# Chitinase 3-Like 1 Regulates IL-8 Expression in Airway Epithelial Cells

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Directed by Professor Myung Hyun Sohn

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Master of Medical Science

Jae Woo Lee

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This certifies that the Master's Thesis of
Jae Woo Lee is approved.

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The Graduate School
Yonsei University

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# Acknowledgement

설레는 마음으로 연세대학교 대학원에 입학한 지도 벌써 2년이라는 시간이 흘러 석사과정을 마무리하게 되었습니다. 그 시간 동안, 많은 분께 도움과 은혜를 받았기에 과학자로서 성장할 수 있었습니다. 감사한 분들께 이 지면을 빌려 감사의 마음을 전합니다.

먼저 저의 학위 과정을 지도해주시고 아낌없는 조언과 가르침을 주셨던 손명현 교수님께 깊은 감사의 말씀을 드립니다. 그리고 연구 결과에 대해 날카로운 지적과 깊은 고찰을 해 주신 김경원 교수님, 저의 주제에 대해 폭넓게 생각할 기회를 주신 윤호근 교수님께도 진심으로 감사의 말씀을 드립니다. 저를 지도해 주신 교수님의 은혜로 제가 한 걸음 더 성장할 수 있었습니다.

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이 재 우

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## ABSTRACT

# Chitinase 3-Like 1 Regulates IL-8 Expression in Airway Epithelial Cells

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***The Graduate School, Yonsei University***

**(Directed by Professor Myung Hyun Sohn)**

**Background:** Viral respiratory infection is a leading cause of inflammatory lung disease and mortality. Interleukin (IL)-8, one of the critical inflammatory mediators, has been suggested to induce inflammation against viral infections. Chitinase 3-like 1 (CHI3L1), which belongs to 18-glycosylhydrolase family, contributes to airway inflammation. However, it remains unclear that the role of CHI3L1 in the expression of IL-8 caused by viral infections. Thus, this study was to investigate the role of CHI3L1 in the regulation of IL-8 expression, which was caused by viral infections in bronchial epithelial cells.

**Methods:** Human bronchial epithelial cell line, BEAS-2B, was stimulated with a synthetic analog of viral double-stranded RNA, polyinosinic : polycytidylic acid (poly(I:C)). To explore the role of CHI3L1 in viral infection, CHI3L1 knock-down was performed in BEAS-2B cells by shRNA lentiviral transduction. The expression of CHI3L1 and pro-inflammatory cytokines such as IL-8, and phosphorylation of mitogen-activated protein kinase (MAPK) pathways were analyzed. BEAS-2B cells were infected with human respiratory syncytial virus (RSV) A2 strain and the expression levels of CHI3L1 and IL-8 were analyzed.

**Results:** Stimulation of BEAS-2B cells with poly(I:C) increased expression of CHI3L1 and IL-8. However, IL-8 expression was abrogated in CHI3L1 knock-down BEAS-2B cells. Poly(I:C) stimulation of BEAS-2B cells resulted in phosphorylation of MAPK pathways, and inhibitions of MAPK pathways significantly abolished IL-8 secretion. The phosphorylation of MAPK pathways was also diminished in CHI3L1 knock-down BEAS-2B cells. In addition to poly(I:C), infection with RSV increased the expression levels of CHI3L1 and IL-8. The expression of IL-8 induced by RSV infection was abrogated in CHI3L1 knock-down BEAS-2B cells.

**Conclusion:** This study suggests that CHI3L1 is involved in IL-8 secretion by regulating MAPK pathways during viral infections in bronchial epithelial cells.

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Key words: airway epithelial cells, chitinase 3-like 1, interleukin-8, MAPK signaling pathways, respiratory syncytial virus

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## I. INTRODUCTION

Viral infections are considered the main cause of various diseases worldwide[1]. Notably, respiratory virus infection induces lower respiratory tract infections such as bronchiolitis, laryngotracheobronchitis, or pneumonia[2]. Moreover, the respiratory virus infection can also exacerbate asthma and chronic obstructive pulmonary disease (COPD)[3-6]. The common respiratory viruses that cause respiratory diseases are known as coronavirus, human rhinovirus, respiratory syncytial virus (RSV), and influenza virus. These are RNA viruses in which nucleic acids are composed of RNA and synthesize double-stranded RNA (dsRNA) during replication in the host cells[6-10]. The viral nucleic acid is one of

pathogen-associated molecular patterns (PAMPs), which is recognized by pattern recognition receptors (PRRs) such as toll-like receptors (TLRs) and retinoic-acid-inducible gene I-like receptors (RLR). For RNA viruses, TLR7 detects single-stranded RNA, besides TLR3 recognizes double-stranded RNA[10,11]. The infected cells that sense viral nucleic acid through their specific receptors induce secretion of pro-inflammatory cytokines which evoke antiviral immunity, especially inflammation[12,13].

Polyinosinic : polycytidylic acid (poly(I:C)) is a synthetic dsRNA analog that has similar molecular patterns with viral nucleic acid and is recognized by TLR3 of host cells[11]. In recent studies, it has been demonstrated that poly(I:C) stimulation induces pro-inflammatory chemokines and cytokines such as Interleukin(IL)-8 and IL-6[10,11,14]. IL-8 is known as a neutrophil-attracting chemokine, and previous studies demonstrated that IL-8 secretion in airway epithelial cells was induced by German cockroach extract and chitinase[15,16]. Also, IL-8 secretion is increased in bronchoalveolar fluids of patients suffering from asthma, COPD, and bronchiolitis which is caused by respiratory viral infections[17-19]. These results indicate that IL-8 plays a significant role in acute lung inflammation[16].

In 18-glycosyl-hydrolase family, there are two main groups based on the activity of chitinase. The one is enzymatically active chitinases containing chitotriosidase (CHIT1) and acid mammalian chitinase (AMCase). The other is called chitinase-

like proteins (CLPs) that cannot degrade chitin and are represented by chitinase-3-like 1 (CHI3L1)[20,21]. CHI3L1 is known as YKL-40 in humans, and breast regression protein-39 (BRP-39) in mice[22]. Previous studies have demonstrated that CHI3L1 has been related to several diseases, such as cancers, rheumatoid arthritis, and allergic diseases[23-28]. Moreover, it has been demonstrated that CHI3L1 plays an important role and can be a biomarker for airway inflammation caused by respiratory virus infections. In a previous study, CHI3L1 expression was elevated in nasopharyngeal inhalation of RSV-infected pediatric patients. Also, *in vivo* studies have demonstrated that airway inflammation by RSV infection was reduced in CHI3L1 knock-out mice by modulating alveolar macrophage polarization[29]. And a recent study demonstrated that CHI3L1 is an attractive therapeutic target in COVID-19[30]. Therefore, CHI3L1 is associated with various inflammatory diseases, and it is now considered to be a biomarker for lung inflammation and a therapeutic target as well[31,32]. Nonetheless, little is known whether CHI3L1 regulates IL-8 secretion against viral infections in bronchial epithelial cells.

Thus, we explored the role of CHI3L1 in IL-8 secretion induced by respiratory viral infections in bronchial epithelial cells.

## II. MATERIALS AND METHODS

### 1. Cell culture

Human bronchial epithelial cell line, BEAS-2B was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in bronchial epithelial cell growth medium, BEGM (Lonza, East Rutherford, NJ, USA). Human lung epithelial cell line, NCI-H292 and A549 (ATCC), and human kidney epithelial cell line, HK2 (ATCC) were grown in RPMI-1640 (Hyclone, Logan, UT, USA) supplemented with 10% fetal bovine serum (FBS) and 100U/mL penicillin/streptomycin (Hyclone). To lentivirus production, human embryonic kidney 293 T cell line, HEK-293 T cell (ATCC) was used. HEK-293 T cells were grown in DMEM (Hyclone) supplemented with 10% FBS and 100U/mL penicillin/streptomycin. To prepare RSV stock, HEp-2 cells (ATCC) were used. HEp-2 cells were grown in MEM (Hyclone) supplemented with 10% FBS and 100U/mL penicillin/streptomycin. The cells were grown in a 37ºC humidified incubator consisting of 5% $CO_2$ and the fresh media was replaced every 3 days.

### 2. Respiratory syncytial virus (RSV) preparation

The RSV A2 strain was propagated in HEp-2 cells and harvested on 4 days post-infection. The harvested virus was stored at -70℃ immediately. Virus titer, plaque-forming units/mL (PFU/mL), was determined with the standard plaque assay[29].

### 3. Treatment

For stimulation with poly(I:C), BEAS-2B cells, A549 cells, NCI-H292 cells, and HK2 cells were plated in 6 well cell culture plates or 60mm dishes. The next day, the media was replaced with fresh media and incubated overnight. After the overnight incubation, media was replaced containing 5μg/mL of poly(I:C)-HMW (Invivogen, San Diego, CA, USA). Then the cells were incubated for up to 24 hr. For RSV infection, BEAS-2B cells were plated in 6 well cell culture plate. The next day, the media was discarded and washed with PBS (Hyclone) to remove serum. After washing, RSV absorption to BEAS-2B cells was performed at multiplicity of infection (MOI) of 0.1 for 1 hr. After absorption of RSV to BEAS-2B cells, the free virus was removed and replaced with fresh media. Then the cells were incubated for up to 24 hr. At specific time points, incubated cells and supernatants were harvested and stored at -70ºC until assayed.

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### 4. Inhibition of mitogen-activated protein kinase (MAPK) pathways

The specific inhibitors of MAPK pathways – extracellular signal-regulated kinases (ERK1/2) inhibitor (PD98059), p38 inhibitor (SB202190), and c-Jun N-terminal kinase (JNK) inhibitor (SP600125) were purchased from Calbiochem (San Diego, CA, USA). Before stimulation with poly(I:C), BEAS-2B cells were pre-incubated for 30 min with the inhibitors. After the pre-incubation, the cells were stimulated with 5μg/mL poly(I:C) along with the inhibitors for 12 hr.

### 5. Lentiviral short hairpin RNA transduction

CHI3L1 knockdown of BEAS-2B cells was performed by short hairpin RNA (shRNA) lentivirus transduction. For the lentivirus production, the pLKO.1-puro vector containing CHI3L1 shRNA (Sigma-Aldrich, St. Louis, USA) was used for CHI3L1 knock-down, besides an empty vector was used for the control cells. The vectors were transduced into HEK-293 T cell using TransIT-X2® Dynamic Delivery System (Mirus Bio, Madison, WI, USA) according to manufactures' protocol. The lentivirus transduction was performed by incubating BEAS-2B cells for 5 hr with the lentivirus and 8μg/mL of polybrene (Sigma-Aldrich) in BEBM. After the incubation, the media was replaced with fresh BEGM and incubated for 3 days. To select shRNA-containing cells, puromycin selection was conducted. For the puromycin selection, the media was replaced with fresh media supplemented with puromycin (Sigma-Aldrich) and incubated for 3 days.

### 6. Real-time polymerase chain reaction (PCR)

Total RNA of the harvested cells was isolated with TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to an RNA isolation protocol. The quantification of the isolated total RNA was accomplished by Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA). The 2μg of total RNA was synthesized to cDNA using ReverTra Ace® qPCR RT Master Mix Kit (Toyobo Co., Ltd., Osaka, Japan). Real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). The primer sequences are as follows: IL-8: 5'-GTG CAG TTT TGC CAA GGA GT-3' (forward) and 5'-CTC TGC ACC CAG TTT TCC TT-3' (reverse); IL-6: 5'-TAC CCC CAG GAG AAG ATT CC -3' (forward) and 5'-TTT TCT GCC AGT GCC TCT TT-3' (reverse); IL-1β: 5'-CTG TCC TGC GTG TTG AAA GA-3' (forward) and 5'-TTC TGC TTG AGA GGT GCT GA-3' (reverse); TNF-α: 5'- AAC CTC TCT GCC ATC AA -3' (forward) and 5'- CCA AAG TAG ACC TGC CCA GA-3' (reverse); GAPDH: 5'-AAG GTG AAG GTC GGA GTC AAC-3' (forward) and 5'-GGG GTC ATT GAT GGC AAC AAT A-3' (reverse). GAPDH was used as endogenous control, and relative expressions of each pro-inflammatory cytokine were analyzed using $2^{-\Delta\Delta CT}$ method.

### 7. Western blot analysis

Total protein of harvested cells was extracted by mammalian protein extraction reagent (MPER) supplemented with Halt™ Protease Inhibitor Cocktail (both from Thermo Fisher Scientific) according to manufacturer's instructions. The extracted total protein was quantified by Bradford protein assay (Bio-Rad, Hercules, CA, USA). In western blot analysis using cell supernatants, the total protein of cell supernatants was concentrated with methanol (Duksan Chemicals, Gyeonggi-do, Korea) and chloroform (Sigma-Aldrich). 15-30μg of the quantified protein samples or concentrated protein samples of cell supernatants were electrophoresed using 10% SDS polyacrylamide gel and electrotransferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). At the end of following every step, the membrane was washed out with 1X tris-based saline buffer supplemented with 1% Tween 20 (Duksan Chemicals) 3 times. Afterward, the membrane was blocked with 5% skim milk (Becton Dickinson, Sparks, MD, USA) and incubated overnight at 4℃ with a specific primary antibody diluted in 5% skim milk. The specific primary antibodies as follows: CHI3L1 (Invitrogen) and phosphorylation (p)- ERK 1/2, total (T)-ERK 1/2, p-p38, T-p38, p-JNK, T-JNK, and β-actin (Cell signaling Technology, Beverly, MA, USA). The membrane was incubated with horseradish peroxidase-conjugated second antibody (Santa Cruz Biotechnology, Dallas, TX, USA) for 1 hr. The protein bands were visualized using ImageQuant™ LAS 4000 Mini Biomolecular Imager

(GE health care, Buckinghamshire, UK) with Chemiluminescence Substrate (Thermo Fisher Scientific). For the densitometric analysis of specific protein bands, Image J software (National Institutes of Health, Bethesda, MD, USA) was used.

### 8. Immunofluorescence staining

BEAS-2B cells were plated in 8-well chamber slides (SPL Life Sciences Co., Gyeonggi-do, Korea) and incubated with 5μg/mL poly(I:C) for 24 hr. After the incubation, the cells were washed with PBS (Hyclone) 3 times and fixed with 4% paraformaldehyde (Biosesang, Gyeonggi-do, Korea) for 15 min at room temperature. The fixed cells were permeabilizated by incubation in PBS with 1% Triton X-100 (Daejung Chemicals & Metals Co. Ltd., Gyeonggi-do, Korea) for 15 min at room temperature. At the end of following every step, the cells were washed with PBS containing 1% Tween 20 (Duksan Chemicals). Afterward, the cells were blocked with 5% bovine serum albumin (Sigma Aldrich) for 1 hr and incubated with anti-CHI3L1 antibody (Invitrogen) and anti-receptor for advanced glycation end products (RAGE) antibody (Santa Cruz Biotechnology) overnight at 4℃. The cells were incubated with Alexa Fluor 647-conjugated donkey anti-rabbit IgG and Alexa Fluor 555-conjugated goat anti-mouse IgG (both from Abcam, Cambridge, UK) for 1 hr at room temperature. After 1 hr incubation, nuclei of the cells were stained with DAPI (4’,6-diamidino-2-phenylindole;

Thermo Fisher Scientific) for 5 min. The cells were mounted with Dako Faramount Aqueous Mounting Medium Ready-to-use (Dako, Santa Clara, CA, USA). The images of immunofluorescence staining were obtained using LSM 700 confocal microscopes (Zeiss, Oberkochen, Germany). The ZEN software was used to obtain images and process.

### 9. Enzyme-linked immunosorbent assay (ELISA)

The expression levels of proinflammatory cytokines and CHI3L1 in the cell supernatant were detected by enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's protocol.

### 10. Statistical analysis

The data are presented as the mean ± standard error of the mean (SEM) of at least three individual experiments. Statistical analysis was evaluated using Student's *t*-test for comparing two groups, and one-way ANOVA with Tukey test for comparing multiple groups. In the statistical significance of differences, *P*-value $<0.05$ was considered statistically significant.

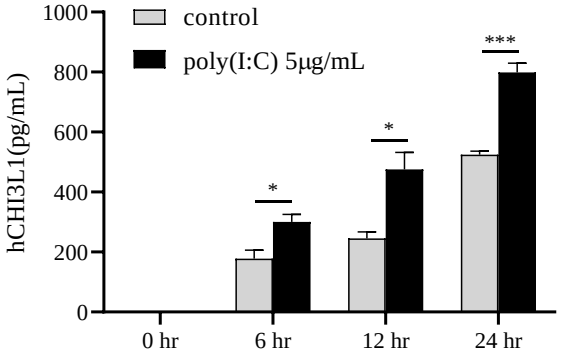
## III. RESULTS

### 1. CHI3L1 expression is elevated by poly(I:C) stimulation in BEAS-2B cells

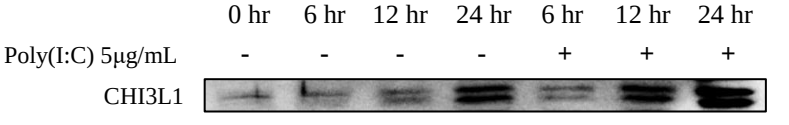
To reveal whether CHI3L1 is induced by stimulation of viral dsRNA in bronchial epithelial cells, we stimulated BEAS-2B cells with 5μg/mL poly(I:C) for 6 hr, 12 hr, and 24 hr, and analyzed expression levels of CHI3L1 by ELISA and western blot. As shown in Fig. 1A and 1B, poly(I:C) stimulation significantly increased CHI3L1 expression in a time-dependent manner (Fig. 1A and 1B). Also, CHI3L1 expression levels in cell lysates were elevated by poly (I:C) stimulation (Fig. 1C). In an immunofluorescence study, CHI3L1 and its receptor, RAGE, were increased by poly(I:C) stimulation. And the co-localization between CHI3L1 and RAGE was detected in poly(I:C) stimulated cells. Therefore, we demonstrated that CHI3L1 is increased in bronchial epithelial cells during viral infections.

Next, we stimulated A549 cells, H292 cells, and HK2 cells with 5μg/mL poly(I:C) to investigate CHI3L1 expression in different cell lines. The expression of CHI3L1 was hardly detected in cell supernatants of A549 cells and NCI-H292 cells but was slightly increased in cell supernatants of HK2 cells by poly(I:C) stimulation (Fig. 1E).

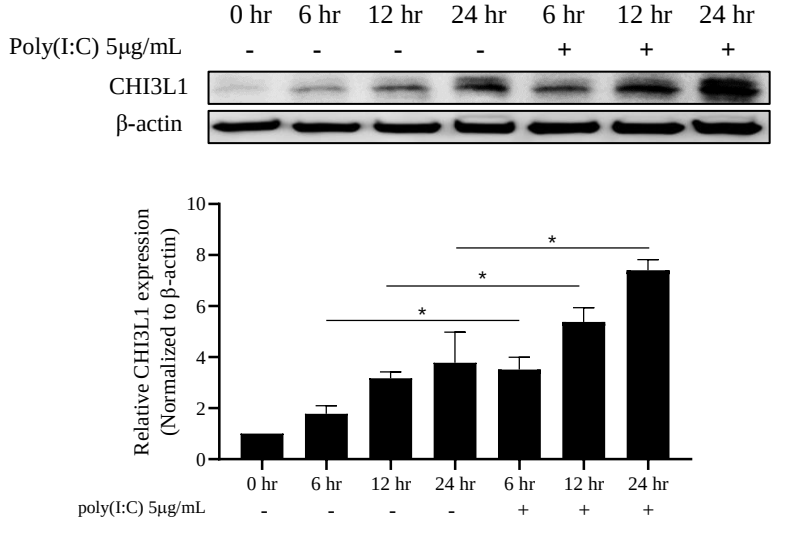
**(A)**




**(B)**




**(C)**



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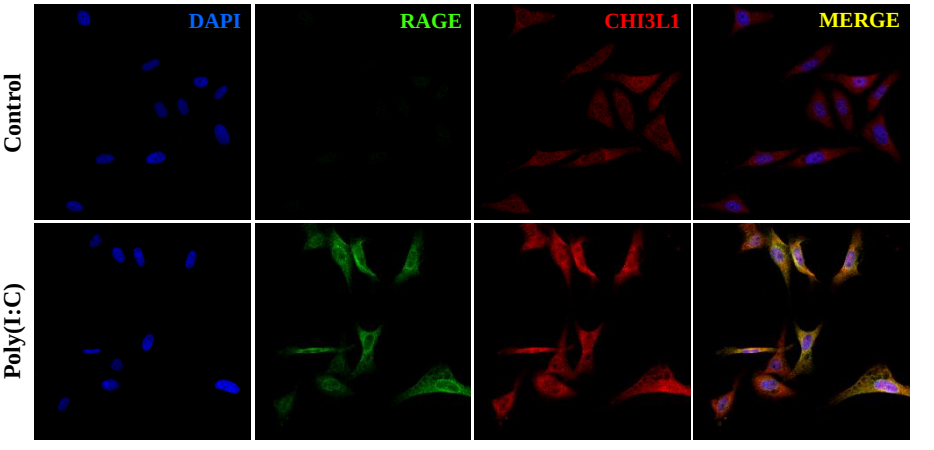
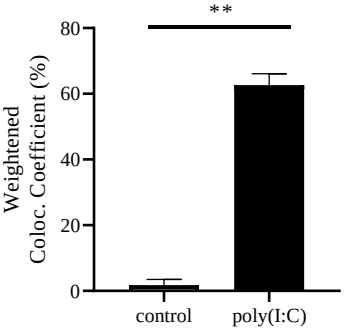



**(E)**

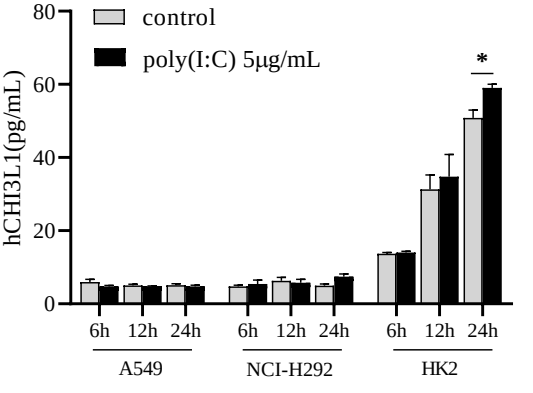
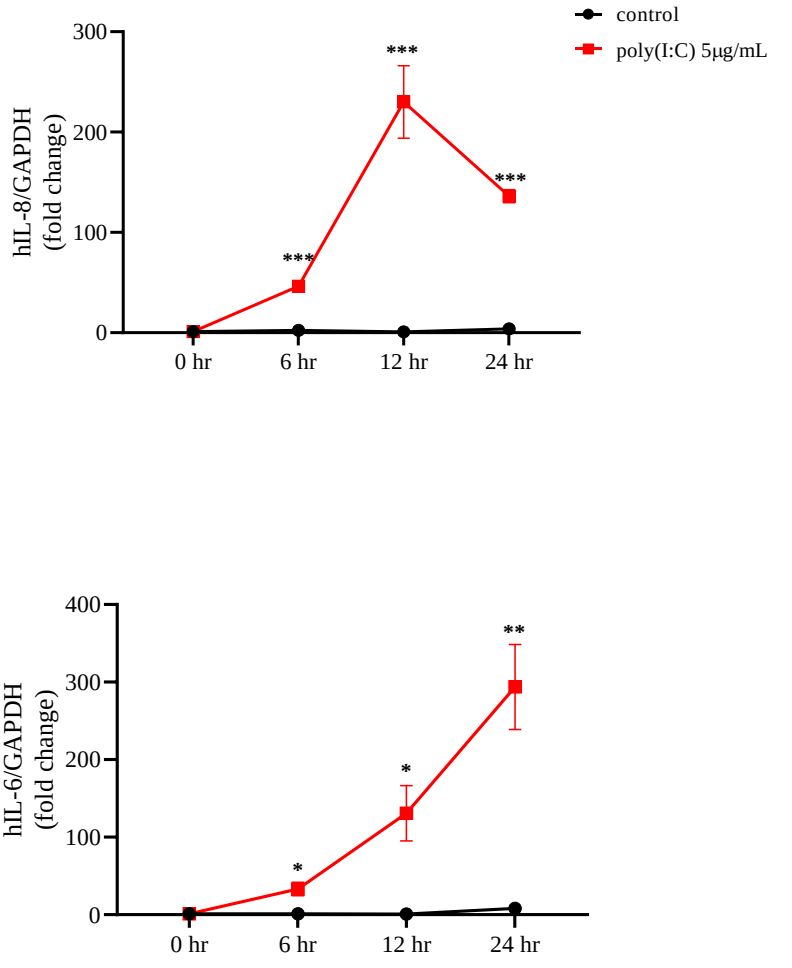


**Figure 1. CHI3L1 expression is increased in poly(I:C) stimulated BEAS-2B cells.** BEAS-2B cells were stimulated with 5μg/mL of synthetic dsRNA, poly(I:C), and the cells were harvested at the end of multiple time points. (A), (B) The expression levels of CHI3L1 in cell supernatants were detected by (A) ELISA and (B) western blot analysis. (C) The protein levels of CHI3L1 in cell lysates were analyzed by western blot analysis (top). Densitometric analysis of CHI3L1 was performed by Image J software (bottom). (D) Immunofluorescence staining images of CHI3L1 and RAGE in BEAS-2B cells were obtained using confocal microscopes (top). Quantification of CHI3L1 with RAGE was measured using the ZEN software (bottom). (E) The protein levels of CHI3L1 in supernatants of A549 cells, H292 cells, and HK2 cells were analyzed by ELISA. The data represents the mean ± SEM of at least three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ comparison between control groups and time-matched poly(I:C) stimulated groups. CHI3L1, chitinase 3-like 1; Poly(I:C), Polyinosinic : polycytidylic acid; RAGE, receptor for advanced glycation end products; ELISA, enzyme-linked immunosorbent assay.

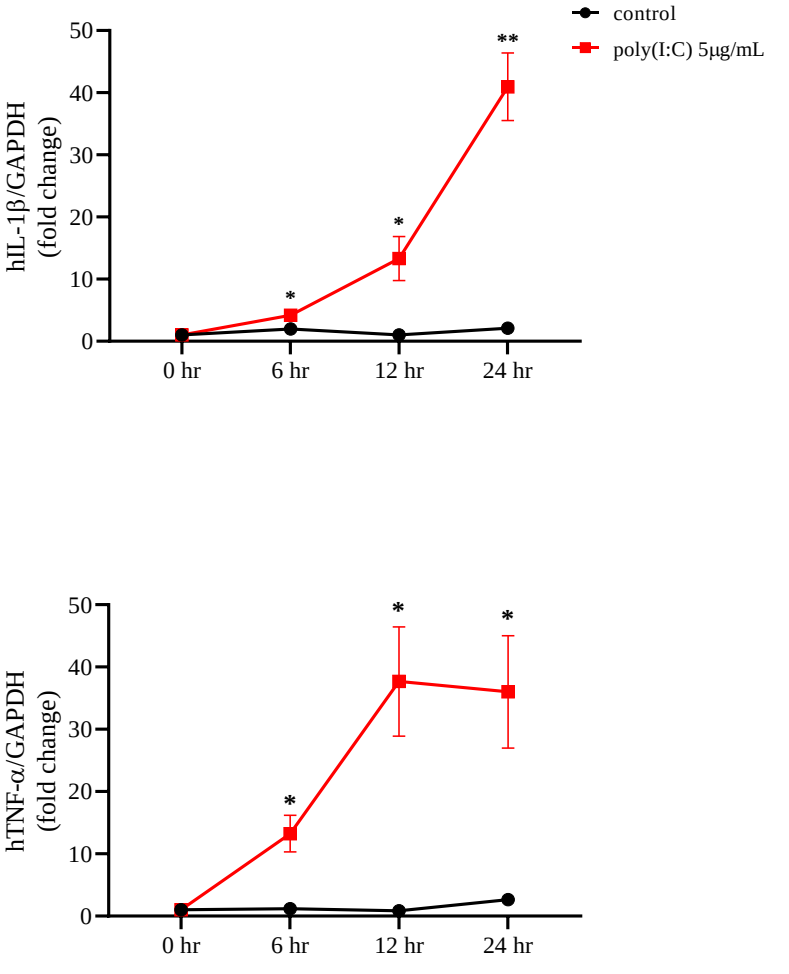
### 2. Expression of pro-inflammatory cytokines is elevated by poly(I:C) stimulation in BEAS-2B cells

Following the increased expression of CHI3L1 in BEAS-2B cells by poly(I:C) stimulation, we also investigated whether pro-inflammatory cytokines were induced by poly(I:C) stimulation. The mRNA expression levels of IL-8, IL-6, IL-1β, and TNF-α were induced by poly(I:C) stimulation (Fig. 2A and 2B). In addition to mRNA expression levels, the secreted protein levels of these cytokines were also increased by poly(I:C) stimulation (Fig. 2C and 2D). In these results, we confirmed that the pro-inflammatory cytokines which are known as important immune mediators in viral infections were induced in bronchial epithelial cells stimulated with poly(I:C).

**(A)**



**(B)**



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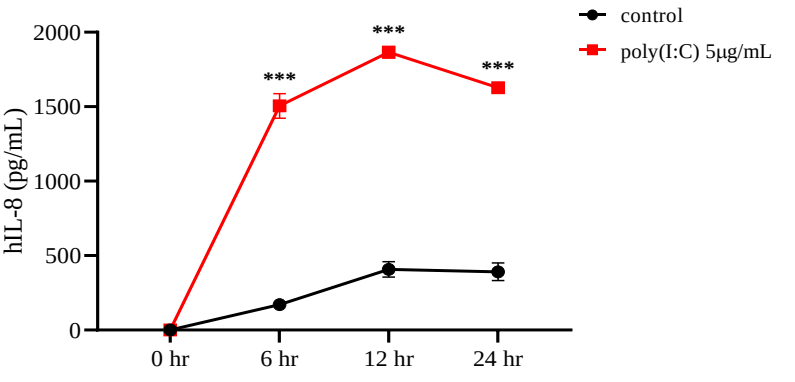
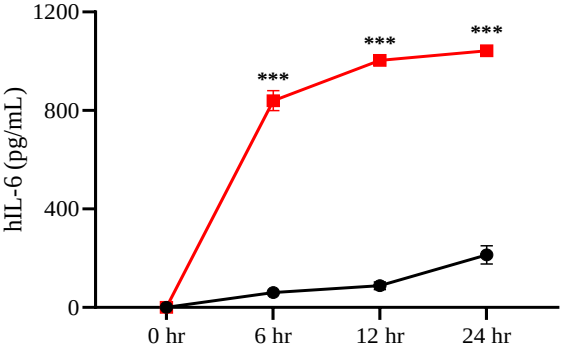


**(D)**

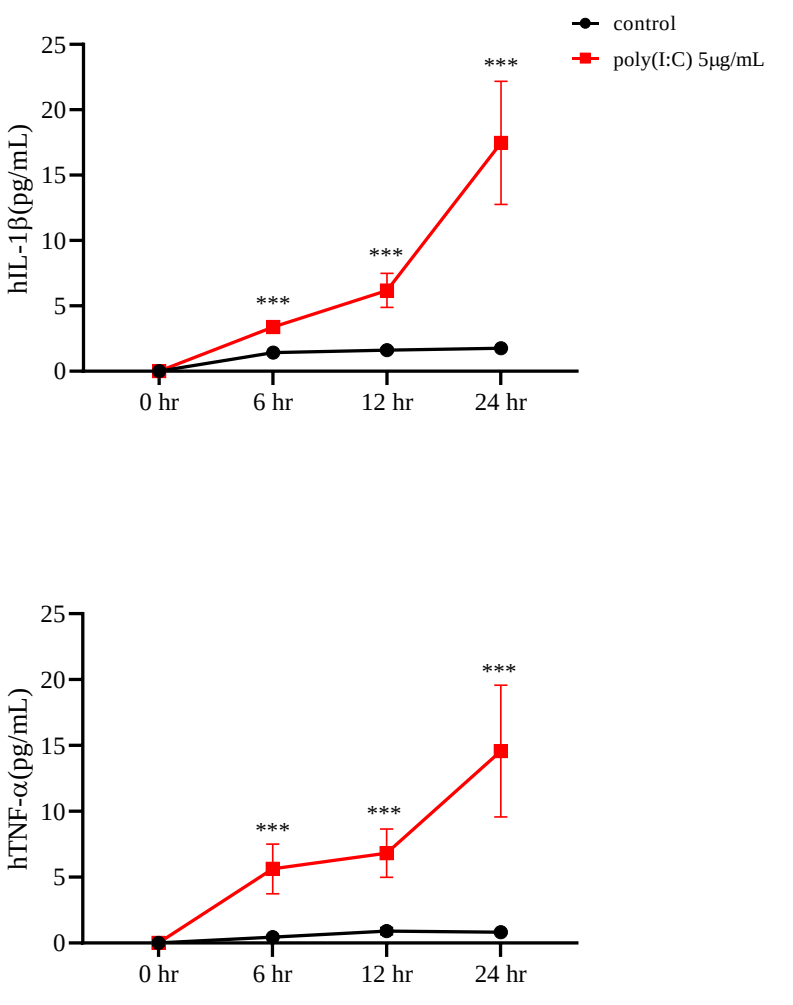


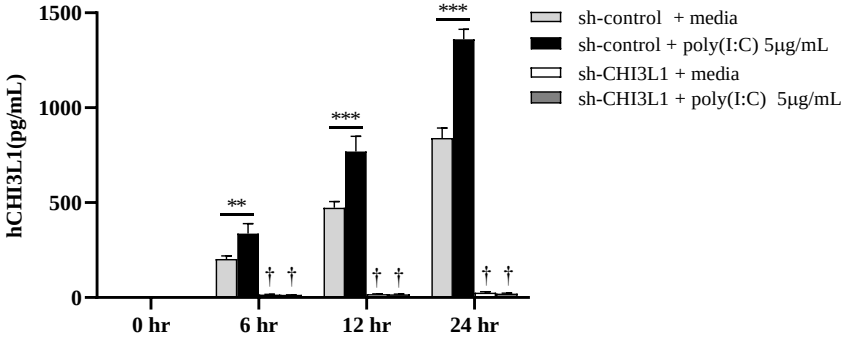
**Figure 2. Pro-inflammatory cytokines are increased in poly(I:C) stimulated BEAS-2B cells.** BEAS-2B cells were stimulated with 5μg/mL poly(I:C). The cells and supernatants were harvested at the end of multiple time points. (A), (B) The mRNA expression levels of IL-8, IL-6, IL-1β, and TNF-α were analyzed by real-time PCR. (C), (D) The expression levels of IL-8, IL-6, IL-1β, and TNF-α in cell supernatants were detected by ELISA. The data represents the mean ± SEM of three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ comparison between control group and time-matched poly(I:C) stimulated group. IL, interleukin; TNF-α, tumor necrosis factor-α; Poly(I:C), Polyinosinic : polycytidylic acid; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay.

### 3. CHI3L1 is involved in poly(I:C) induced IL-8 expression

To elucidate whether CHI3L1 contributes to pro-inflammatory responses by poly(I:C) stimulation, CHI3L1 expression of BEAS-2B cells was silenced by shRNA lentiviral transduction. As shown in Fig. 3A and 3B, CHI3L1 expression levels of BEAS-2B cells were dramatically abolished by CHI3L1 shRNA. But in cells transfected with control shRNA, CHI3L1 expression levels were increased by poly(I:C) stimulation compared to media-treated cells (Fig. 3A and 3B).

Next, we analyzed the expression levels of IL-8 in shRNA-containing BEAS-2B cells by poly(I:C) stimulation. The mRNA and protein expression levels of IL-8 were diminished in CHI3L1 knock-down BEAS-2B cells, compared to time-matched control cells (Fig. 3C and 3D). In these results, we suggest that CHI3L1 contributes to IL-8 secretion induced by poly(I:C) in bronchial epithelial cells.

**(A)**

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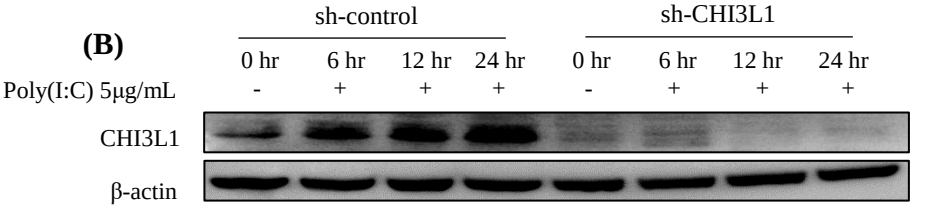


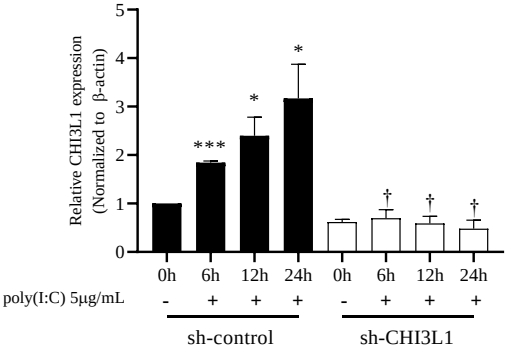

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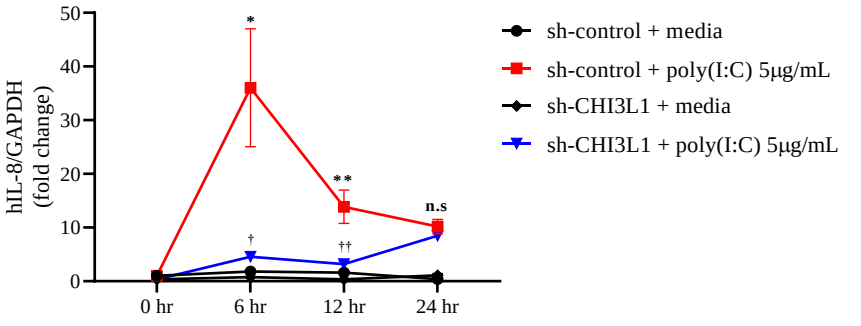



**(D)**

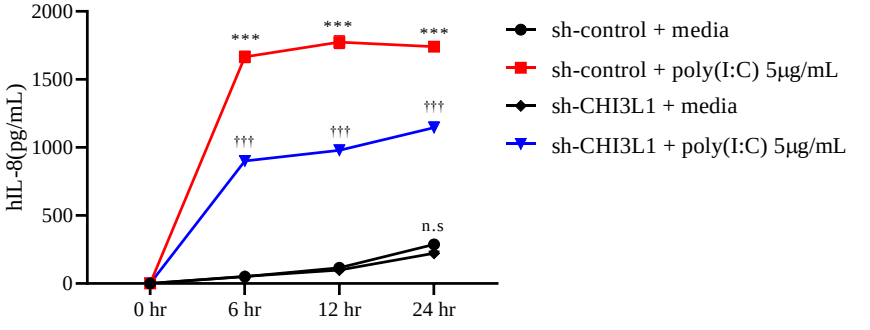
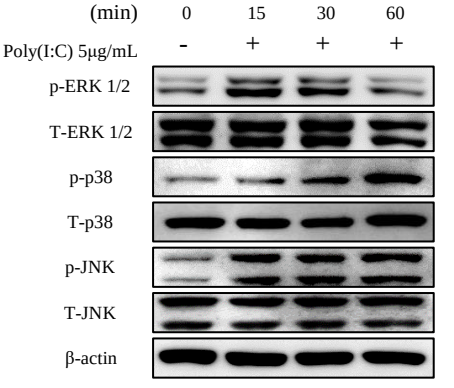


**Figure 3. CHI3L1 deficiency diminished IL-8 expression induced by poly(I:C) stimulation.** BEAS-2B cells transfected with control shRNA (sh-control) or CHI3L1 shRNA (sh-CHI3L1) were stimulated with 5μg/mL poly(I:C). (A) The secreted CHI3L1 levels in supernatants were analyzed by ELISA. (B) The protein levels of CHI3L1 in cell lysates were analyzed by western blot analysis (top). Densitometric analysis of CHI3L1 was performed by Image J software (bottom). (C) The mRNA expression levels of IL-8 were analyzed by real-time PCR (D) The expression levels of IL-8 in cell supernatants were detected by ELISA. The data represents the mean ± SEM of at least three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ comparison between sh-control cells treated with media and poly(I:C). † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$ time-matched comparison between poly(I:C) treated sh-control cells and sh-CHI3L1 cells. CHI3L1, chitinase 3-like 1; shRNA, short hairpin RNA; IL-8, interleukin-8; n.s, not significant; Poly(I:C), Polyinosinic : polycytidylic acid; PCR, polymerase chain reaction; ELISA, enzyme-linked immunosorbent assay.

### 4. MAPK pathways contribute to IL-8 expression induced by poly(I:C) stimulation

According to our previous observations, we showed that the expression levels of pro-inflammatory cytokines were increased by poly(I:C) stimulation. In previous studies, it has been demonstrated that phosphorylation of MAPK signaling pathways induce the expression of pro-inflammatory cytokines against various stimuli[33,34]. Thus, we further investigated that which signaling pathways were involved in IL-8 secretion induced by poly(I:C). As shown in western blot analysis, MAPK signaling pathways – ERK 1/2, p38, and JNK were phosphorylated by poly(I:C) stimulation (Fig. 4A and 4B). Following this data, we examined the inhibitory effect of MAPK pathways in IL-8 production. The expression levels of IL-8 were decreased by the specific inhibitors - PD98059 (ERK1/2), SB202190 (p38), and SP600125 (JNK) in a dose-dependent manner. Among these inhibitors of MAPK pathways, SB202190 most effectively diminished IL-8 secretion (Fig. 4C). In these results, we suggest that IL-8 expression induced by poly(I:C) is related to the phosphorylation of MAPK signaling pathways.

**(A)**

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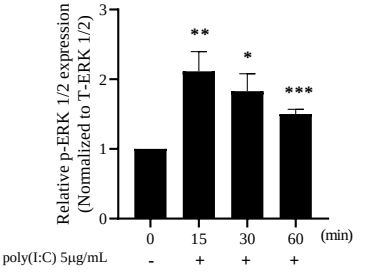


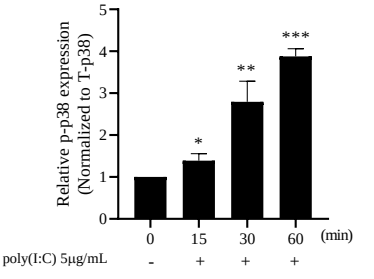


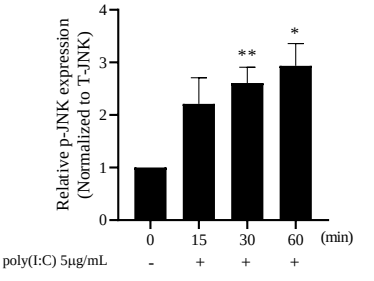

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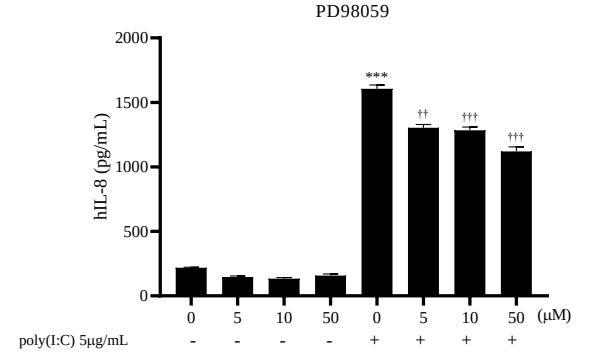
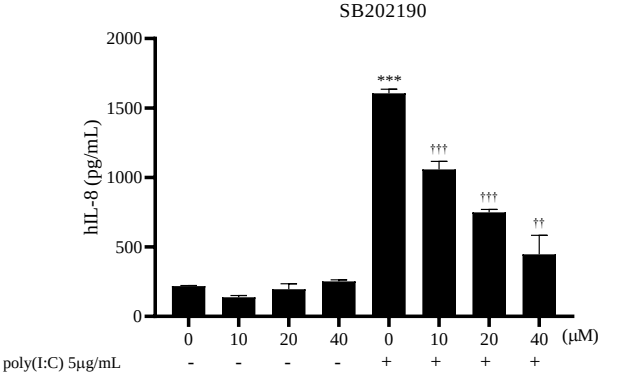


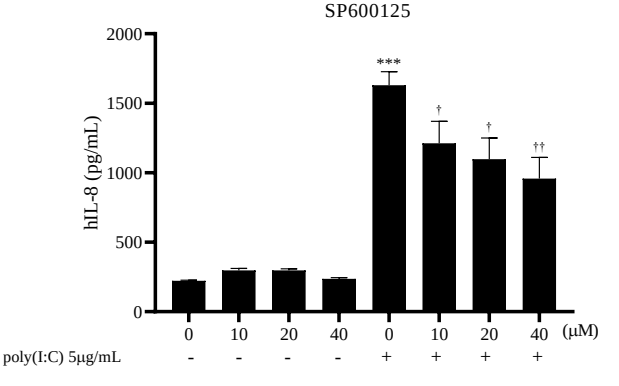



**Figure 4. Inhibition of MAPK pathways decreased IL-8 expression induced by poly(I:C) stimulation.** BEAS-2B cells were stimulated with 5μg/mL poly(I:C) for multiple time points. (A) Phosphorylation of MAPK signaling pathways by poly(I:C) was detected by western blot analysis. (B) Densitometric analysis of p-ERK 1/2 (top), p-p38 (middle), and p-JNK (bottom) was performed by Image J software. (C) The expression levels of IL-8 in cell supernatants were analyzed by ELISA. The specific inhibitors for ERK 1/2 (top), p38 (middle), and JNK (bottom) were pre-incubated for 30 min and stimulated with 5μg/mL poly(I:C) for 12 hr. The data represents the mean ± SEM of at least three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ comparison between control group and poly(I:C) stimulated groups. † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$ comparison between the groups treated with inhibitors and poly(I:C) and the groups treated with poly(I:C) alone. Poly(I:C), Polyinosinic : polycytidylic acid; P, phosphorylation; T, total; ERK 1/2, extracellular signal-regulated kinases 1/2; JNK, c-Jun N-terminal kinase; IL-8, interleukin-8; MAPK, mitogen-activated protein kinase; ELISA, enzyme-linked immunosorbent assay.

### 5. CHI3L1 regulates phosphorylation of MAPK pathways induced by poly(I:C) stimulation

In our above observations, MAPK pathways are phosphorylated by poly(I:C) stimulation and regulate the expression of IL-8. Moreover, the interaction between CHI3L1 and MAPK pathways has been revealed in previous studies[26,35,36]. Therefore, we hypothesized that CHI3L1 regulates IL-8 secretion induced by poly(I:C) stimulation through mediating the phosphorylation of MAPK pathways. To investigate our hypothesis, we stimulated poly(I:C) to control cells or CHI3L1 knock-down cells and compared the phosphorylation of MAPK pathways. In control cells, phosphorylation of ERK 1/2, p38, and JNK was observed as non-transfected BEAS-2B cells shown in Fig. 4A. However, the phosphorylation of the MAPK pathways by poly(I:C) stimulation was significantly abrogated in CHI3L1 knock-down cells. These results suggest that CHI3L1 regulates phosphorylation of MAPK signaling pathways which mediates IL-8 secretion during the viral infections of bronchial epithelial cells.

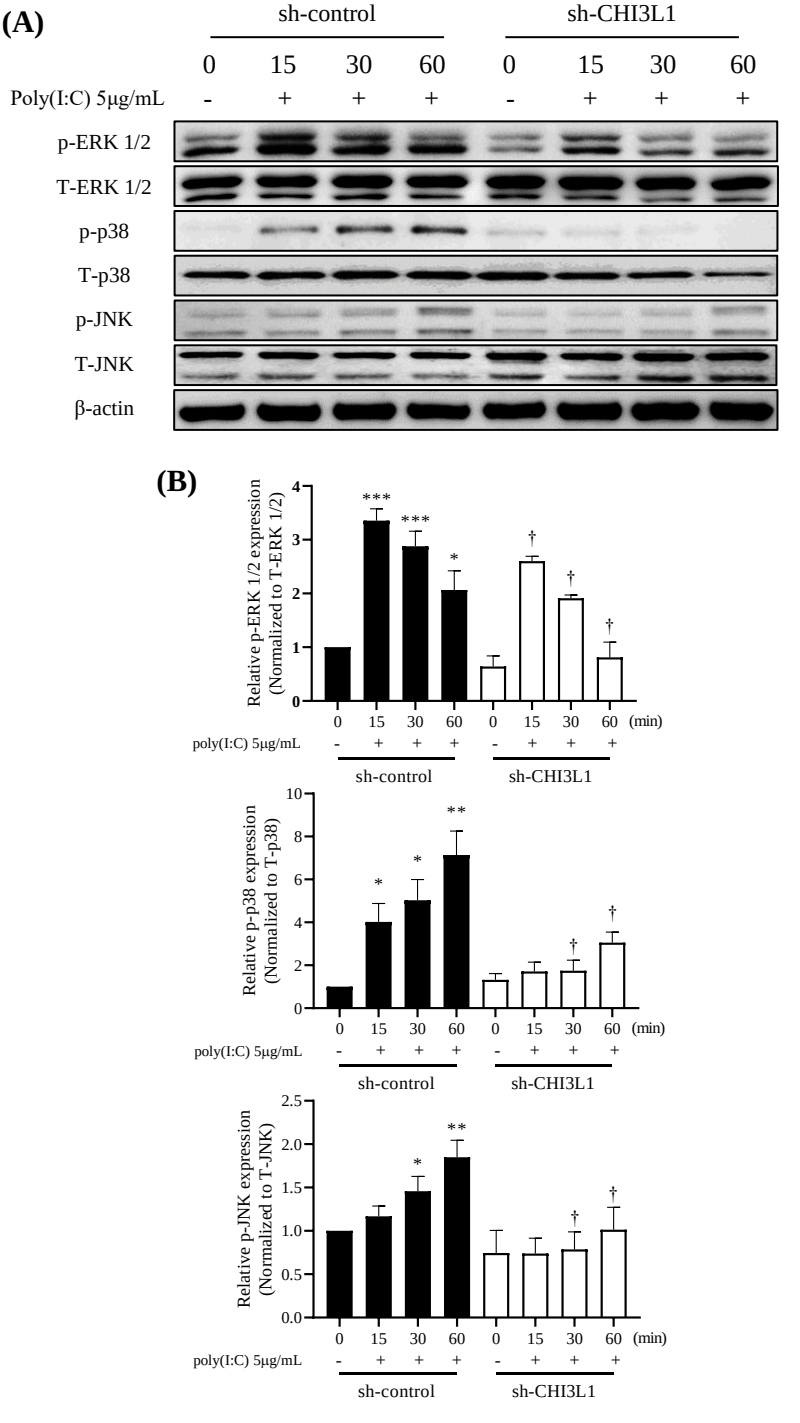
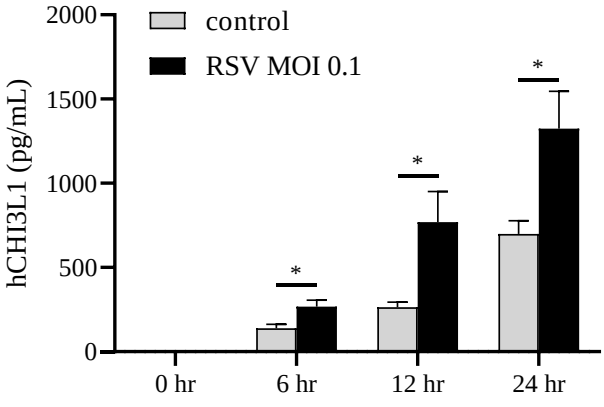


**Figure 5. CHI3L1 deficiency diminished phosphorylation of MAPK pathways induced by poly(I:C) stimulation.** BEAS-2B cells transfected with control shRNA (sh-control) or CHI3L1 shRNA (sh-CHI3L1) were stimulated with 5μg/mL poly(I:C) for multiple time points. (A) The phosphorylation of MAPK pathways – ERK 1/2, p38, and JNK was analyzed by western blot analysis. (B) Densitometric analysis of p-ERK 1/2 (top), p-p38 (middle), and p-JNK (bottom) was performed by Image J software. The data represents the mean ± SEM of at least three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ comparison between sh-control cells treated with media and poly(I:C). † $P < 0.05$ time-matched comparison between poly(I:C) treated sh-control cells and sh-CHI3L1 cells. shRNA, short hairpin RNA; CHI3L1, chitinase 3-like 1; Poly(I:C), Polyinosinic : polycytidylic acid; P, phosphorylation; T, total; ERK 1/2, extracellular signal-regulated kinases 1/2; JNK, c-Jun N-terminal kinase; MAPK, mitogen-activated protein kinase.

### 6. The expressions of CHI3L1 and IL-8 are elevated by RSV infection in BEAS-2B cells

We showed that the expression levels of CHI3L1 and IL-8 were induced by poly(I:C) stimulation. To investigate whether CHI3L1 and IL-8 are induced by RSV infection of bronchial epithelial cells, BEAS-2B cells were infected with RSV at MOI of 0.1. As shown in Fig. 6A and 6B, RSV infection significantly increased expression levels of CHI3L1 and IL-8 in a time-dependent manner, which is similar to poly(I:C) stimulation (Fig. 6A and 6B). Therefore, we demonstrated that the expressions of CHI3L1 and IL-8 in bronchial epithelial cells are also induced by RSV infection, which is comparable to that of synthetic dsRNA stimulation.

**(A)**



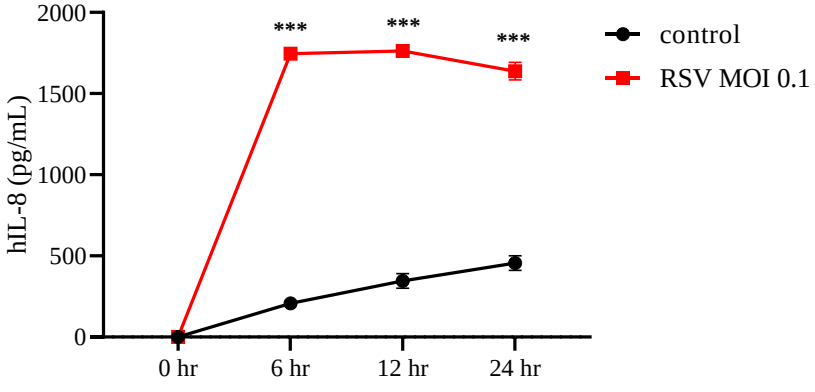
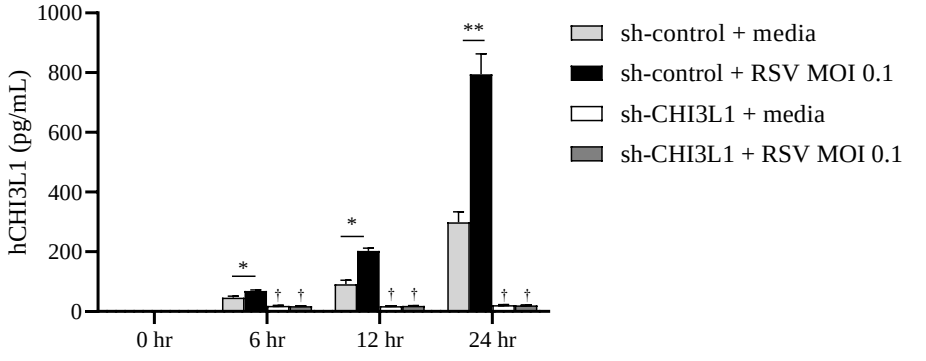



**Figure 6. The expressions of CHI3L1 and IL-8 are increased in RSV infected BEAS-2B cells.** BEAS-2B cells were infected with RSV at MOI of 0.1, and cells were harvested at the end of multiple time points. (A), (B) The expression levels of (A) CHI3L1 and (B) IL-8 in cell supernatants were detected by ELISA. The data represents the mean ± SEM of the three independent experiments. * $P < 0.05$, *** $P < 0.001$ comparison between media treated control groups and time-matched RSV infected groups. CHI3L1, chitinase 3-like 1; RSV, respiratory syncytial virus; MOI, multiplicity of infection; IL-8, interleukin-8; ELISA, enzyme-linked immunosorbent assay.

### 7. CHI3L1 is involved in RSV induced IL-8 secretion

As shown in Figure 6, the infection with RSV increased expression of CHI3L1 and IL-8, which is consistent with poly(I:C) stimulation. Finally, we investigated the relation between CHI3L1 and IL-8 in RSV infection using shRNA transfected BEAS-2B cells. The shRNA transfected BEAS-2B cells were infected with RSV at MOI of 0.1 and the expression levels of CHI3L1 and IL-8 were analyzed. We confirmed that CHI3L1 expression levels were also increased by RSV infection in control cells (Fig. 7A). Interestingly, IL-8 expression levels induced by RSV infection were diminished in CHI3L1 knock-down BEAS-2B cells, compared to the control cells (Fig. 7B). In these results, we demonstrated that CHI3L1 regulates IL-8 expression induced by RSV infection in bronchial epithelial cells.

**(A)**

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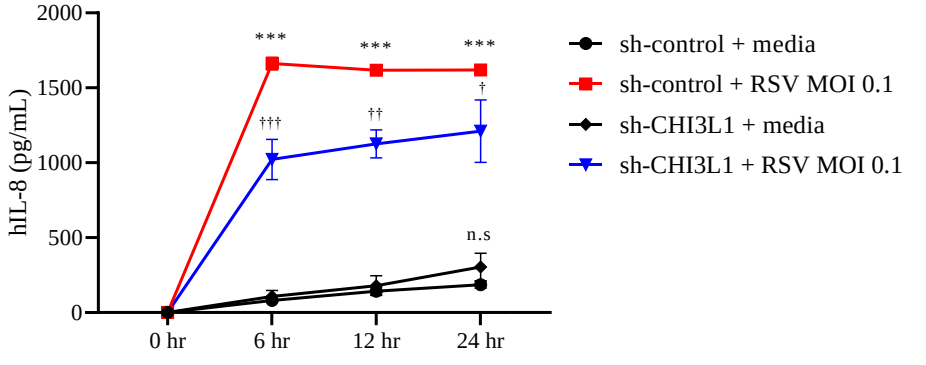

**Figure 7. CHI3L1 deficiency diminished IL-8 expression induced by RSV infection.** Cells transfected with either control shRNA or CHI3L1 shRNA were infected with RSV at MOI of 0.1. (A), (B) The expression levels of (A) CHI3L1 and (B) IL-8 in cell supernatants were analyzed by ELISA. The data represents the mean ± SEM of the three independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ comparison between sh-control cells treated with media and infected with RSV. † $P < 0.05$, †† $P < 0.01$, ††† $P < 0.001$ time-matched comparison between RSV infected sh-control cells and sh-CHI3L1 cells. CHI3L1, chitinase 3-like 1; shRNA, short hairpin RNA; RSV, respiratory syncytial virus; MOI, multiplicity of infection; IL-8, interleukin-8; poly(I:C), ELISA, enzyme-linked immunosorbent assay.

## IV. DISCUSSION

In this study, we focused on the regulatory roles of CHI3L1 in IL-8 expression induced by viral infections of bronchial epithelial cells and demonstrated the following results. First, poly(I:C) stimulation results in the expression of CHI3L1 and pro-inflammatory cytokines in BEAS-2B cells. Poly(I:C) stimulation induces the phosphorylation of MAPK signaling pathways which are associated in the expression of IL-8. Second, CHI3L1 deficiency results in diminished IL-8 release induced by poly(I:C) stimulation. CHI3L1 deficiency abolished the phosphorylation of MAPK pathways compared to that in control cells. Finally, RSV infection results in the expression of CHI3L1 and IL-8 as in poly(I:C) stimulation. And CHI3L1 deficiency diminished IL-8 expression induced by RSV infection. Thus, we suggest that CHI3L1 regulates IL-8 expression induced by viral infections in bronchial epithelial cells.

In humans, CHI3L1 is secreted in various cells throughout the body, including inflammatory cells (e.g., neutrophils and macrophages) and structural cells (e.g., chondrocytes, tumor cells, endothelial cells, and epithelial cells)[37-39]. Chitinase 3-like 1 is known to be important in homeostasis. CHI3L1 regulates cell survival and proliferation and also has a mitogenic effect on human lung fibroblasts. Therefore CHI3L1 expression can be found in healthy subjects but increased in several diseases[40]. In the pathogenesis of CHI3L1, increased CHI3L1 mediates cell apoptosis, tumor metastasis, and inflammation[26-28,35,41-43]. Also, previous

studies demonstrated that CHI3L1 is related to lung function and respiratory diseases[27,29,44]. Furthermore, CHI3L1 expression is increased in airway epithelial cells and alveolar macrophages of RSV-infected C57BL/6 mice[29]. Based on these studies, we stimulated bronchial epithelial cells with synthetic viral dsRNA analog, poly(I:C), and RSV to explore the role of CHI3L1 in viral infection of airway epithelial cells. Our results revealed that CHI3L1 expression was elevated by both poly(I:C) stimulation and RSV infection of BEAS-2B cells. CHI3L1 deficiency results in diminished secretion of IL-8 induced by poly(I:C) or RSV infection. IL-8 induces airway inflammation and has been shown to be increased in patients with inflammatory lung diseases[17-19]. Previous studies demonstrated that blockade of IL-8 reduced neutrophil infiltration and tissue damage[45,46]. Taken together, we suggest that CHI3L1 may be a therapeutic target for the inflammation induced by respiratory viral infections[29].

The mechanisms by which CHI3L1 regulates downstream signaling in viral infection have not been clearly elucidated. According to previous studies, it has shown that CHI3L1 combines with RAGE during inflammation[47]. Also, RAGE regulates the expression of pro-inflammatory cytokines by altering the phosphorylation of MAPK pathways[34]. Therefore, we explored the interaction between CHI3L1 and RAGE in viral infections of bronchial epithelial cells. In our immunofluorescence study, we confirmed that poly(I:C) stimulation resulted in increased expression and co-localization of CHI3L1 and RAGE. This result

may suggest that increased CHI3L1 mediates the phosphorylation of the MAPK signaling pathway through RAGE, finally resulting in IL-8 secretion. In addition to RAGE, the receptors that bind with CHI3L1 are known as follows: Interleukin-13 receptor subunit alpha-2 (IL-13Rα2), galectin-3 (Gal-3), and chemoattractant receptor-homologous molecule expressed on Th2 cells (CRTH2)[48-50]. The interaction between CHI3L1 and other receptors in inflammation caused by viral infection needs to be further elucidated.

Airway epithelial cells are the first line of the innate immune system since exogenous antigens including viruses, bacteria, and pollens enter the airway through respiration[51]. Hence, in airway epithelial cells, there are numerous pathogen recognition receptors (PRRs) that recognize exogenous antigens. The PRRs that recognized antigens can activate downstream signaling pathways which induce the secretion of pro-inflammatory cytokines. In this study, we have confirmed that pro-inflammatory chemokines and cytokines were increased by poly(I:C) stimulation in BEAS-2B cells. In addition, we showed the relation between IL-8 and MAPK signaling pathways using specific inhibitors of MAPK pathways. Interestingly, we revealed that SB202190, p38 MAPK inhibitor, most effectively suppressed poly(I:C) induced IL-8 secretion. Through these results, we suggest that airway epithelial cells which are infected with respiratory viruses can induce pro-inflammatory cytokines, and IL-8 expression was induced through MAPK signaling pathways.

In our study, we demonstrated that CHI3L1 deficiency diminished the phosphorylation of MAPK signaling pathways. Based on our observations, we suggest that CHI3L1 contributes to the phosphorylation of MAPK pathways which eventually mediates the expression of IL-8. In addition to MAPK pathways, NF-κB is also known as the regulator of inflammation against several stimuli. In previous *in vitro* studies, it has been demonstrated that the expression of pro-inflammatory cytokines by chitinase, German cockroach extract, and lipopolysaccharide in airway epithelial cells was induced via NF-κB pathways[15,16,52]. Moreover, recombinant CHI3L1 treatment to BEAS-2B cells induced IL-8 secretion via phosphorylation of NF-κB pathways[36]. For this reason, CHI3L1 is also expected to regulate inflammation through NF-κB pathway in bronchial epithelial cells. Therefore, the interaction between CHI3L1 and NF-κB in inflammation of the airway epithelial cells needs to be further elucidated.

## V. CONCLUSION

In this study, we revealed that CHI3L1 secretion and pro-inflammatory cytokines are associated with inflammation of bronchial epithelial cells induced by viral infections. We also showed that CHI3L1 deficiency reduced IL-8 expression which is associated with the activation of MAPK signaling pathways.

Therefore, CHI3L1 is involved in IL-8 expression by regulating activation of MAPK pathways during viral infections in bronchial epithelial cells.

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## ABSTRACT (IN KOREAN)

# 인체 기도 상피 세포의 IL-8 발현에서 Chitinase 3-like 1의 역할

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**배경:** 호흡기 바이러스는 여러 호흡기 질환을 유발하거나 악화시키는 원인 중 하나이다. 호흡을 통해 들어온 호흡기 바이러스는 기도 상피 세포를 감염시키고, 인터루킨-8 분비를 유도하여 폐 염증을 유발한다. Chitinase 3-like 1 (CHI3L1)은 인간에서는 YKL-40, 쥐에서는 BRP-39 으로, 여러 염증성 질환과 연관이 있지만 그중에서도 폐 염증 및 기능과의 상관관계가

밝혀지며 폐 기능의 바이오 마커로 알려진 물질이다. 최근 연구 결과, 여러 호흡기 바이러스 감염에 의한 염증 반응에 있어서 CHI3L1 과의 관련성이 규명되었다. 하지만 바이러스 감염에 의해 유도된 면역 반응에 있어서 CHI3L1 이 어떠한 역할을 하는지 밝혀진 바 없다. 따라서 본 연구에서, 기도 상피 세포의 호흡기 바이러스 감염으로 인해 유도된 인터루킨-8 의 발현에 있어서 CHI3L1 의 역할을 규명하고자 하였다.

**방법:** 본 연구에서는 인위적으로 합성한 바이러스 리보핵산인 polyinosinic : polycytidylic acid (poly(I:C))을 기관지 상피 세포주인 BEAS-2B 세포에 처리하였고, CHI3L1 및 전염증성 사이토카인 발현, MAPK 신호 전달 경로를 분석하였다. 또한 유전 물질이 리보핵산으로 구성된 호흡기세포융합바이러스를 BEAS-2B 세포에 감염시키고, CHI3L1과 인터루킨-8의 발현을 분석하였다.

**결과:** 대조군에 비해 poly(I:C)를 처리한 군에서 CHI3L1 및 전염증성 사이토카인의 발현 증가를 확인하였다. 반면 BEAS-2B 세포의 CHI3L1 발현을 억제하였을 때, poly(I:C)에 의한 인터루킨-8의 발현이 감소하는 것을 확인하였다. 또한, poly(I:C)에 의해 유도되는 인터루킨-8의 발현에 있어서 MAPK 신호 전달 경로가

관여하였음을 밝혔고, CHI3L1 유전자의 발현을 억제한 세포에서 야생형 세포에 비해 MAPK 신호 전달 경로의 활성화가 감소하는 것을 확인하였다. 이어서 호흡기세포융합바이러스에 감염된 BEAS-2B 세포에서, CHI3L1과 인터루킨-8의 발현이 poly(I:C) 자극과 비슷한 경향성을 나타내며 증가하였다. 마지막으로, BEAS-2B 세포의 CHI3L1 발현을 억제하였을 때 야생형 세포에 비해 호흡기세포융합바이러스 감염으로 인한 IL-8의 발현이 감소한 것을 확인하였다.

**결론:** 기도 상피 세포에서 CHI3L1이 호흡기 바이러스 감염으로 인한 인터루킨-8의 분비를 MAPK 신호 전달 경로의 활성화에 영향을 미침으로써 조절하는 역할을 밝혔다.

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핵심되는 말: 기도 상피 세포, chitinase 3-like 1, 인터루킨-8, MAPK 신호 전달 경로, 호흡기세포융합바이러스