The role of activated leukocyte cell adhesion molecule (ALCAM/CD166) in respiratory syncytial virus induced airway inflammation

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The role of activated leukocyte cell adhesion molecule (ALCAM/CD166) in respiratory syncytial virus induced airway inflammation

Directed by Professor Myung Hyun Sohn

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Seung Min Baek

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This certifies that the Master's Thesis of
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감사의 글

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저를 아껴 주시는 모든 친인척 분들과, 자주 찾아뵙지 못함에도 진심으로 응원해 주시고, 항상 애틋하게 대해 주시는 외할머니에게도 감사드립니다. 마지막으로, 이 학위 논문 한 편이 나오기까지 늘 철부지였던 저를 믿어 주시고, 옆에서 조건없는 사랑으로 헌신해 주신 아버지, 어머니께 이 작은 결실을 바칩니다. 대학원 입학 때부터 지금까지 많은 고민이 있을 때마다 항상 저보다도 더 많이 걱정해 주시느라 마음 아프셨을 부모님께 죄송하고 감사했습니다. 제가 이루어 낸 모든 것들이 부모님의 사랑 덕분임을 잊지 않고 늘 감사한 마음으로 살아가겠습니다. 그리고 어릴 때나 지금이나 항상 착한 마음씨로 동생을 위해주고, 매번 어려운 일이 생길 때마다 명쾌한 해결사처럼 나서서 도와주는, 나의 멘토와도 같은 오빠에게 너무나도 감사합니다. 이제는 새신랑이 된 오빠가 앞으로는 예쁜 소영언니와 행복하게 잘 살아갈 것임을 확신하고, 또 기도하겠습니다.

이 지면에 미처 다 표현하지 못한, 저에게 도움 주신 모든 분들께 감사의 말씀을 드립니다. 곁에 계신 분들에게 감사하며, 늘 겸손한 마음가짐으로 정진하는 사람이 될 수 있도록 노력하겠습니다.

2023년 12월
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ABSTRACT

The role of activated leukocyte cell adhesion molecule (ALCAM/CD166) in respiratory syncytial virus induced airway inflammation

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(Directed by Professor Myung Hyun Sohn)

Background : Respiratory syncytial virus (RSV) is a common respiratory virus that cause acute lower respiratory tract infectious (LRTI) diseases. Interleukin (IL)-33, a critical inflammatory mediator which activates type 2 responses, is expressed in airway epithelial cells in response to RSV infection. Activated leukocyte cell adhesion molecule (ALCAM) involves in inflammatory responses and allergic diseases. However, the role of ALCAM in RSV infection remains unclear. This study aims to investigate the association of ALCAM in regulating IL-33 expression derived from RSV infected airway epithelial cells.

Methods : A549 cells were also exposed to RSV at a multiplicity of infection (MOI) of 0.1 for 1 hour. To investigate the specific involvement of ALCAM, knockdown of ALCAM in the A549 cells was established using short hairpin RNA lentivirus. The expression of ALCAM and inflammatory cytokines including IL-33 induced by RSV infection were investigated. In order to verify the role of ALCAM in regulating expression of IL-33, anti-ALCAM antibody assay was conducted. Moreover, the phosphorylation of mitogen

activated protein kinase (MAPK) pathways was analyzed. To further investigate the involvement of ALCAM in RSV induced inflammation in vivo, C57BL/6 wild-type mice were infected with $8x10^6$ plaque forming units (PFU) of RSV.

Results : The increased expression levels of ALCAM and inflammatory cytokines were observed both in A549 cells and mice upon RSV infection. The viability of A549 cells was decreased in a time-dependent manner following RSV infection. Interestingly, IL-33 expression showed significant alleviation in ALCAM knockdown cells compared to control cells following RSV infection. Moreover, anti-ALCAM antibody treatment also reduced IL-33 expression after RSV infection. RSV infection induced phosphorylation of MAPK signaling pathways such as extracellular signal-regulated kinase 1/2 (ERK1/2), p38, and c-Jun-N-terminal kinase (JNK). Notably, the phosphorylation of MAPK pathways was diminished in ALCAM knockdown cells compared to control cells following RSV infection. Moreover, inhibition of these MAPK pathways remarkably decreased the expression of IL-33.

Conclusion : These findings indicate that ALCAM contributes IL-33 expression by regulating MAPK signaling pathways induced by RSV infection.

Key words : activated leukocyte cell adhesion molecule, airway epithelial cells, interleukin-33, MAPK signaling pathways, respiratory syncytial virus

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I. INTRODUCTION

Respiratory syncytial virus (RSV) is an enveloped, negative-sense, single-stranded RNA virus.[1,2] The symptoms of RSV infection are characterized by airway obstruction, nasal congestion, wheezing, cough, and tachypnea, which can progress to bronchiolitis and viral pneumonia in severe cases.[3] RSV infection is one of the major causes of lower respiratory tract infections (LRTI), particularly among young children and the elderly worldwide, often resulting in hospitalization.[4,5] It has been reported that there are approximately 33 million RSV-related LRTI episodes reported worldwide, with more than 118,200 fatalities in 2015.[6] However, despite constant efforts for several decades, no approved vaccine for young children or effective cure for RSV infection is available.[7,8]

Interleukin (IL)-33, a member of the IL-1 family, functions as a crucial cytokine in type

2 immunity and allergic airway diseases.[9] In response to RSV infection, the expression of IL-33 is triggered, activating type 2 innate lymphoid cells. This activation leads to the secretion of type 2 cytokines, including IL-5 and IL-13, which play a role in promoting type 2-mediated pulmonary disorders such as asthma.[10] Additionally, a previous study has demonstrated that inflammation exacerbation induced by RSV infection upregulates IL-33 levels in asthmatic patients.[11] Although it is evident that IL-33 plays a crucial role in virus-induced diseases, the mechanism underlying the regulation of IL-33 expression in airway inflammation induced by RSV infection has not been cleared yet.[9]

The activated leukocyte cell adhesion molecule (ALCAM), also known as CD166, is a 100-105 kDa membrane glycoprotein belonging to the immunoglobulin superfamily, characterized by the presence of five immunoglobulin-like domains.[12,13] ALCAM is expressed in various cell types, including epithelial cells, leukocytes, hematopoietic stem cells, bone marrow precursors, endothelial cells, neurons, and liver cells.[14] ALCAM is involved in various physiological processes, such as tumor progression, tumor metastasis, and T cell activation.[15,16] Additionally, it is associated with cell adhesion, autoimmune diseases, and inflammatory diseases.[16-18] Previous studies have reported an upregulation of ALCAM expression in the tissues or serum of patients of asthma, rhinitis, food allergy, and atopic dermatitis indicating its potential involvement in allergic diseases.[19-23] Moreover, a recent study has reported that ALCAM deficiency regulated the inflammatory responses during acute lung injury induced by lipopolysaccharide (LPS).[24] Although various studies have verified the role of ALCAM in different cancers, and allergic diseases, and inflammatory responses, the specific association between ALCAM and RSV-induced airway inflammation remains unclear.

Mitogen-activated protein kinase (MAPK) signaling pathways, including extracellular signal-regulated kinase 1/2 (ERK1/2), p38, and c-Jun-N-terminal kinase (JNK) subfamilies, play essential roles in various cellular functions, such as proliferation, differentiation, and apoptosis.[25] It has been demonstrated that MAPK pathways can also play an important role in regulating the expression of inflammatory cytokines and chemokines.[26] In addition,

previous researches shown that ALCAM is implicated in MAPK signaling pathways in various disease contexts, including TGF-β-induced lung fibrosis in type 2 alveolar epithelial cells, mice exposed to cocaine treatment, and a rat model of myocardial infarction.[19,27,28]

This study aimed to investigate the role of ALCAM in RSV-induced inflammation in airway epithelial cells, and assessed ALCAM expression under the conditions of RSV infection. ALCAM knockdown was established in airway epithelial cells, and the differences in RSV induced inflammatory responses between ALCAM knockdown cells and control cells were examined. Additionally, this study sought to identify the associated signaling pathways.

II. MATERIALS AND METHODS

1. Cell culture

The human alveolar epithelial cell line, A549 were obtained from American Type Culture Collection (ATCC, Manassas, VA, USA) and cultured in RPMI-1640 media (Hyclone, Logan, UT, USA) containing 10% fetal bovine serum and 100 U/ml penicillin/streptomycin (Hyclone). HEK-293 T cell, the human embryonic kidney 293T cell line (ATCC) was used to produce lentivirus particles. HEK-293 T cells were cultured in DMEM (Hyclone) supplemented with 10% fetal bovine serum and 100 U/ml penicillin/streptomycin. For RSV production, Human epithelioma-2 cell, HEp-2 (ATCC) were used. Hep-2 cells were grown in MEM (Hyclone) containing with 10% fetal bovine serum and 100 U/ml penicillin/streptomycin. Cells were grown in the humidified condition that consists of 95% air and 5% CO_2 at 37℃.

2. RSV production

To establish RSV production, HEp-2 cells seeded in 150mm cell culture dishes (SPL Life Sciences Co., Pocheon, South Korea) were inoculated with RSV and cultured for 4 days. RSV was harvested from the cultured cells using an ultracentrifuge at 25,000 rpm for 1 hour at 4℃. The harvested RSV stocks were stored in -70℃ immediately. To determine the titer of RSV stocks, a standard plaque assay was conducted. HEp-2 cells were placed in 6 well cell culture plates (VWR Funding Inc, Radnor, PA, USA), at a density of 1×10^6 cells per well. The cells were infected with RSV for 1 hour 30 minutes and immobilized by covering them with MEM medium containing a 0.6% concentration of low melting agar (Lonza, Basel, Switzerland) and incubated for 4 days. After incubation, the cells were stained with Neutral Red solution (Millipore, Bedford, MA, USA) in MEM medium. Cells were incubated under conditions consisting of 95% air and 5% CO_2 at 37℃. The plaque forming units/ml (PFU/ml) was determined by counting the number of total plaques which were made after RSV infection.

3. RSV infection

For RSV infection of airway epithelial cells, A549 cells were placed at a density of 7 x 10^5 cells per well in 6 well cell culture plates, or at a density of 2 x 10^4 cells per well in 96 well cell culture plates (Thermo Fisher Scientific, Waltham, MA, USA). The following day, the culture medium was aspirated, and the cells were gently washed with phosphate-buffered saline (PBS) to remove any residual serum. The cells were inoculated with RSV at a multiplicity of infection (MOI) of 0.1 for 1 hour. After RSV infection, RSV inoculum was removed and fresh serum-free media was added to the cells. The cells were then incubated for up to 72 hours. At 24 hours, 48 hours, and 72 hours post infection, both the incubated cells and culture supernatants were collected and stored at -70℃ until further assay.

4. Cell viability

Cell viability was determined by using a Cell Counting Kit-8 (CCK-8, Dojindo, Kumamoto, Japan). A549 cells were seeded at a density of 2 x 10^4 cells per well in 96 well cell culture plates and incubated overnight at 37℃. Subsequently, the cells were infected with RSV or serum-free media for 1 hour. After 72 hours incubation, a CCK-8 solution, WST-8[2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] was added to the cell culture media, and incubated for 1 hour. The absorbance was then measured at 450nm.

5. Lentivirus transduction

ALCAM knockdown in the A549 cells was estabilshed using a short hairpin RNA (shRNA) lentivirus transfection. To produce lentivirus particles, 4.5 x 10^6 of HEK-293 T cells were seeded in 100mm cell culture dishes (Corning Inc, Corning, NY, USA) and cultured in DMEM (Hyclone). HEK-293 T cells were transfected with ALCAM plasmid DNA using a Lipofectamine 2000 (Thermo Fisher Scientific) following the manufacturer's protocol. After 2 days of incubation, lentivirus particles were obtained from the

supernantant of the cells. For lentivirus trnasduction, A549 cells were treated with lentivirus particles containing 7 μg/ml of polybrene (Sigma-Aldrich, St. Louis, MO, USA) for 5 hours. The cells were then replaced wtih complete media and incubated at 37℃ for 3 days. To isolate the A549 cells containing the vectors, puromycin selection was conducted. A549 cells were incubated in media containing 1.5 μg/ml of puromycin (Sigma-Aldrich) for 3 days, and live cells were selected.

6. Anti-ALCAM antibody neutralization assay

To neutralize ALCAM expression, A549 cells were pre-incubated with a concentration of 0.1 or 0.5 μg/ml of anti-ALCAM antibody (Santa Cruz Biotechnology, Dallas, TX, USA) for 1 hour at 37°C before RSV infection. The RSV inoculum was removed, and post-incubation was conducted using the same concentration of anti-ALCAM antibody for an additional 72 hours.

7. Small interfering RNA (siRNA) transfection

The downregulation IL-33 gene expression in A549 cells was achieved through IL-33 siRNA transfection. 4 x 10^5 A549 cells were seeded in 6 well cell culture plates, and incubated in RPMI-1640 media supplemented with 10% fetal bovine serum for 24 hours. For siRNA transfection, the cells were transfected with 6 ug of IL-33 siRNA or scrambled siRNA (Santa Cruz Biotechnology) for 5 hours, following the manufacturer's protocol. After 5 hours incubation, the cells were added with 1 ml of the normal growth media containing 2 times the normal serum and antibiotics concentration, and incubated for additional 24 hours. Subsequently, the cells were replaced wtih fresh normal growth media and infected with RSV, as described above.

8. MAPK inhibition assay

In order to inhibit the signaling of MAPK pathways, A549 cells were pre-incubated with the serum-free media containing specific MAPK inhibitors before RSV infection. The

ERK1/2 inhibitor (PD98059), p38 inhibitor (SB202190) and JNK inhibitor (SP600125) were obtained from Calbiochem (San Diego, CA, USA). After 1 hour of RSV infection, the media containing respective inhibitors was replaced to the cells. The cells were then incubated at 37℃ for 72 hours.

9. Mouse RSV infection and *in vivo* experiments.

Female wild type C57BL/6 mice purchased from Orient Bio Inc. (Seongnam, South Korea) were used at 6 weeks of age. For the RSV infection of mice, the mice were intranasally inoculated with 50μl of RSV (8 x 10^6 PFU/ml) or an equal volume of PBS. At 7 days postinfection, the mice were sacrificed and analysed. All of animal experiments were approved by the Institutional Animal Care & Use Committee (IACUC) of the Yonsei University (protocol No. 2023-0011; Seoul, Korea).

10. Inflammatory cell count in bronchoalveolar lavage (BAL) fluid

During the sacrifice, 2 x 0.9 ml of PBS was injected to lung throughout the blunted trachia. The lavaged fluid was collected and centrifuged at 3000 rpm for 5 minutes at 4℃ to seperate the BAL fluid cells. The supernatant was frozen at -70℃ for later analysis. Cell pellet was resuspended in PBS for total and differential cell counts. Total cells mixed with an equal volume of Trypan blue solution were counted using a hemocytometer. BAL cells were centrifuged on the slide glass using a Cytospin centrifuge (Thermo Fisher Scientific). Differential cell counts were conducted by Diff-Quik stain kit (ITW Reagents, Catalunya, Spain).

11. Histological analysis and Immunohistochemistry

The lung tissues were fixed in 4% formalin for 3 days and embedded in paraffin. 5-μm sectioned lung specimens were stained with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS) to evaluate the inflammation and mucus production. To observe the expression of ALCAM in lung tissues, immunohistochemistry was conducted. Unstained

lung sections were deparaffinized by washing twice with xylene for 10 minutes each, followed by immersion in 100% ethanol for 5 minutes (repeated twice), 95% ethanol for 5 minutes, and 70% ethanol for 5 minutes. Lung tissues were rehydrated with retrieval buffer (Dako Denmark A/S, Glostrup, Denmark) for 20 minutes in the bolied steamer. After cooling at room temperatrue for 30 minutes, lung sections were washed three times with PBS containing 0.1% Tween 20 (PBST) for 5 minutes. Next, the tissues were blocked wtih peroxidase blocking solution (Dako Denmark A/S) for 5 minutes and protein block solution (Dako Denmark A/S) for 1 hour. After then, the anti-ALCAM antibody or normal rabbit IgG (Santa Cruz Biotechnology) dileuted 1:200 in antibody diluent (Dako Denmark A/S) was added on the lung sections and overnight incubated at 4℃. The slides were washed with PBST for three times and applied with labelled polymer HRP (Dako Denmark A/S) for 30 minutes. The staining was performed using a 3,3'-diaminobenzidine solution (Dako Denmark A/S), and the reaction was terminated by washing with deionized water. The stained lung tissues were fixed with Faramount Mounting Medium (Dako Denmark A/S).

12. Immunofluorescence

For two color immunoflurescence, lung tissue slides were rehydrated with xylene and ethanol, and retrievaled using a retrieval buffer as described above. The slides were subjected to anti-ALCAM, anti-clara cell 10 kDa protein (CC10, Santa Cruz Biotechnology), and anti-prosurfactant protein C (SPC, Millipore) antibodies and incubated at 4℃ overnight. The following day, Alexa Fluor 555, 488, and 647-conjugated secondary antibodies targeting mouse, goat, and rabbit seperately were added. After 1 hour incubation at room temperature, the nucleis of the lung tissues were stained with 4',6-diamidino-2-phenylindole (Thermo Fisher Scientific) for 5 minutes. The slides were mounted with mounting medium (Dako Denmark A/S) and the images were captured by the LSM 700 confocal microscope (Carl Zeiss, Oberkochen, Germany). The acquired images were analyzsd using a ZEN software.

13. Real-time polymerase chain reaction (PCR)

Total RNA was extracted from harvested cells or mouse lung tissues using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) following the RNA extraction protocol. The isolated total RNA was quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific). For reverse-transcription PCR, a calculated amount of 2 ug of total RNA was synthesized to the cDNA by using ReverTra Ace ® qPCR RT Master Mix Kit (Toyobo Co.,Ltd., Osaka, Japan). Then real-time PCR was accomplished using SYBR Green PCR Master Mix and StepOnePlus™ Real Time PCR System (Applied Biosystems, Foster City, CA, USA). The primer sequences used for human samples were as follows : hALCAM: 5'-ACT TGA CGT ACC TCA GAA TCT CA-3' (forward) and 5'-CAT CGT CGT ACT GCA CAC TTT-3' (reverse); hIL-1β: 5'-CTG TCC TGC GTG TTG AAA GA-3'(forward) and 5'-TTC TGC TTG AGA GGT GCT GA-3'(reverse); hIL-6: 5'-TAC CCC CAG GAG AAG ATT CC-3'(forward) and 5'-TTT TCT GCC AGT GCC TCT TT-3'(reverse); hIL-8: 5'-GTG CAG TTT TGC CAA GGA GT-3'(forward) and 5'-CTC TGC ACC CAG TTT TCC TT-3'(reverse); hIL-33: 5'- GTG ACG GTG TTG ATG GTA AGA T-3'(forward) and 5'-AGC TCC ACA GAG TGT TCC TTG-3'(reverse); hTNF-α: 5'-AAC CTC CTC TCT GCC ATC AA-3'(forward) and 5'-CCA AAG TAG ACC TGC CCA GA-3'(reverse); hGAPDH: 5'-AAG GTG AAG GTC GGA GTC AAC-3'(forward) and 5'-GGG GTC ATT GAT GGC AAC AAT A-3'(reverse). The primer sequences used for mouse samples were as follows : mIL-1β: 5'-AAG GAG AAC CAA GCA ACG ACA AAA- 3'(forward) and 5'-TGG GGA ACT CTG CAG ACT CAA ACT-3'(reverse); mIL-6: 5'-CTG CAA GAG ACT TCC ATC CAG-3'(forward) and 5'-AGT GGT ATA GAC AGG TCT GT-3'(reverse); mIL-13: 5'-CCT GGC TCT TGC TTG CCT T-3' (forward) and 5'- GGT CTT GTG TGA TGT TGC TC-3' (reverse); mIL-33: 5'-TCC AAC TCC AAG ATT TCC CCG-3'(forward) and 5'-CAT GCA GTA GAC ATG GCA GAA-3' (reverse); mMUC5AC: 5'-GGA CTT CAA TAT CCA GCT AC-3' (forward) and 5'-CAG CTC AAC AAC TAG GGC CAT-3' (reverse); mTNF-α: 5'-GGC AGG TCT ACT TTG GAG TCA TTG C-3'(forward) and (reverse); 5'- ACA TTC GAG GCT CCA GTG AAT TCG G-3' mGAPDH: 5'-TTG ATG

GCA ACA ATC TCC AC-3'(forward) and 5'-CGT CCC GTA GAC AAA ATG GT-3'(reverse); The mRNA expression levels were normalized using GAPDH as a reference gene. The fold increases were calculated using $2^{-\Delta\Delta CT}$ method.

14. Enzyme linked immunosorbent assay (ELISA)

The protein expression levels of inflammatory cytokines and ALCAM were detected from cell supernatant or cell lysates using ELISA kits (R&D systems, Minneapolis, MN, USA) following the manufacturer's protocol.

15. Western blot

The total protein of cells was isolated using Mammalian Protein Extraction Reagent (M-PER™, Thermo Fisher Scientific) following the manufacturer's protocol. A549 cells were centrifuged at 8000 rpm for 3 minutes at 4℃. The cell pellet was resuspended in 200μl of M-PER solution containing 1% Halt™ Protease and Phosphatase Inhibitor Cocktail (100X, Thermo Fisher Scientific,) and shaken for 10 minutes at room temperature. Aftter then, the cells were centrifuged at 15000 rpm for 10 minutes at 4℃. The supernatant was collected and stored at -70℃ until further analysis. The total protein from the mouse lung tissues was isolated using RIPA Lysis and Extraction Buffer (Thermo Fisher Scientific) according to the manufacturer's protocol. Mouse lung tissues were homogenized using a T10 Basic Ultra-Turrax homogenizer (IKA Labortechnik, Staufen, Germany) in RIPA lysis buffer containing inhibitor cocktail (Thermo Fisher Scientific) and shaken for 10 minutes at room temperature. The supernatant was collected and stored at -70℃ until further analysis. The protein concentration was measured by bradford assay. And equal amount of protein were mixed with 4X Laemmli Sample Buffer (Bid-Rad, Hercules, CA, USA). Protein sample were loaded into 10% sodium dodecyl sulfate polyacrylamide gel and electrotransfered into a polyvinylidene fluoride membrane (Millipore). The membrane was washed in tris-based saline containing with 0.1% Tween 20 (TBST) and blocked with 5% skim mlik in TBST for 1 hour. The membrane was incubated overnight at 4℃ with specific primary antibodies

targetting ALCAM (Santa Cruz Biotechnology) and p-ERK1/2, t-ERK1/2, p-p38, t-p38, p-JNK, t-JNK, GAPDH, β-actin (Cell Signaling Technology, Beverly, MA, USA). Next day, the membrane was incubated with HRP-conjugated secondary antibody (Santa Cruz Biotechnology) at room temperature for 1 hour. The protein bands were visualized by Amersham ImageQuant 800 (Cytiva, Marlborough, MA, USA) using Amersham™ ECL select Western Blotting Detection Reagent (Cytiva) and Amersham™ ECL prime Western Blotting Detection Reagent (Cytiva). ImageJ software program (National Institutes of Health, Bethesda, MD, USA) was utilized for quantification of protein bands.

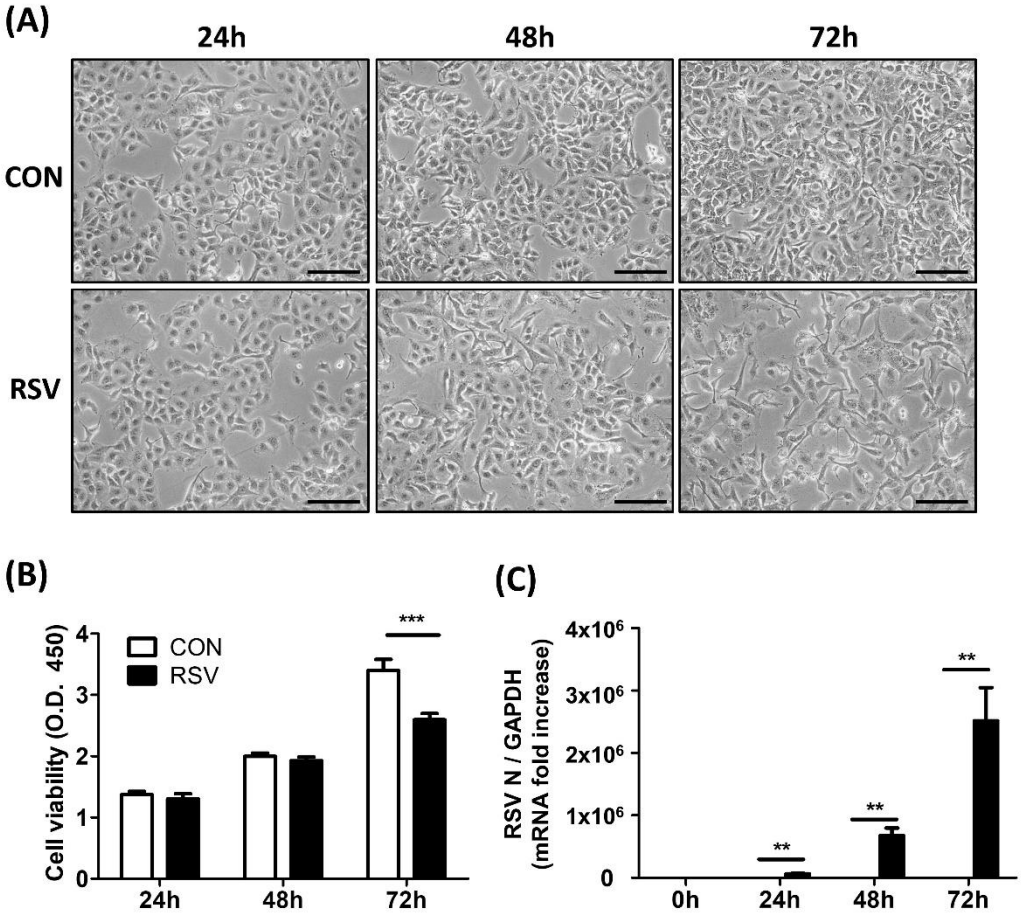
16. Statistical analysis

In figure 1B, the data of cell viability were presented as the mean ± standard deviation (SD). All other data were presented as the mean ± standard error of the mean (SEM) of at least three independent experiments. Statistical analysis was conducted using Student's *t*-test to compare two groups, and one-way ANOVA with Turkey test to compare multiple groups.

III. RESULTS

1. RSV infection increases the expression levels of ALCAM and inflammatory cytokines in airway epithelial cells (A549)

This study investigated the contribution of ALCAM in RSV induced inflammation using alveolar epithelial cell line A549 cells. The cells were infected with RSV at a MOI 0.1 and incubated for 24, 48, and 72 hours. RSV infection resulted in reduced viability of A549 cells in a time dependent manner (Figure 1A-B). To assess the replication of RSV, the expression of RSV nucleoprotein (N) gene was measured, and RSV infected cells showed a significant increase in RSV N gene expression compared to the control cells. (Figure 1C). In addition, elevated mRNA and protein expression levels of inflammatory cytokine IL-1β, IL-6, IL-8, and TNF-α were observed after RSV infection (Figure 1D-E). Moreover, mRNA and protein expression of type 2 innate cytokine, IL-33, were also increased by RSV infected cells (Figure 1F-G). Along with increased cytokines after RSV infection, RSV infection significantly upregulated mRNA and protein expression of ALCAM in a time dependent manner (Figure 1H-K). These findings inferred that ALCAM is implicated in RSV induced airway inflammation.



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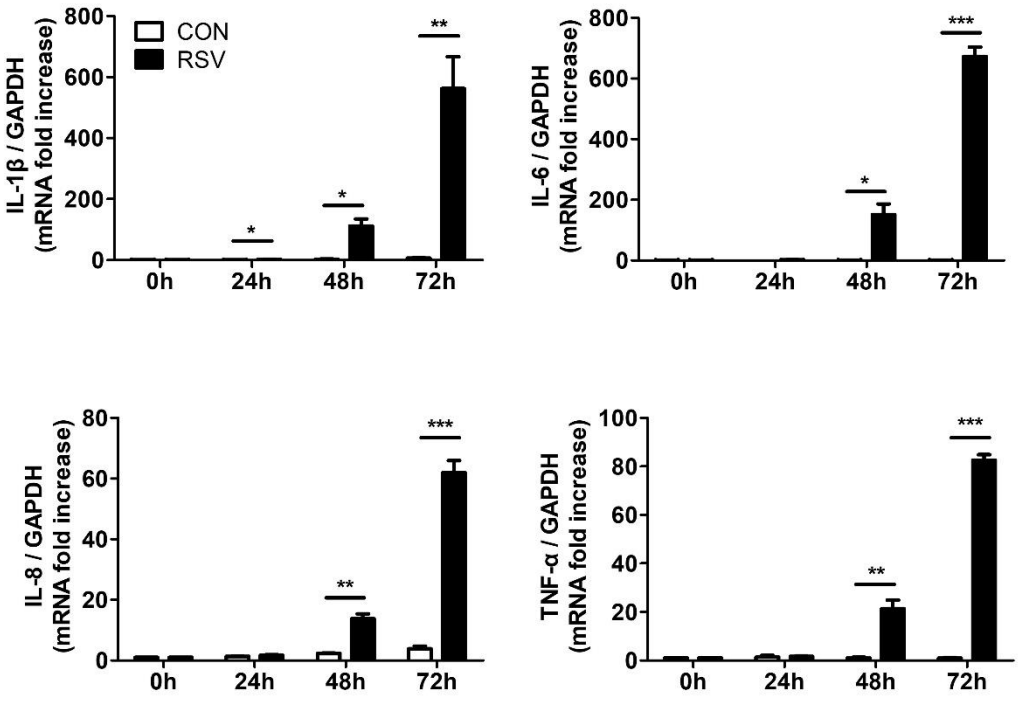

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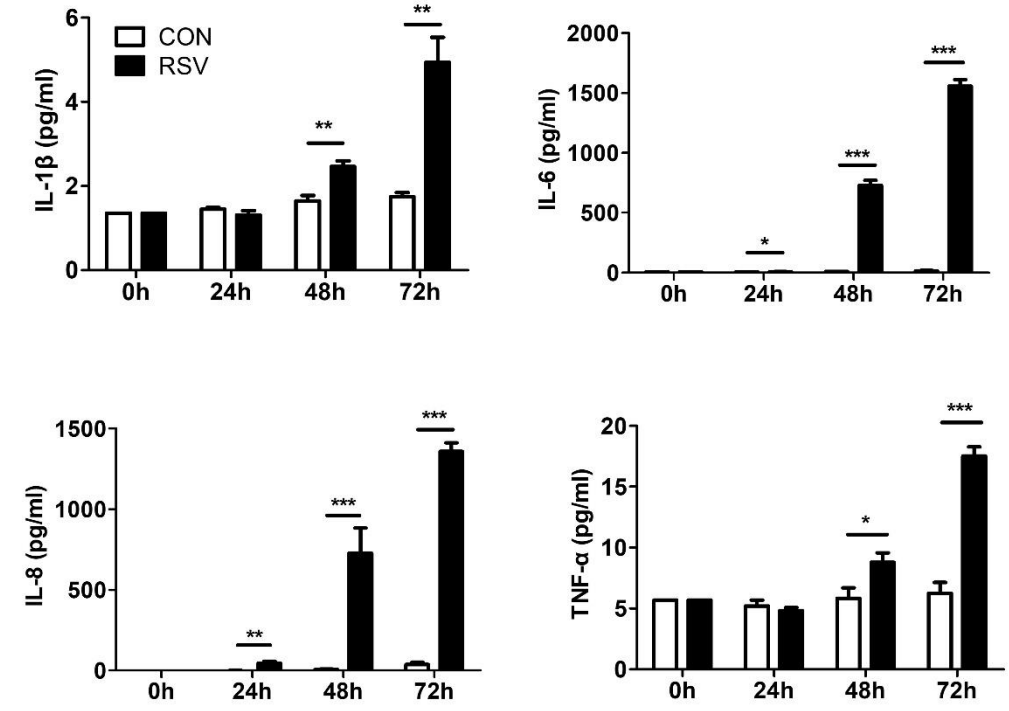
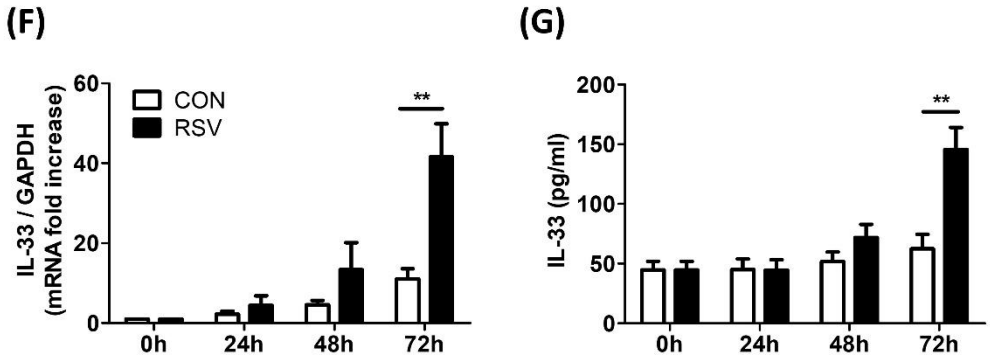




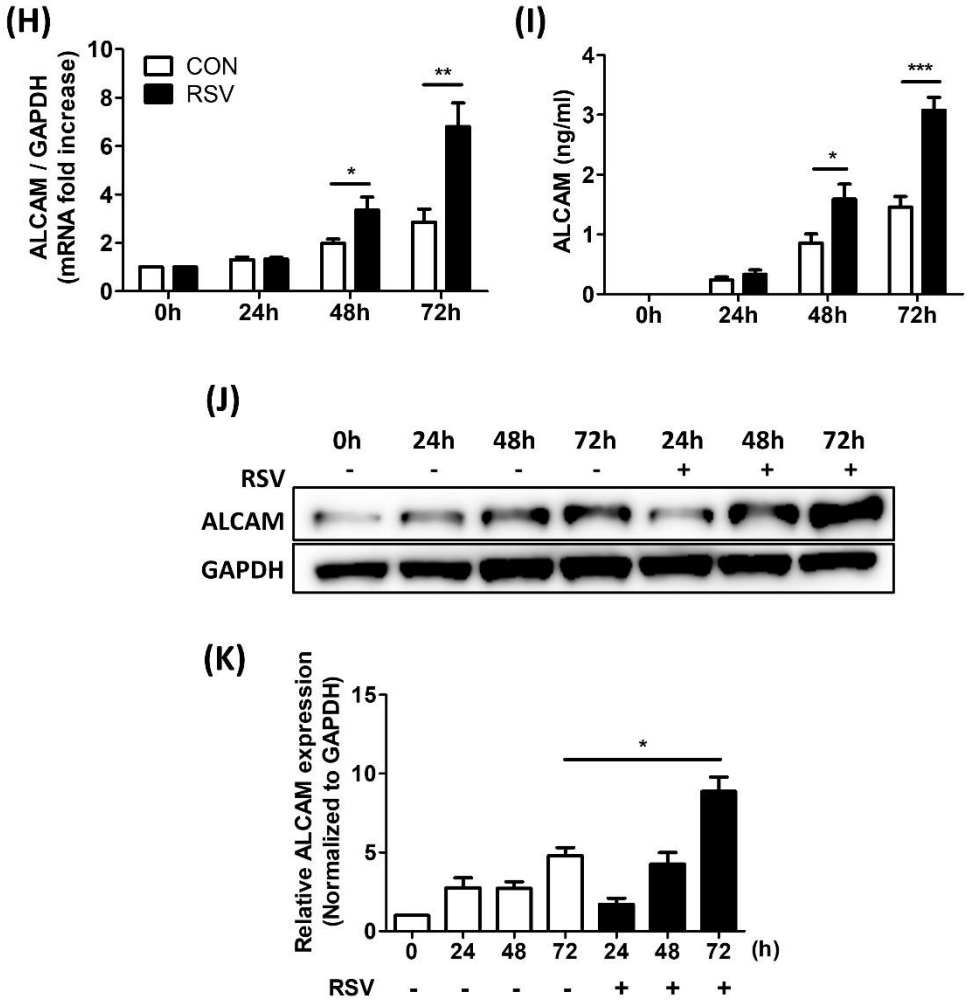
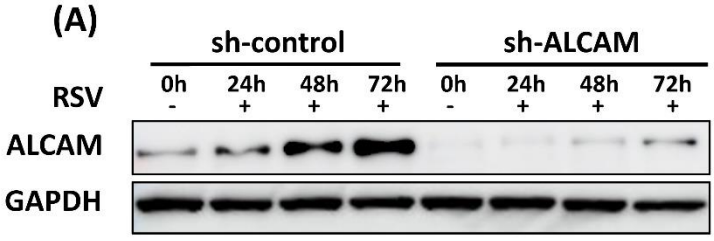


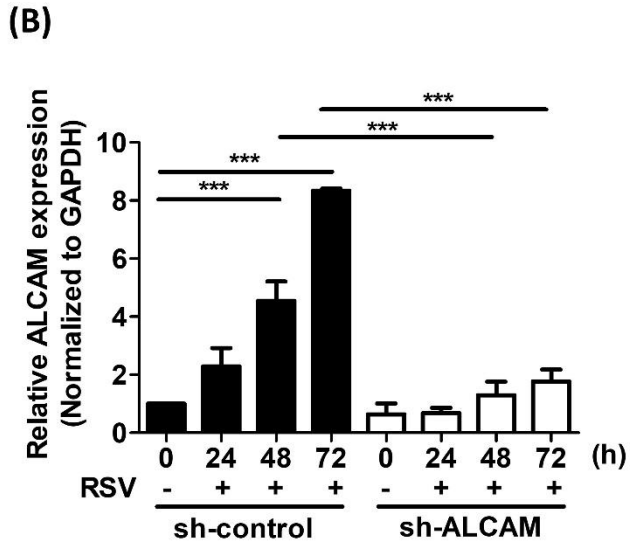
Figure 1. ALCAM and inflammatory cytokines are increased in RSV infected airway epithelial cells. A549 cells were infected with RSV at a MOI 0.1. (A) The morphology of A549 cells was observed by optical microscope. Scale bar, 100 μm. (B) Cell viability was measured using CCK-8 assay in a time dependent manner. (C) The mRNA expression of RSV N gene was detected by real time PCR. (D) The expression levels of inflammatory cytokines in cell lysates and supernatant were assessed by real time PCR and (E) ELISA analysis. (F) IL-33 expression in cell lysates was analyzed by real time PCR and (G) ELISA. (H) mRNA expression of ALCAM in cell lysates was measured by real time PCR. (I) The protein expression of ALCAM in cell supernatants and cell lysates was evaluated by ELISA and (J) western blot analysis. (K) Normalization of ALCAM to GAPDH was performed by Image J software. Data of (B) are presented as the mean ± SD, and others are presented as the mean ± SEM of at least three of independent experiments. * P <0.05, ** P < 0.01, *** P < 0.001.

2. ALCAM regulates IL-33 expression induced by RSV infection in A549 cells

To identify the contribution of ALCAM in RSV induced inflammation, ALCAM knockdown in A549 cell line was performed by using a shRNA transfection. The expression of ALCAM was increased by RSV infection in a time dependent manner, but significantly abolished in ALCAM knockdown cells (Figure 2A-B). Moreover, the expression of the RSV N gene exhibited no significant difference between ALCAM knockdown cells and control cells (Figure 2C), indicating that there was no disparity in virus replication between the two cell lines. Next, this study investigates the expression levels of inflammatory cytokines. Notably, the mRNA and protein expression of IL-33 expression levels was dramatically diminished in ALCAM knockdown cells compared to control cells (Figure 2D-E). To verify these results, ALCAM expression in A549 cells was blocked by using an anti-ALCAM antibody. Interestingly, IL-33 expression induced by RSV infection was also dose-dependently downregulated in cells treated with the anti-ALCAM antibody compared to normal IgG (Figure 2F-G). These results confirmed that ALCAM modulates IL-33 expression after RSV infection.

Further study was performed to determine whether inhibition of IL-33 expression would affect expression levels of ALCAM. A549 cells were transfected with either IL-33 interfering RNA (siRNA) or scramble siRNA followed by RSV infection. The elevated expression of IL-33 induced by RSV infection was downregulated in IL-33 siRNA transfected cells compared to the scramble siRNA transfected cells (Figure 2H). However, no significant changes were observed in the expression level of ALCAM when IL-33 expression was inhibited (Figure 2I).





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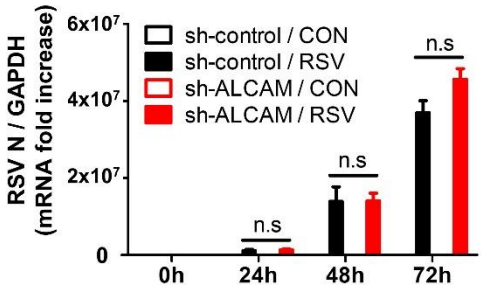

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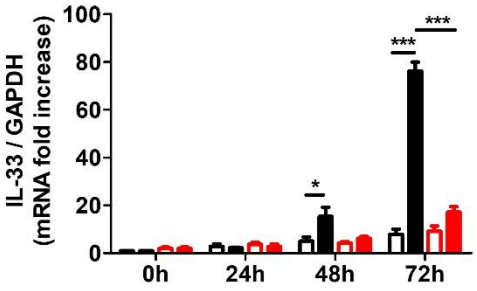

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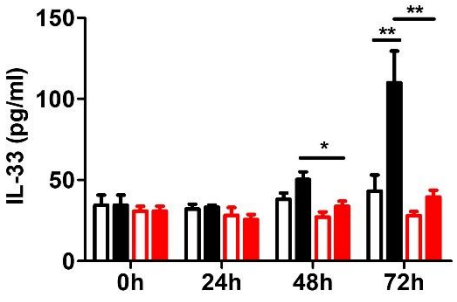


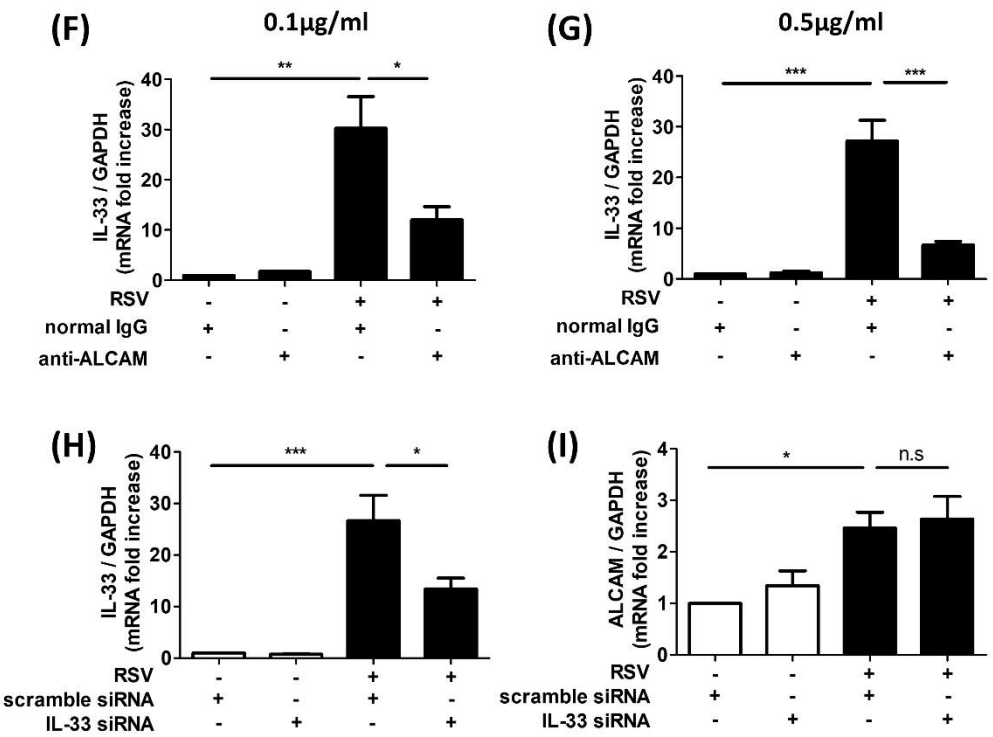
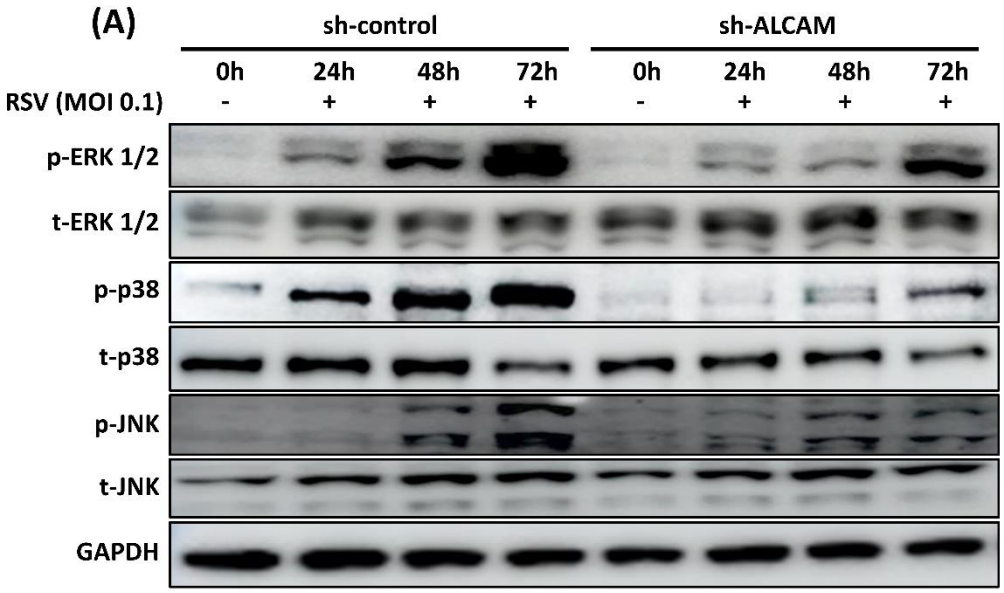


Figure 2. ALCAM deficiency attenuates IL-33 expression induced by RSV infection. (A) ALCAM gene expression in A549 cells transfected with control shRNA (sh-control) or ALCAM shRNA (sh-ALCAM) was assessed by western blot analysis. (B) Normalization of ALCAM to GAPDH was performed by Image J software. (C) The expression of RSV N gene was measured by real time PCR both in RSV infected sh-control and sh-ALCAM cells. (D) The mRNA and (E) protein expression of IL-33 was measured by real time PCR and ELISA in cell lysates of both sh-control and sh-ALCAM cells. (F) 0.1 and (G) 0.5 μg/ml of anti-ALCAM antibody or normal IgG was applied to A549 cells and the expression of IL-33 at 72 hours after infection was measured by real time PCR. (H) IL-33 and (I) ALCAM gene expression in A549 cells transfected with scramble siRNA or IL-33 siRNA was measured by real time PCR. Data are presented as the mean ± SEM of at least three of independent experiments. * P <0.05, ** P < 0.01, *** P < 0.001. n.s = not significant.

3. The upregulated expression of IL-33 induced by RSV is regulated via MAPK signaling pathway in A549 cells

This research tried to identify the signaling pathway through which ALCAM influences IL-33 expression in RSV induced airway inflammation. It has been revealed that viral stimulation, including RSV infection, activates MAPK signaling pathways involved in various cellular functions and the production of inflammatory cytokines.[29,30] Therefore, the phosphorylation of MAPK pathways including ERK 1/2, p38, and JNK was examined both in ALCAM knockdown and control cells to determine whether there are differences upon RSV infection. As expected, the phosphorylation of ERK1/2, p38, and JNK was upregulated by RSV infection in a time dependent manner in the control cells. However, the phosphorylation was significantly downregulated in ALCAM knockdown cells compared to control cells after RSV infection (Figure 3A-D).

To verify the association of the MAPK signaling pathway and IL-33 expression, MAPK inhibitor experiments were performed. Each of inhibitors for ERK1/2, p38, and JNK was treated to A549 cells and expression levels of IL-33 was measured. And our results showed that IL-33 expression was significantly reduced upon treatment with ERK1/2, p38, and JNK inhibitors (Figure 3E-G). These findings confirmed that ALCAM regulates IL-33 through the MAPK pathway during RSV infection.



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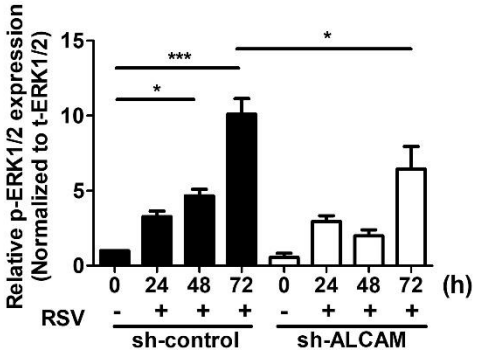

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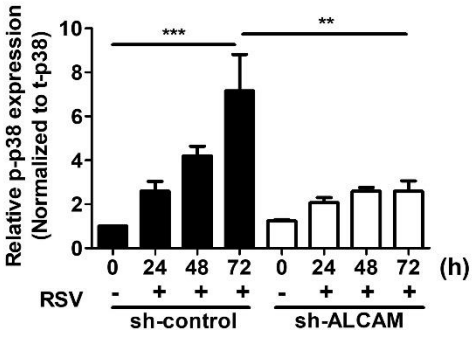

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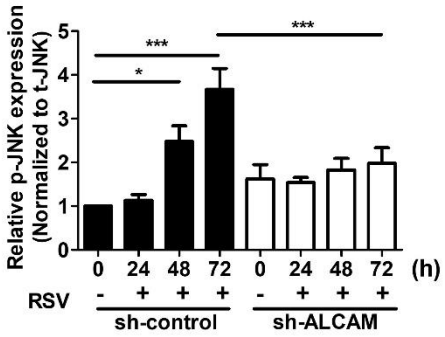


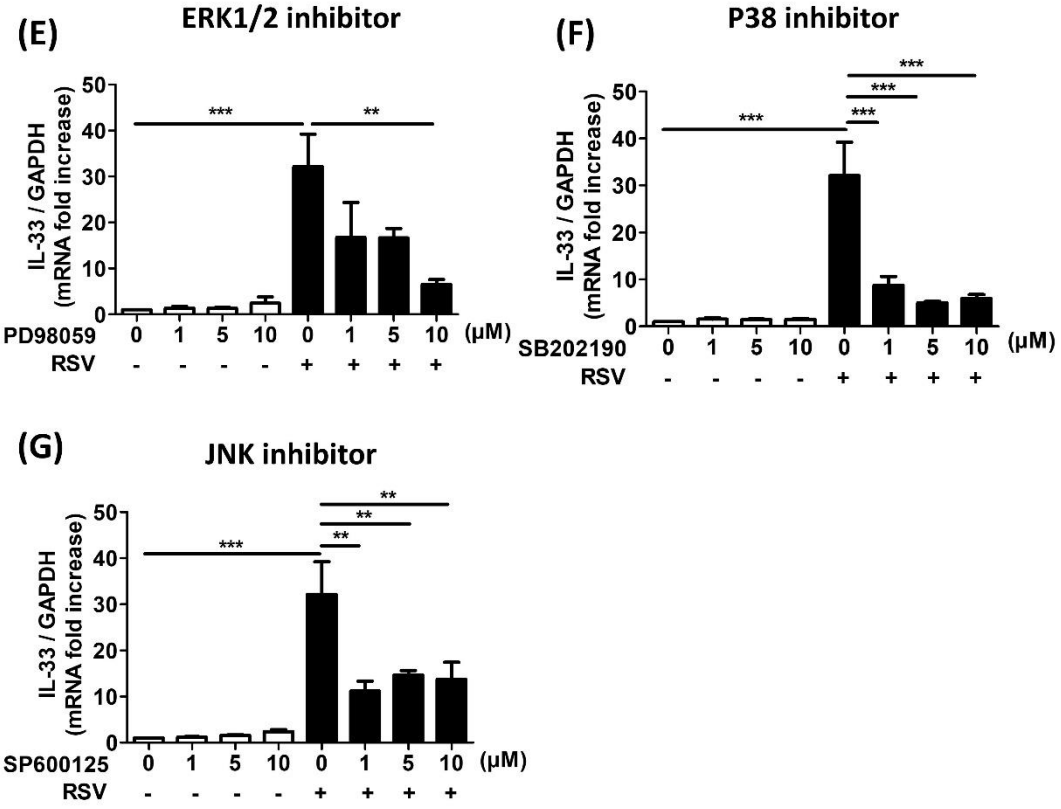
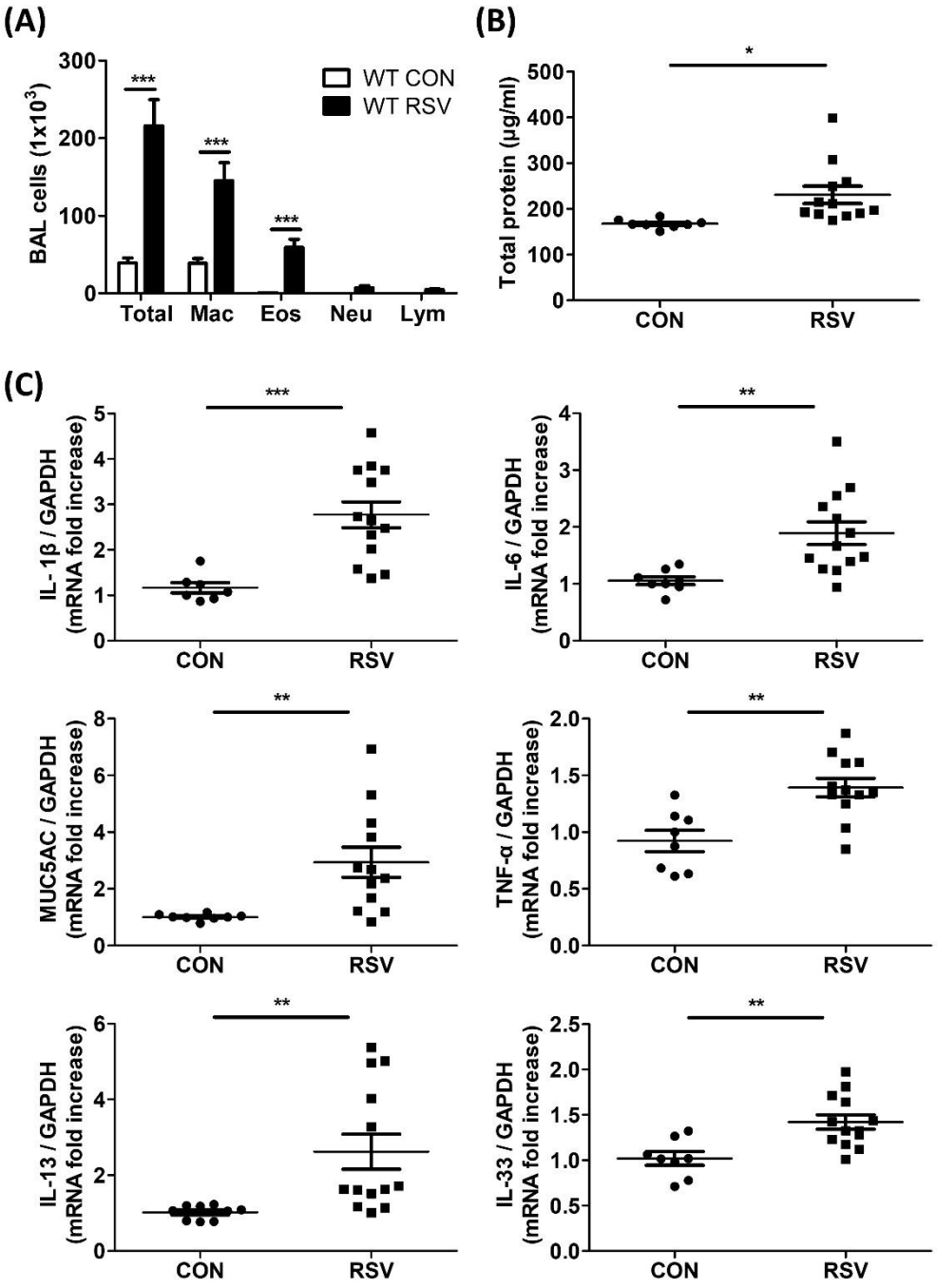


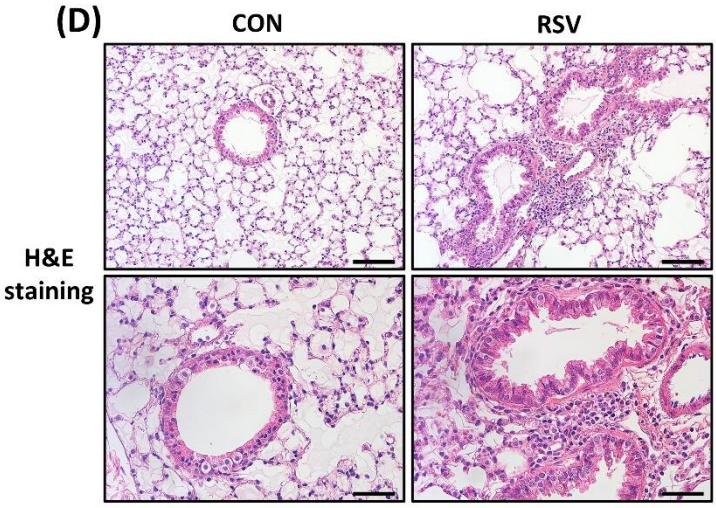
Figure 3. MAPK signaling pathways regulate IL-33 expression induced by RSV infection. (A) Phosphorylation of MAPK signaling pathways induced by RSV infection was examined by western blot analysis. (B-D) Normalization of p-ERK1/2, p-p38, and p-JNK to t-ERK1/2, t-p38, and t-JNK was performed by Image J software. (E-G) The specific MAPK inhibitors targeting ERK1/2 (PD98059), p38 (SB202190), and JNK (SP600125) applied to A549 cells and the expression levels of IL-33 in the cell lysates at 72 hours after infection was assessed by real time PCR. Data are presented as the mean ± SEM of at least three of independent experiments. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

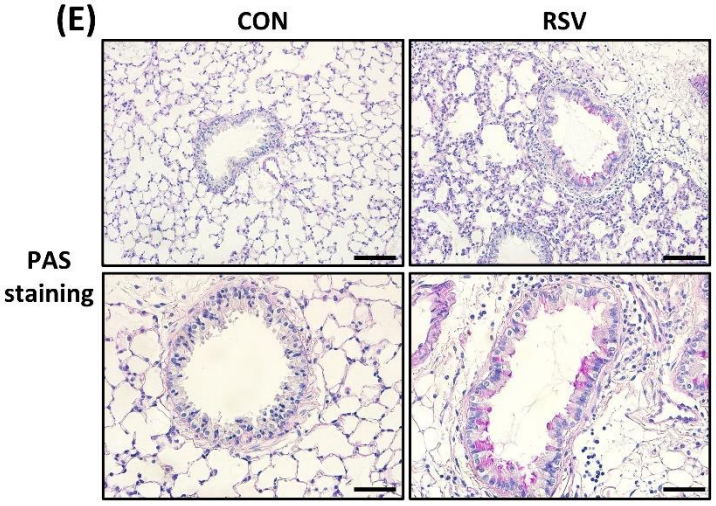
4. ALCAM and inflammatory cytokines are elevated in RSV infected mice

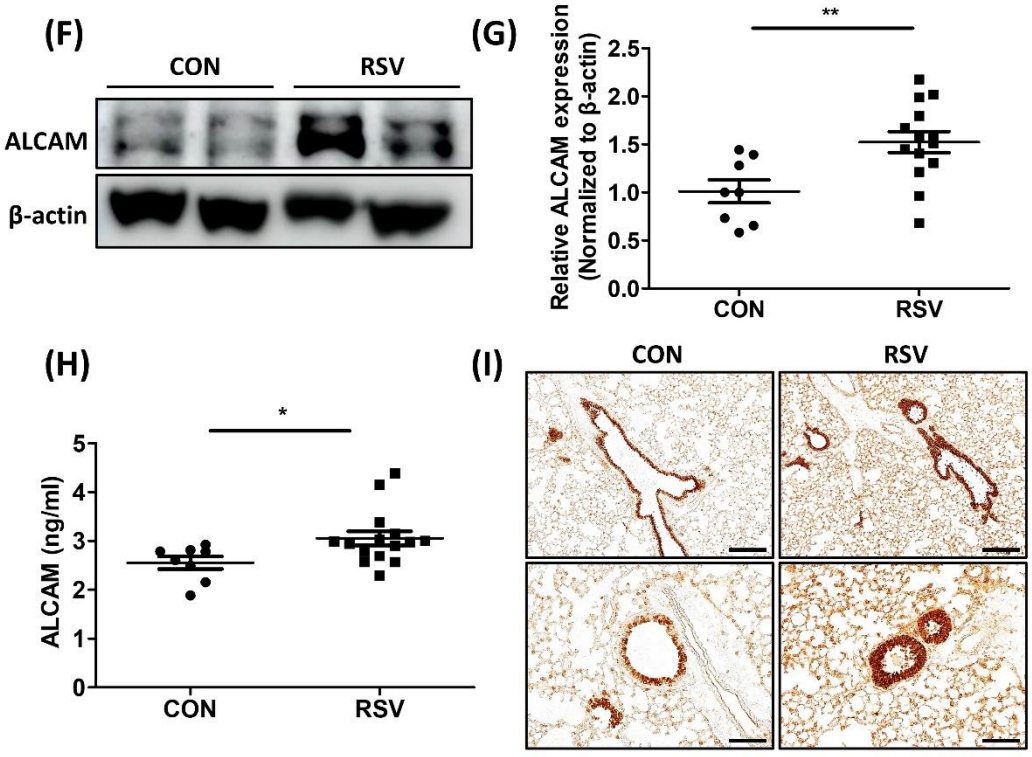
For further investigation of the relationship between ALCAM and RSV induced inflammation in vivo, this study intranasally administered RSV (8×10^6 PFU/ml) to wild-type C57BL/6 mice and measured the expression of ALCAM. RSV infection resulted in increased number of total cells and inflammatory cells in BAL fluid (Figure 4A). Total protein concentration in BAL fluid was also increased by RSV infection (Figure 4B). Moreover, the mRNA expression levels of inflammation cytokines such as IL-33, IL-1β, IL-6, IL-13, MUC5AC and TNF-α were upregulated in RSV infected mice compared to control mice. (Figure 4C). A histological analysis performed via H&E staining exhibited increased inflammation as shown by an increase in cell infiltration (Figure 4D), and PAS staining showed the increased mucus production in RSV infected mice (Figure 4E).

The expression levels of ALCAM were examined in the RSV infected mice. The protein expression of ALCAM in lung tissue lysates and BAL fluid was upregulated in RSV infected mice compared to control mice (Figure 4F-H). Immunohistochemistry staining also demonstrated that ALCAM expression was increased in lung tissues (Figure 4I). In an immunofluorescence study, ALCAM colocalized with CC10, a marker of epithelial cells, and SPC, a marker of type 2 alveolar epithelial cells. (Figure 4J)









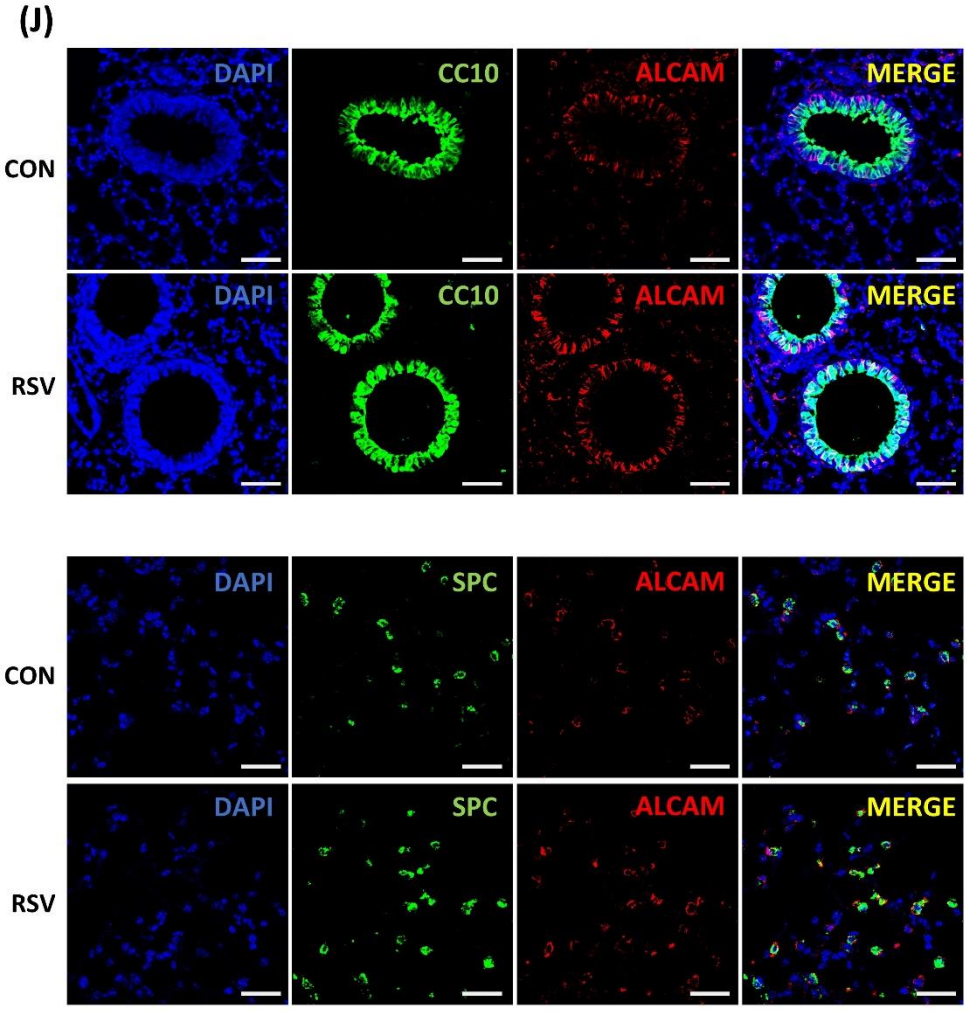


Figure 4. RSV infection increases the expression levels of ALCAM and inflammatory cytokines in RSV infected mice. Wild-type C57BL/6 mice were infected with RSV. (A) Total and differentiated inflammatory cells from BAL fluid were counted. (B) Total protein of BAL fluid was measured by bicinchoninic acid assay. (C) The mRNA expression levels of inflammatory cytokines were measured by real-time PCR in lung lysates. (D) Lung sections were histologically assessed by optical microscope after staining with H&E or (E) PAS staining. Scale bars, (D), 100 μm (top) and 50 μm (bottom), (E), 100 μm (top), and 50 μm (bottom). (F) ALCAM protein expression in lung lysates was analyzed by western blot. (G) Normalization of ALCAM to β-actin was performed by Image J software. (H) ALCAM protein expression in BAL fluid was measured by ELISA. (I) ALCAM protein expression in lung tissues was analyzed in Immunohistochemistry (IHC). Scale bars, 200 μm (top) and 100 μm (bottom). (J) Colocalization of ALCAM with CC10 and SPC was assessed in lung sections. Scale bars, 200 μm (CC10) and 50 μm (SPC). Data are presented as the mean ± SEM of at least three of independent experiments (n=8-13 mice per group). * P <0.05, ** P < 0.01, *** P < 0.001.

IV. DISCUSSION

This study investigated the role of ALCAM in the inflammatory response triggered by RSV infection in airway epithelial cells, and found that inflammation and ALCAM expression were increased in both RSV-infected mice and human airway epithelial cells. The expression of IL-33 induced by RSV infection was downregulated in ALCAM-deficient A549 cells compared to the control cells. The present study also confirmed that decreased IL-33 expression is associated with decreased phosphorylation of ERK1/2, p38, and JNK.

ALCAM has been extensively researched as a cancer biomarker that modulates cancer by interacting with the CD6 ligand.[14] Increased ALCAM expression has been observed in various cancers including melanoma, breast and clonal cancer patients.[14,30,31] Furthermore, recent studies showed a potential association between ALCAM and inflammatory diseases. Chalmers SA reported an increase in ALCAM in lupus nephritis.[32] Additionally, the association of ALCAM with allergic disease such as allergic asthma, atopic dermatitis, and food allergy was also described.[20,22,23] In these studies, ALCAM was reported to contribute to allergic diseases by stimulating $CD4^+$ T cell activation and proliferation. Serum levels of ALCAM were also upregulated in patients with allergic asthma, atopic dermatitis, and food allergy, respectively. Considering the results of previous studies, the importance of ALCAM in cancer has been recognized, but its impact on inflammatory diseases has recently been revealed. ALCAM is considered a biomarker for various diseases and a potential molecule that requires further research.

Here, this study demonstrated that the expression of ALCAM was upregulated in the RSV-infected A549 cells which are representative of type II alveolar epithelial cells and are known to be susceptible to RSV infection.[29,33] Moreover, the in vivo experiments also confirmed the elevated expression of ALCAM in RSV infected mice. The previous study reported that ALCAM colocalizes with CC10, a marker of airway epithelial cells, and SPC, a marker of type 2 alveolar epithelial cells, in the lungs of mice with bleomycin-induced inflammation.[19] Likewise, present study also confirmed that both CC10 and SPC markers

co-localized with ALCAM expression respectively in mice with RSV infection.

Airway epithelial cells serve as the primary target of respiratory viruses, acting as both the initial physical barrier against tissue infiltration and the immunological barrier that triggers inflammatory responses.[34] Upon RSV infection, epithelial cell-derived cytokines and chemokines are released from infected airway epithelial cells.[34,35] Previous studies have demonstrated the increased expression of IL-33 in RSV-infected mice and patients infected with RSV.[11,35] Consistent with these results, the present study verified that RSV infection increased the expression of proinflammatory cytokines, including IL-33, at both the mRNA and protein levels. Notably, the expression of IL-33 induced by RSV infection was rapidly downregulated in ALCAM-deficient cells compared to the control cells. Additionally, blocking ALCAM in A549 cells using an anti-ALCAM antibody resulted in a significant reduction in IL-33 expression, confirming our earlier observations.

In this study, the signaling pathways involved in regulation of IL-33 expression was investigated. It has been demonstrated that various stimuli can trigger the activation of MAPK cascades, and previous studies have verified that RSV and influenza virus infection can activate the phosphorylation of these pathways in airway epithelial cells.[26,36,37] And there are several studies suggesting the possibility that ALCAM is associated with these signaling pathways.[19,27,28] It has been reported that the phosphorylation of MAPK was involved in enhanced expression of ALCAM induced by cocaine treatment both in human and mice brain, and these activations of MAPK signaling are also important for ALCAM expression.[27] Another study demonstrated that ALCAM/CD6 interaction activates MAPK cascades.[38] Therefore, this study sought to investigate the MAPK signaling pathways and found that RSV infection induced the phosphorylation of ERK1/2, p38, and JNK. Furthermore, phosphorylation was significantly downregulated in ALCAM-deficient A549 cells compared to the control cells.

The activated MAPK pathways are known to be involved in the expression and release of inflammatory mediators.[26,39,40] IL-33 expression has been reported to be modulated through the MAPK pathway in macrophages and dendritic cells.[41,42] Another study

indicated that the toll-like receptor-TGF-β activated kinase 1-MAPK signaling pathway regulates IL-33 expression in airway epithelial cells infected with *Pseudomonas aeruginosa* in a model of cystic fibrosis.[43] Consistent with these findings, present study confirmed that RSV infection induced activation of the MAPK pathway in A549 cells, and that inhibitors targeting each of ERK1/2, p38, and JNK significantly diminished IL-33 expression.

It has been reported that type 2 immune responses were alleviated in ALCAM-knockout mice and mice treated with anti-ALCAM antibodies in an ovalbumin-induced experimental asthma model.[20] Additionally, a recent study revealed that the inflammatory response caused by LPS induced acute lung injury was alleviated in ALCAM knockout mice.[24] Here, this research revealed that the expression of IL-33 induced by RSV infection decreased in ALCAM-knockdown A549 cells. It is well known that IL-33 plays a significant role in regulating immune responses and is important in pulmonary diseases. In fact, one study has reported that neutralization of IL-33 alleviated type 2 and type 3 inflammatory responses induced by viral stimulation.[44] Another investigation has demonstrated that airway hyperreactivity and eosinophila were reduced in IL-33 knockout mice.[45] Taken together, thease findings suggest that ALCAM, which plays an important role in regulating IL-33 expression induced by RSV infection, may be an important therapeutic target for lung disease involving IL-33 regulation.

V. CONCLUSION

This study demonstrated that ALCAM expression was increased both *in vivo* and *in vitro* due to RSV infection, and it was found that IL-33 expression was greatly inhibited in ALCAM deficient A549 cells. Additionally, it was verified that the phosphorylation of the MAPK singaling pathways was greatly reduced in ALCAM deficient cells. Moreover, this research found that the expression of IL-33 was regulated by MAPK pathways using MAPK inhibitors. Taken together, these results provide evidence for the role of ALCAM in mediating RSV-induced inflammation through the regulation of MAPK signaling pathway phosphorylation in airway epithelial cells. Notably, the observed impact of moderate MAPK phosphorylation on IL-33 expression highlights the potential of targeting ALCAM as a therapeutic strategy for mitigating IL-33-associated lung diseases. These findings contribute to our understanding of the underlying mechanisms involved in IL-33-associated lung pathologies and offer promising avenues for future therapeutic interventions.

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ABSTRACT(IN KOREAN)

호흡기 세포융합 바이러스로 유도되는 기도 염증에서 ALCAM의 역할

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배경: 호흡기 세포융합 바이러스는 급성 하부 호흡기 감염 질환의 주요 원인이며 주로 영유아 및 노인에게 취약한 호흡기 바이러스이다. 호흡기 세포융합 바이러스에 감염되었을 때 기도 상피세포에서 인터루킨-33의 발현이 유도되며, 이는 2형 면역 반응을 활성화시키는 중요한 염증 매개체이다. Activated leukocyte cell adhesion molecule (ALCAM)은 세포막 내 존재하는 당단백질의 일종으로, 염증 반응의 조절과 알레르기 질환에 관여한다고 알려져 있다. 최근 연구 결과, 알레르기성 천식으로 인한 염증 반응과 ALCAM과의 연관성이 규명된 바 있다. 그러나 호흡기 세포융합 바이러스 감염으로 유발되는 인터루킨-33 발현에 ALCAM이 미치는 영향은 아직 명확하지 않다. 본 연구는 호흡기 세포융합 바이러스에 감염된 기도 상피세포에서 유래한 인터루킨-33 발현 조절에서 ALCAM의 연관성을 조사하고자 한다.

방법: 본 연구에서는 A549 세포에 호흡기 세포융합 바이러스를 시킨 후 ALCAM의 역할을 확인하기 위해 A549 세포에서 ALCAM의 발현을 억제하여

대조군과 비교하였다. 호흡기 세포융합 바이러스 감염으로 인해 유도되는 ALCAM 및 염증성 사이토카인의 발현을 조사하였다. 또한, 인터루킨-33의 발현을 조절하는 ALCAM의 역할을 검증하기 위해 항-ALCAM 항체를 이용한 실험을 실시하였다. 이후 호흡기 세포융합 바이러스에 감염된 A549 세포에서 mitogen activated protein kinase (MAPK) 신호 전달 경로를 분석하였다. 또한, 호흡기 세포융합 바이러스로 C57BL/6 야생형 마우스를 감염시켜 ALCAM의 발현을 분석하여, 생체 내에서 호흡기 세포융합 바이러스로 인한 염증과 ALCAM 간의 연관성을 추가적으로 확인하였다.

결과: 본 연구에서는 C57BL/6 야생형 마우스 및 A549 세포에서 호흡기 세포융합 바이러스 감염으로 인한 ALCAM 및 염증성 사이토카인의 발현이 모두 증가되었음을 확인하였다. 호흡기 세포융합 바이러스 감염으로 인해 A549 세포의 생존율은 시간에 따라 감소하였다. 또한, ALCAM의 발현을 억제시킨 세포에서는 호흡기 세포융합 바이러스 감염으로 인해 유도되는 인터루킨-33의 발현이 현저하게 저해됨을 확인하였다. 항-ALCAM 항체를 처리한 A549 세포에서도 호흡기 세포융합 바이러스 감염으로 인한 인터루킨-33의 발현이 감소하였다. 호흡기 세포융합 바이러스에 감염된 A549 세포에서는 MAPK 신호 경로의 활성화가 유도되었지만, ALCAM의 발현이 억제된 A549 세포에서는 호흡기 세포융합 바이러스 감염 후 대조군에 비해 MAPK 신호 경로의 인산화가 감소하였다. 또한, A549 세포에 MAPK 경로 억제제를 처리한 결과, 호흡기 세포융합 바이러스로 유도되는 인터루킨-33의 발현이 유의미하게 감소됨을 확인하였다.

결론: 본 연구에서는 기도 상피세포에서 ALCAM이 호흡기 세포융합 바이러스 감염에 의해 유발되는 MAPK 신호 전달 경로에 영향을 미침으로써 인터루킨-33 발현을 조절한다는 것을 밝혔다.

핵심되는 말 : ALCAM, 기도 상피 세포, 인터루킨-33, MAPK 신호 전달 경로, 호흡기 세포융합 바이러스