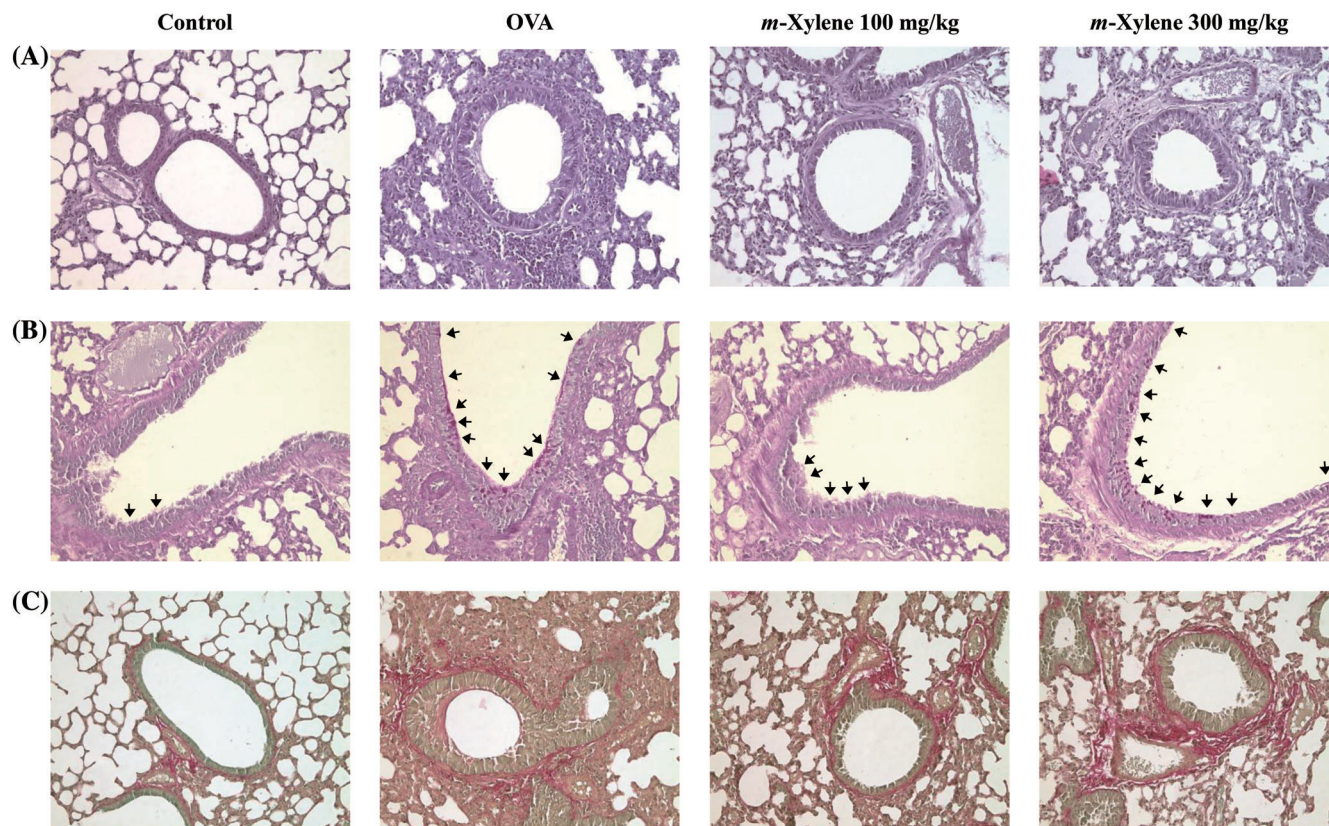


FIGURE 6 Effects of *m*-xylene on the histological changes. Lung tissue was collected 24 hours after the last challenge. The images were magnified by $\times 200$. A, Lung tissues stained with H&E, showing inflammatory cells infiltration; B, lung tissues stained with PAS, showing mucous cells (reddish purple color stain); and C, lung tissues stained with Picrosirius red, showing collagen deposition (red color stain). H&E, hematoxylin and eosin; PAS, periodic acid-Schiff [Color figure can be viewed at wileyonlinelibrary.com]

blood from the pulmonary circulation and the systemic circulatory system and supplies blood to the parenchyma and airways.²⁸ Measuring the number of eosinophils and neutrophils in the blood is a major means of evaluating airway inflammation or asthma.^{29,30} Both *m*-xylene exposure of 100 and 300 mg/kg significantly increased the number of total inflammatory cells in the blood and BALF. In addition, the number of eosinophils and neutrophils significantly increased in both the 100 and 300 mg/kg of *m*-xylene exposure groups. These findings confirmed that exposure to *m*-xylene induced airway inflammation or asthma.

Th2 cytokines are known to play an important role in asthma.³¹ IL-4 and IL-5, which are Th2 type cytokines, induce class switching and produce IgE by B cells. IL-13 induces allergic responses by switching from IgM to IgE in B cells when IL-4 production is low or absent.^{31,32} IFN- γ secreted by Th1 cells blocks the differentiation into Th2 cells and inhibits IgE synthesis and allergic inflammation.³³ IgE is a major antibody in allergic diseases, and combination of the IgE and IgE receptor (Fc ϵ RI) stimulates mast cells. Activated mast cells induce asthma through the secretion of histamine IL-4 and IL-5.^{34,35} In this study, we demonstrated that exposure to *m*-xylene induces Th2 immune responses by increasing the production of IL-4, IL-5, IL-13, and IgE.

Inflammatory cells, such as CD4⁺ T cells, eosinophils, and mast cells act on the resident cells of the airway and cause airway remodeling. Airway remodeling is characterized by thickening of the

basement membrane, subepithelial fibrosis, goblet cell and submucosal gland enlargement, and an increased smooth muscle.^{36,37} IL-4 and IL-5 promote airway remodeling by supporting inflammatory cell the filtration and the activation of other inflammatory cells. IL-13 induces the production of mucus in airway epithelial cells and that it plays a role in excessive production of goblet cells. Furthermore, transforming growth factor- β secreted by IL-13 induces fibrosis by stimulating fibroblasts to produce collagen and fibronectin.³⁶ In the present study, in terms of immunohistochemistry, we demonstrated that *m*-xylene exposure increases the infiltrations of inflammatory cells, production of mucus, lung fibrosis.

5 | CONCLUSION

The relationship between exposure to VOCs and asthma is well known. There is a lack of research on the alone effects of *m*-xylene and the mechanism of asthma onset. We indirectly confirmed that exposure to *m*-xylene increases airway inflammation by increasing the number of eosinophils and neutrophils in the blood of mice. *m*-Xylene induced the Th2 immune response by increasing the production of IL-4, IL-5, IL-13, and total IgE. Moreover, AHR was increased in the mice exposed to *m*-xylene, and characteristics of airway remodeling were observed with infiltration of inflammatory cells in the lung,

production of mucus, and lung fibrosis. Therefore, we suggest that *m*-xylene, one type of VOCs, may result in the onset of asthma and the onset of asthma is caused by Th2 cytokines.

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CONFLICT OF INTEREST

The authors declared no conflicts of interest.

AUTHOR CONTRIBUTIONS

S.G.H. conceived the study, designed experiment, performed experiments, analyzed data, and wrote manuscript; S.T.Y. and M.J.P. conceived the study and designed experiment; Y.H.H. designed experiment and performed experiments; S.K.M., S.J.K., and H.Y.J. contributed reagents, materials, and analysis tools; H.K. was involved in revising the work critically for important intellectual content.

ORCID

Sung-Tae Yee  <https://orcid.org/0000-0002-5108-8140>

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