Role of breast regression protein-39 (BRP-39) in Respiratory Syncytial Virus-induced Airway Inflammation

Min Jung Kim

Department of Medicine

The Graduate School, Yonsei University

Role of breast regression protein-39 (BRP-39) in Respiratory Syncytial Virus-induced Airway Inflammation

Directed by Professor Myung Hyun Sohn

The Doctoral Dissertation
submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of requirement
for the degree of Doctor of Philosophy

Min Jung Kim

June 2017

This certifies that the Doctoral Dissertation of Min Jung Kim is approved.

Thesis Supervisor: Myung Hyun Sohn

Thesis Committee Member #1: Jae Myun Lee

Thesis Committee Member #2: Chul Hoon Kim

Thesis Committee Member #3: Ho-Geun Yoon

Thesis Committee Member #4: Man Ki Song

The Graduate School

Yonsei University

June 2017

ACKNOWLEDGEMENTS

2013 년 3 월 처음 시작한 실험이 4 년이 지나서야 결실을 맺어 드디어 마무리하게 되었습니다. 호기롭게 시작한 학위 과정이 이토록 힘들고 길어지게 될 줄 미쳐 몰랐지만 돌이켜보면 하나하나 의미 있고 소중한 시간이었습니다.

먼저 사고뭉치 저를 여기까지 이끌어주시고 기다려주신 손명현 지도교수님, 전공의 1 년차 3 월에 처음 만나 현재까지 지도해주시는 김경원 교수님, 그리고 은퇴하신 후에도 못난 제자의 진로를 걱정해주시는 김규언 교수님께 감사드립니다. 저에게 실험할 수 있는 기회를 주시고 모든 실험 과정을 전폭적으로 지지해주신 미생물학 교실 이재면 교수님과 애정 어린 조언을 아끼지 않으신 박전한 교수님께도 진심으로 감사드립니다. 그리고 바쁘신 와중에도 제 학위 논문 심사에 참여해주시고 많은 조언 주신 김철훈 교수님, 윤호근 교수님, 송만기 박사님께도 깊이 감사드립니다.

지난 4 년 동안 동고동락한 소중한 인연들. 저의 실험 선생님이자 연구 동료이면서 또 벤틸레이션 창구인 워킹맘 3 인방 (하나부터 열까지 다 가르쳐준 심두희 박사님과 차혜란 박사님, 씩씩한 막내 국영이까지) 덕분에 그 많은 삽질과 스트레스를 견딜 수 있었습니다. 한결같이 성실한 모습으로 감동을 준 양창모 선생님, 이제 곧 졸업할 조용근 선생님, 박필구 박사님, 항상 친절한 혜미, 훌륭한 논문으로 졸업하는 수지, 막내 수진이까지 모든 실험실 식구에게도 진심으로 감사합니다. 그리고 훌륭한 선배로서 많은 격려와 위로를 해준 박광환 선생님께도 감사드립니다. 또 서로 걱정하고 위로하며 이 길을 헤쳐온 동병상련 홍정연 박사님과 김미나 박사님 그리고 각자 자신의 길을 열심히 가고 있는 알레르기 동우회 선배님들과 후배들에게도 감사의 말을 전합니다.

자주 만날 수는 없지만 항상 서로를 응원하고 있는 소녀시절 스터디 모임 (나래언니, 현지언니, 주현언니, 경숙이), 인턴 동기이자 소중한 동반자 (지현, 지수, 여훈, 유미, 지희, 선영)과 든든한 소아과 동기 (이희, 쩡아, 이슬, 지현언니, 리타언니, 윤수언니, 은샘언니), 그리고 육아동지 영주언니와 해린에게도 특별히 고마운 마음 전합니다. 모두 건강하고 앞으로 꽃길만 걷길 진심으로 바랍니다.

그 누구보다 저의 졸업을 기다리셨을 부모님. 무한 지지와 사랑을 주신 아빠, 선배 연구자로서 인생 선배로서 저를 받쳐주고 다독여주신 엄마 덕분에 결실을 보게 되었습니다. 너무 사랑하고 죄송합니다. 또 저를 믿고 자랑스러워하는 예지엄마 영정이, 같은 길을 걸어가고 있는 막내 민재와 제부에게도 진심으로 감사합니다. 비록 지금은 곁에 안 계시지만 할아버지, 외할머니께도 늦게나마 이 논문을 보여드릴 수 있게 되어 기쁘게 생각합니다.

부족한 며느리 때문에 하루도 편할 날 없이 지내신 아버님과 어머님께 무엇보다 감사 드립니다. 아들, 손자, 며느리까지 다 거두시느라 고생하시는 아버님, 며느리 뒷바라지에 당신 생활도 다 접으시고 손자까지 키워주시는 어머님, 정말 죄송하고 또 감사 드립니다. 조카라면 끔찍하게 위해주는 도련님께도 감사의 말을 전합니다.

결혼하고 많은 일이 있었지만 티격태격하면서도 묵묵히 곁을 지켜준 신랑과 엄마의 힘든 학위 과정을 씩씩하게 함께 버텨준 3 살 아들 진휘, 그리고 10 월이면 태어날 진동이(=진휘 동생), 세상에서 가장 사랑하고 감사합니다. 항상 변함없이 저를 아껴주고 격려해주는 가족들이 있어 중심을 잃지 않을 수 있었고 이 모든 것이 가능했습니다. 앞으로도 모두 건강하고 즐겁게 행복하게 살아요.

전공의를 마치고 펠로우를 시작하고, lab 에 오게 되고, 최근 5 년은 저에게 가장 다사다난했습니다. 실패한 일도 많았고 불가능한 일들도 있었지만 저를 믿고 기다려주는 가족들과 여러 선생님들 덕분에 하나하나 해나갈 수 있었습니다. 모든 분들께 다시 한 번 감사의 마음을 전합니다.

감사합니다.

2017 년 6 월

김 민 정

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLE

ABSTRACT

Role of breast regression protein (BRP-39) in Respiratory Syncytial Virus-induced airway inflammation

Min Jung Kim

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Myung Hyun Sohn)

Chitinase 3-like 1 (CHI3L1), has been shown to be necessary for optimal allergen sensitization and Th2 inflammation in various chronic inflammatory diseases including asthma. However, the role of CHI3L1 in airway inflammation induced by respiratory viruses has not been proved yet. The purpose of this study is to determine the relationship between breast regression protein-39 (BRP-39), a mouse chitinase 3-like 1 protein, and airway inflammation followed by respiratory syncytial virus (RSV) infection.

In this study, C57BL/6 mice and C57BL/6-background BRP-39 null mice were used. Mice were inoculated with live A2-strain RSV and control PBS. A methacholine challenge test to measure airway resistance worked on day 7 after inoculation. Bronchoalveolar lavage fluid (BALF) samples were obtained and lung specimens were also harvested on day 7 after inoculation to assess lung inflammation, cytokine expression, and BRP-39 production. BRP-39 expression was evaluated by ELISA. RSV loads were assessed by culture and by real-time polymerase chain reaction (PCR). Histological evaluations of H&E and PAS staining were used to evaluate inflammation and tissue remodeling.

Expression of BRP-39 in BALF and lung lysates was significantly increased in wild-type (WT) mice after RSV infection, but was not observed in BRP-39 null mice. Inflammatory changes induced by RSV infection were less in BRP-$39^{-/-}$ mice rather than in WT mice. In WT mice, RSV infection caused loss of body weight and a significant increase in total cells, macrophages, neutrophils, and eosinophils in BALF. Exaggerated AHR was also noted in WT mice after RSV infection. BRP-$39^{-/-}$ mice, however, showed

decreased responses in each of these parameters. Between RSV infection groups, histological tissue inflammation was also decreased in BRP-39$^{-/-}$ mice. Expression of Th2 cytokines in the lungs was increased in RSV-infected mice. IL-13 was the most prominent cytokine changed by RSV infection in this study. BRP-39 also regulated M2 macrophage activation in RSV-infected mice.

To investigate the therapeutic effect of anti-CHI3L1 antibody in RSV infection, anti-CHI3L1 was administered intraperitoneally to RSV-infected WT mice. Mice given anti-CHI3L1 treatment showed less inflammatory changes compared to WT RSV mice. Expression of BRP-39 and Th2 cytokines were also significantly decreased in mice treated with anti-CHI3L1. When measuring the human homologue of CHI3L1, YKL-40, in nasopharyngeal aspirates (NPA) from hospitalized children presenting with acute respiratory symptoms, YKL-40 and IL-13 levels were significantly higher in children with RSV infection than in control subjects. In addition, YKL-40 levels were positively correlated with symptom scores in patients and with IL-13 levels in NPA.

RSV-related respiratory illness has been a leading cause of hospitalization in young childhood. RSV is also known as a significant risk factor for asthma that extends into adolescence and adults, and its mechanism is still under investigation. In this study, expression of BRP-39 increased by RSV infection in mice. And inflammatory changes and AHR induced by RSV were decreased in BRP-39 null mice and in anti-CHI3L1-treated mice. These findings suggest that CHI3L1 could contribute to airway inflammation induced by RSV infection in mice and that BRP-39 could be a potential therapeutic target for attenuating Th2-associated immunopathology during RSV infection.

Key words: Bronchiolitis; Chitinase 3-like 1 protein; Respiratory Syncytial Viruses; Type 2 immunity

Role of breast regression protein (BRP-39) in Respiratory Syncytial Virus-induced airway inflammation

Min Jung Kim

Department of Medicine
The Graduate School, Yonsei University

(Directed by Professor Myung Hyun Sohn)

I. INTRODUCTION

Viral bronchiolitis is a very common clinical syndrome affecting infants and young children due to viral infection of the lower respiratory tract. The peak incidence of bronchiolitis occurs between 3 and 6 months of age. The clinical symptoms and signs are quite variable with presentations ranging from low-grade fever, cough, and rhinorrhea to tachypnea, grunting, increased respiratory effort with retractions, and even apnea. Various risk factors have been associated with progression to severe bronchiolitis, such as the presence of chronic lung disease of prematurity, immunodeficiency or neuromuscular disorders. Young infants aged < 2-3 months also could be at high risk for progression and presenting apnea suddenly without other clinical findings.[1] In the United States, between 2% and 3% of all children younger than 12 months of age are hospitalized with a diagnosis of bronchiolitis, which accounts for between 57,000 and 172,000 hospitalizations annually.[2]

Respiratory syncytial virus (RSV) is the most commonly identified virus associated with bronchiolitis. RSV is found to constitute 50–80% of isolated viruses in nasopharyngeal aspirates from hospitalized children with bronchiolitis[2] and is the leading cause of infant hospitalization.[3] RSV shows the annual epidemics usually begin in November, peaking in January or February and ending in May in the temperate region.[4] Once virus-containing droplets come into the airway and descend into the lungs, the virus primarily infects the epithelial cells. RSV could induce lower respiratory tract illnesses (LRTIs) ranging

from bronchiolitis to viral pneumonia, which can lead to death in severe cases.[5] In addition, all children have been infected with RSV by the age of 2 years, and nearly half of those will experience recurrent infections.[4] However, current therapeutic options are limited to supportive treatments focusing on fluid and respiratory maintenance. Widely available and cost-effective preventative options are also lacking. In addition, the mechanism by which RSV evades host defenses is not fully understood.[2]

When investigating the consequences of early-life RSV infections, a large proportion of young patients with RSV bronchiolitis are found to have recurrent post-bronchiolitis episodes of lower airway obstruction, which may continue for years after the acute infection has resolved.[2] And several prospective birth cohort studies have been reported that early-life RSV LRTI has been considered to play an important causative role in the pathogenesis of recurrent wheezing, and asthma further.[4,6,7] Therefore, efforts to understand RSV pathogenesis in humans and to discover prophylactic or therapeutic strategies for RSV infection in the respiratory tract are required.

Breast regression protein 39 (BRP-39) and its human homologue YKL-40 (also called chitinase 3-like 1 protein [CHI3L1] and human cartilage glycoprotein 39) are the prototypes of enzymatically deficient chitinase-like proteins (CLPs) produced by the *CHI3L1* gene on chromosome 1 in mouse and human as 39-kD proteins. BRP-39 and YKL-40 are expressed by a variety of cells including neutrophils, monocytes, macrophages, chondrocytes, synovial cells, smooth muscle cells, endothelial cells, and tumor cells.[8] In many disorders, the levels of YKL-40 reflect the activity and natural history of the disease. YKL-40 was suggested as a new biomarker for joint injury based on analysis of YKL-40 in serum and synovial fluid. And serum YKL-40 was introduced as a new potential marker for the prognosis and location of metastases in patients with recurrent breast cancer. It has also been shown to be increased in patients with hepatic fibrosis.[9] In chronic lung inflammation, circulating levels of YKL-40 have been shown to be increased in patients with asthma and correlated with the severity of disease. Serum YKL-40 levels correlated positively with the level of expression of YKL-40 in the airway, the thickness of the subepithelial basement membrane, and clinical indexes of disease severity and correlated inversely with lung function.[10,11] In a study using transgenic and null mutant mice, the biologic properties of BRP-39 and YKL-40, the mouse and human versions of this prototypic CLP were proved as an important regulator in the development of allergic responses and tissue remodeling.[8] And several studies have examined the relationship between CHI3L1 and other chronic lung inflammation or acute lung injury.[12-14] The role of CHI3L1 in respiratory viral infection, however, has not yet been investigated.

In this study, I hypothesized that CHI3L1/BRP-39 might be involved in airway inflammation induced by RSV infection in a murine model. To test this hypothesis, I used BRP-39 null mice and compared the immune responses induced by RSV between wild-type (WT) mice and BRP-39 null mice. I was able to identify that RSV infection could induce IL-13 dominant immune responses in a murine model and that CHI3L1/BRP-39 might augment RSV-induced airway hyperresponsiveness (AHR), inflammatory cell

recruitment, and mucus production in virus-infected mice. Moreover, treatment with anti-CHI3L1 antibody in RSV infected WT mice could attenuate RSV-induced airway inflammation and the production of Th2 cytokines, suggesting CHI3L1 as a potential target to treat RSV-associated LRTI.

II. MATERIALS AND METHODS

1. Mice

C57BL/6 wild-type (WT) mice, 6 - 8 weeks old, 18 - 20 g of body weight, were purchased from Orient-Bio (Seoul, Korea). And C57BL/6 background BRP-39 null mice generated as previously described,[8] were a generous gift from Jack Elias (Brown University, Providence, RI, USA). Animals were housed under specific pathogen-free conditions. And all animal experiments were approved and conducted under the guidelines of the Institutional Animal Care and Use Committee of Yonsei University College of Medicine [Seoul, Korea, IACUC No. 2013-0305].

2. Preparation of RSV stock

RSV A2 strain was propagated in HEp-2 cells (ATCC, Manassas, VA, USA) in MEM (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 20 mM HEPES, nonessential amino acids, penicillin, and streptomycin. Virus was harvested at day 4 after infection when the infected HEp-2 cells exhibited maximal cytopathic effects. In brief, cells were disrupted by three cycles of freeze-thawing, then sonicated for 1 min, and centrifuged at 300 *g* for 10 min. The supernatants were collected, combined with cleared supernatants from the infected HEp-2 culture, and centrifuged at 75,000 g for 1 h. The pellets were resuspended with serum-free MEM by using a 25-gauge needle and brief sonication, and the final titer was determined by a standard plaque assay.[15]

3. Animal model of RSV infection

6 to 8-week-old WT mice and age-matched BRP-39 null mice were anesthetized by intraperitoneal injection of Zoletil^{TM} 50 (Virbac Laboratory, Carros, France) and Rompun® *inj*. (BayerKorea, Korea) and inoculated intratracheally with a 4 x 10^7 plaque-forming unit (PFU) suspended with 50 μl of serum-free MEM. Control mice were treated with the same volume of sterile PBS or serum-free MEM through the same route. Animals were weighed every day until the day of tissue harvest. Illness severity was measured daily based on weight loss. Daily weight loss was displayed as a percentage of original body weight before infection.

On days 1, 3, 5, 7, 10 and 14 after the inoculations, bronchoalveolar lavage (BAL) fluid and whole-lung specimens were harvested. After mice were cannulated using IV catheter (BD Bioscience, San Jose, CA, USA), lung and airways were washed twice with 0.9 ml of sterile PBS. The bronchoalveolar lavage fluids (BALF) were centrifuged at 300 *g* for 5 min, and then supernatants were frozen for later analysis. After collection of BALF, lungs were perfused with 10 ml of sterile PBS injected into the right ventricle, then

removed and rinsed in sterile PBS. Whole mouse lungs were homogenated using a disposable homogenizer (BioMasher-II®; Nippi, Tokyo, Japan). A tissue protein extraction reagent (T-PER®; Thermo Fischer Scientifics, Waltham, MA, USA) was then added, supplemented with protein inhibitor cocktail tablets (cOmplete™, Mini; Roche, Basel, Switzerland). Lysates were centrifuged at 10,000 *g* for 5 min and supernatants were frozen until further experiments.

4. Total and differential counting of cells in BALF

On day 7 post infection (dpi), the collected BALF cells were resuspended in sterile PBS for total and differential cell counts. Total BALF leukocyte counts were determined for each mouse via light microscopy using tryptan blue exclusion. BALF cells were centrifuged onto slides using a cytospin centrifuge (Thermo Fischer Scientifics). Air-dried and fixed cytospin slides were stained with Diff-Quik® staining reagent (Merck, Darmstadt, Germany). Manual differential cell counts were made on 200 cells per each slide. The total number of each cell population was determined by multiplying the percentage of each cell population by the total cell counts.

5. Measurement of AHR in mice

AHR was determined at 7 dpi using Flexivent (SCIRECQ, Montreal, QC, Canada). Mice were anesthetized, tracheotomized, and intubated with an IV catheter (BD Bioscience, San Jose, CA). Mice were mechanically ventilated using Flexivent and challenged with aerosolized saline or methacholine (Sigma-Aldrich) in increasing concentrations (0, 3.125, 6.25, 12.5, 25, and 50 mg/ml). Lung resistance (R_r) was calculated from measures of pressure and flow, and expressed as cm/H_2O/ml/s. R_r was determined as the average of three measurements at each dose per mouse over three independent experiments.

6. Histological analysis and Immunohistochemistry

Lungs from each group were fixed in 4% formaldehyde overnight at 4℃. The tissue was dehydrated by being gradual soaked in alcohol and xylene and, then was embedded in paraffin. The paraffin-embedded specimens were cut into 5 μm sections and stained with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS) to show inflammation or mucus production, respectively.

For immunohistochemistry (IHC), paraffin tissue sections were dewaxed, and blocked with Fc block antibody (BD Pharmingen) for 1 h at room temperature. Sections were incubated with antibody against GP-39 (Santa Cruz Biotechnology, Dallas, TX, USA) for detection BRP-39 at 4℃ overnight. The next day,

after incubation with goat anti-mouse IgG for 1 h at room temperature, slides were developed by REAL EnVision™ Detection System (DakoCytomation A/S, Copenhagen, Denmark).

7. Measurement of cytokines

Levels of IFN-γ, IL-6, IL-10, MCP-1, and RANTES were measured in BALF using cytokine bead assay (CBD flexset kit) according to the manufacturer's instructions (BD Pharmingen, San Diego, CA, USA).

And IL-4, IL-5, and IL-13 were measured in BALF and lung lysates using ELISA kits as directed by the manufacturer (R&D Systems, Minneapolis, MN, USA).

8. Quantification of BRP-39

The levels of BRP-39 in BALF and lung lysates were evaluated by ELISA kit for mouse CHI3L1 (R&D systems).

9. Quantification of mucus production

Muc5AC protein was measured using ELISA, as described by Lee et al.[16] Lung lysates were prepared with PBS and incubated at 37℃ in a 96-well plate overnight until dry. After being washed with PBS and blocked with 2% BSA for at least1 h at room temperature. Then the plate was incubated with a mouse monoclonal Muc5AC antibody (Thermo Fisher Scientific) (1:100) in PBS for 1 h at room temperature and goat anti-mouse IgG-HRP (Santa Cruz Biotechnology) (1:5000) was added for 1 h at room temperature. The plate was washed well and incubated with substrate, tetramethylbenzidine (TMB) solution (KPL, Gaithersburg, MD, USA) in a dark place and stopped by adding 2 N H_2SO_4. The absorbance was read at 450 nm.

10. Real-time polymerase chain reaction

Total RNA from the lung was extracted using the TRIZOL Reagent (Life Technologies, Carlsbad, CA, USA). The purity of the RNA was checked by measuring the absorbance ratio at 260/280 nm, and values ranging from 1.8 to 2.0 were accepted. 5 μg of RNA was converted into cDNA using Superscript III Reverse Transcriptase (Invitrogen Life Technologies, Carlsbad, CA, USA). TaqMan Universal PCR Master Mix and TaqMan primer-probe sets for IL-4, IL-5, IL-13, CHI3L1 and GAPDH were purchased from Applied Biosystems (Foster, CA, USA). Samples were tested in duplicate. The PCR threshold cycle (C_T) for each target product was set in relation to the amplification of the housekeeping gene, GAPDH.

The C_T value was defined as the number of PCR cycles required for the fluorescent signal to exceed the detection threshold value. The relative mRNA expressions of IL-4, IL-5, IL-13 and CHI3L1 were calculated using the comparative $\Delta\Delta C_T$ method. Briefly, the relative quantization of the target value, normalized to the GAPDH gene and relative to controls, is expressed as $2^{-\Delta\Delta CT}$ (*n*-fold induction compared to control animals). For quantification of RSV, known concentrations of RSV A2 were used to derive a standard curve. Standards and negative controls were run together with each PCR assay. Customized TaqMan primer-probe set of RSV N gene, the well-conserved region of the RSV A2. Forward (5'-AGA TCA ACT TCT GTC ATC CAG CAA) and reverse (5'-TTC TGC ACA TCA TAA TTA GGA GTA TCA AT) primers amplified an 85-bp region containing the 25-mer FAM labeled probe (5'-CACCATCCAACGGAGCACAGGAGAT).[17] The gene expression quantification was performed using StepOne™ Real-Time PCR System (Applied Biosystems).

11. Flow cytometry

After whole lung from the mouse was chopped and homogenized, tissues were digested using a collagenase IV (CSL4, Worthington Biochemicals, fraction IX; Sigma-Aldrich, St. Louis, MO, USA) and DNase I (50 U/mL, Sigma-Aldrich) in a RPMI 1640 medium containing 10% FBS. Samples were incubated in a shaking incubator for 60 min at 37℃. Then total lymphocytes were washed and enriched by a discontinuous density gradient of Percoll (Amersham Biosciences, Pittsburgh, PA, USA).[18] Isolated cells were incubated with Fc block antibody (2.4G2, BD Pharmingen) and stained with fluorescein isothiocyanate (FITC)-conjugated rat anti-mouse CD4 (H129.19, BD Pharmingen), allophycocyanin (APC)-conjugated rat anti-mouse CD8 (53-6.7, BD Pharmingen), phycoerythrin (PE)-conjugated F4/80 (BM8, eBioscience, San Diego, CA, USA), APC/Cy7-conjugated anti-mouse CD45 (30-F11, BioLegend), PE-Cyanine7 anti-mouse CD11b (M1/70, BD Bioscience), Alexa Fluor 780-conjugated hamster anti-mouse CD11c (HL3, BD Bioscience), and PerCP-Cy5.5 rat anti-mouse I-A/I-E (M5/114.15.2, BD Pharmingen).

BALF cells were collected and stimulated for 4 h with 50 μg/mL phorbol12-myristate 13-acetate and 500 ng/mL in the presence of a protein transport inhibitor. Cells were stained for cell-surface proteins, fixed/permeablized, and stained for intracellular antigen, a PE-Cyanine7-conjugated anti-mouse IL-13 (eBio13A, eBioscience). All samples were analyzed on LSR II (BD Bioscience) using Flowjo V Software (Tree Star, Ashland, OR, USA).

12. Assessment of macrophage activation

For assessment of alternative macrophage activation, the surface expression of CD206 on alveolar

macrophages in BALF and lung cells was evaluated. In addition, arginase activity was measured in BALF and lung lysates using the QuantiChrom™ Arginase Assay Kit according to the manufacturer's instructions (BioAssay Systems, Hayward, CA, USA).[19]

13. Anti-CHI3L1 treatment in wild-type mice

WT RSV mice were treated 24 h after RSV infection via the intraperitoneal route with either a single 50 μg dose of anti-mCHI3L1 (AF2649, R&D Systems)[20] or with vehicle only. Mice were euthanized at 7 dpi, and inflammatory changes and immune responses induced by RSV infection were analyzed. Production of Th2 cytokines was compared using ELISA and RT-PCR between RSV-infected mice and antibody-treated mice. The change of BRP-39 expression was also compared. Lungs from each group were stained with H&E or PAS to show inflammation or mucus production, respectively.

14. Study population

From 2013 to 2016, nasopharyngeal aspirates were collected from hospitalized children in Severance Children's Hospital due to acute respiratory symptoms such as cough, wheeze, or tachypnea. Among those patients, children with congenital heart disease, history of prematurity, chronic lung disease of prematurity or recurrent infections were excluded. Patients with other respiratory infection detected by multiplex virus PCR panel or serological test were also excluded. A total of 40 children were enrolled in this study. 32 children belonged to the group of RSV where RSV A only was detected from nasopharyngeal aspirates. The control group consisted of 8 patients with mild respiratory symptoms, and without any detected pathogens. To evaluate the severity of each child's respiratory illness, respiratory symptom scores were recorded at admission.[21]

YKL-40, the human homologue of CHI3L1, and IL-13 were measured in nasopharyngeal aspirates from enrolled patients using ELISA kits (YKL-40; R&D systems, IL-13; Abcam, Cambridge, United Kingdom).

This study was approved by the institutional review board of Severance Hospital [Seoul, Korea, IRB No. 4-2012-0880]. All protocols and methods in this study were carried out in accordance with relevant guidelines and regulations.

15. Statistical analysis

Experimental data were expressed as means ± SD. Groups were compared via unpaired Student's t-test using GraphPad Prism (version 6.04). $P < 0.05$ was considered statistically significant.

Patients' baseline characteristics were compared using Mann-Whitney U test or Fisher's exact test, as

appropriate. Data were reported as numbers (percentages) or medians (inter-quartile range, [IQR]). And the correlation between levels of YKL-40 and IL-13 measured in nasopharyngeal aspirates was determined using the Spearman rank correlation test. A p value < 0.05 was considered statistically significant. Statistical software (SPSS, version 20.0; SPSS Inc; Chicago, IL) was used for all analyses.

III. Results

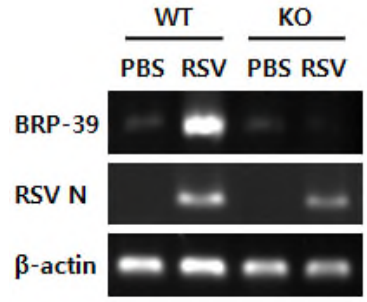
Expression of BRP-39 in RSV infection

To evaluate the role of BRP-39 in RSV infection, the expression of BRP-39 was compared between control and RSV-infected mice at 7 dpi. In RSV-infected mice, the expression of BRP-39 in the lungs and BALFs were increased compared to control mice. The levels of BRP-39 mRNA and protein were significantly increased after RSV infection (Fig. 1, A-D). This increase was not found in BRP-39 knock-out (KO) mice, even after RSV infection (Fig.1, A and D). BRP-39 was induced predominantly in airway epithelial cells and alveolar macrophages (Fig.1, E). Thus, BRP-39 is prominently induced during RSV infection in a murine model.

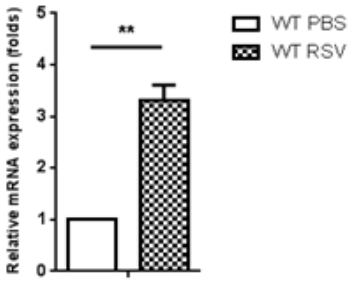
Attenuated RSV-induced airway inflammation in BRP-39 null mice

In WT mice, RSV infection induced significant inflammatory changes at 7 dpi, including weight loss, increased AHR in methacholine test, and increased inflammatory cell recruitments in BALF (Fig.2 A-C). Total cells, neutrophils, lymphocytes, and eosinophils were significantly increased in BALF after RSV infection (Fig.2 D). Tissue inflammation and recruited inflammatory cells were also found in the histological analysis (Fig.2 E). In addition, all of these inductive inflammatory responses were significantly decreased in BRP-39 null mice, even after RSV infection. In BRP-39-/- mice, RSV infection did not induce significant changes in weight loss, airway resistance, and inflammatory cell recruitments (Fig.2 A-D). Lung inflammation in RSV-infected BRP-39 null mice was less severe than in RSV-infected WT mice (Fig.2 E). Mucus production was also increased after RSV infection, but decreased in RSV-infected BRP-39 null mice (Fig.2 F and G). No significant differences were observed in viral load followed by RSV infection between WT and BRP null mice (Fig.2 H and I). These data suggested that the absence of BRP-39 could attenuate airway inflammation induced by RSV infection, ever, without significant changes in viral load.

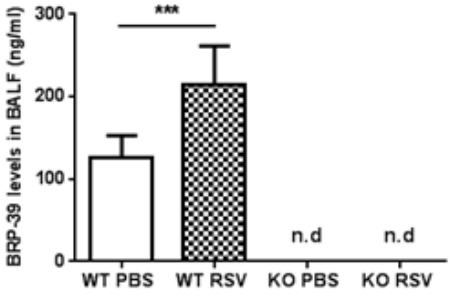
A



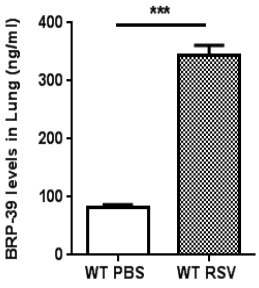
B



C



D



E

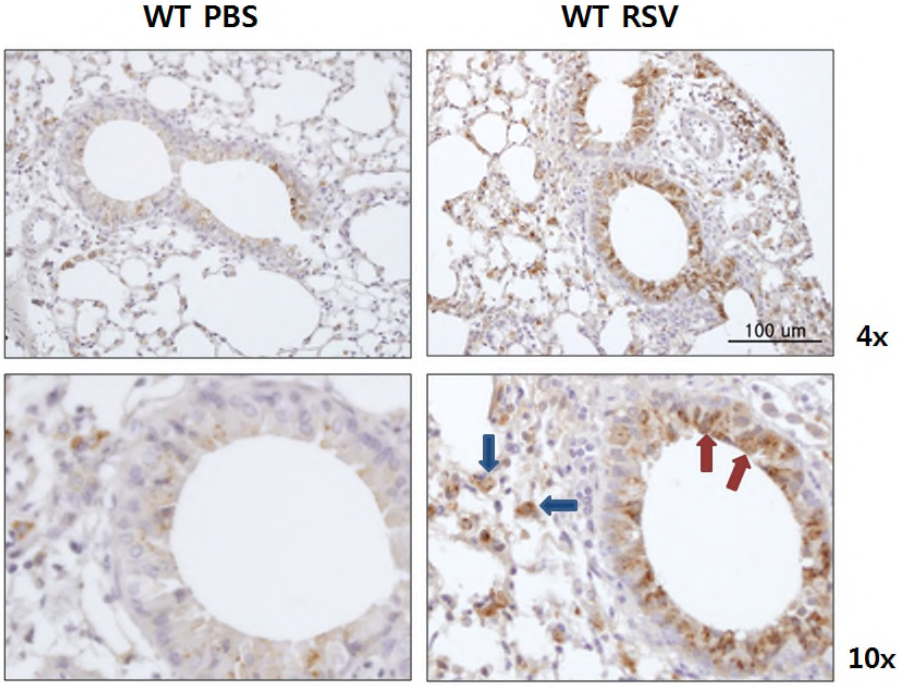
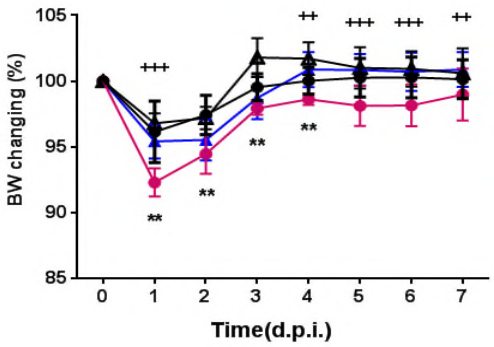


Figure 1. BRP-39 expression in an RSV-infected murine model

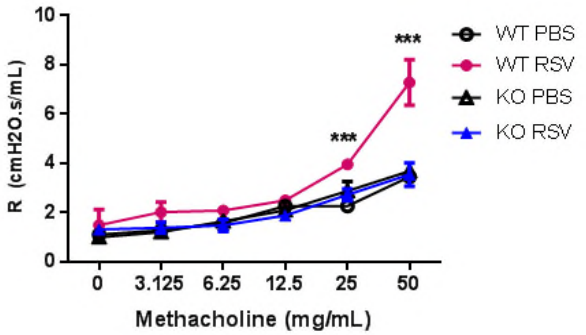
C57BL/6 wild-type (WT) mice were infected with RSV (4 x 10^7 PFU/mice) in an intratracheal route. (A and B) When comparing the expression of BRP-39 between WT control mice and RSV-infected mice at 7 dpi, BRP-39 mRNA expression levels were increased. (C and D) According to BRP expression assessed by ELISA in BALF and lung lysates, RSV infection induced increased levels of BRP-39. (E) Immunohistochemistry was used to localize BRP-39 in lung sections (red arrows, airway epithelial cells; blue arrows, alveolar macrophages). Values in B, C, and D are the mean ± SD of at least 3 independent experiments. n.d., not detected.

$^{**}p < 0.01$, $^{***}p < 0.001$.

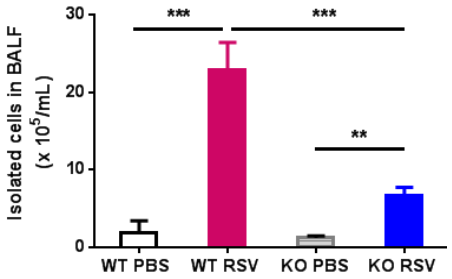
A



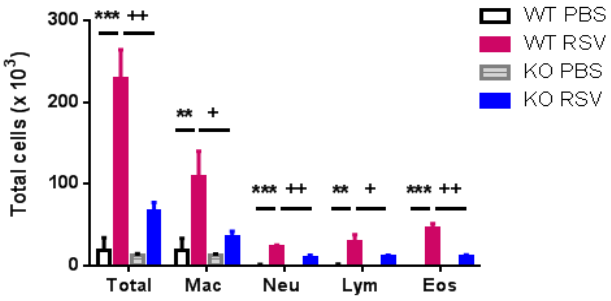
B



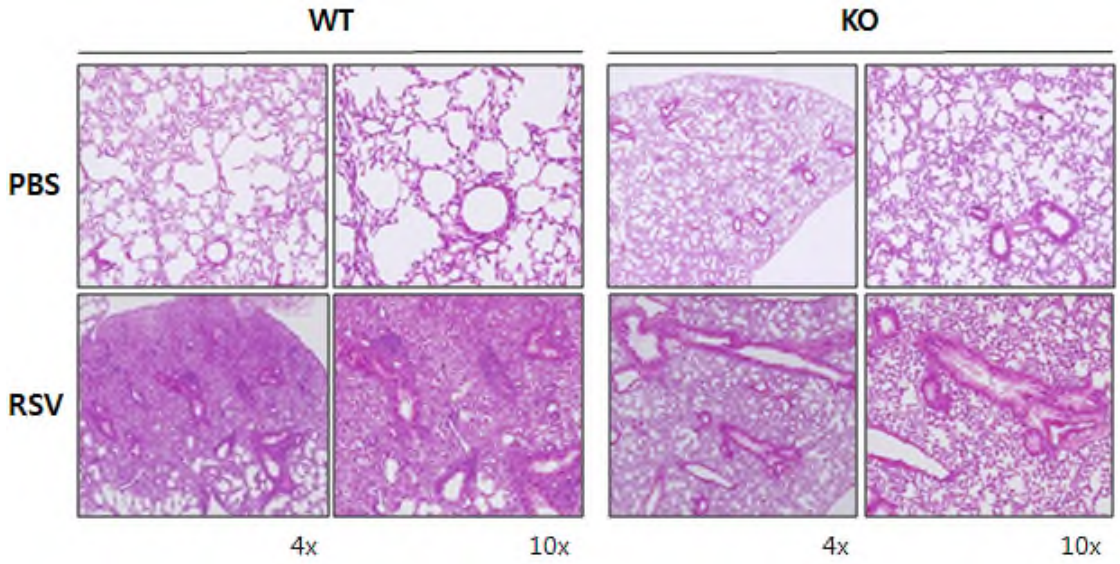
C



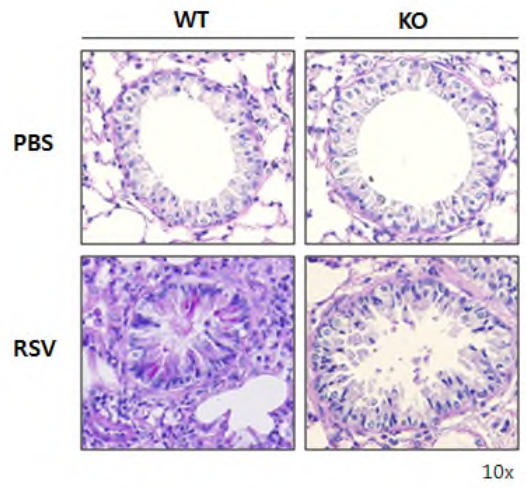
D



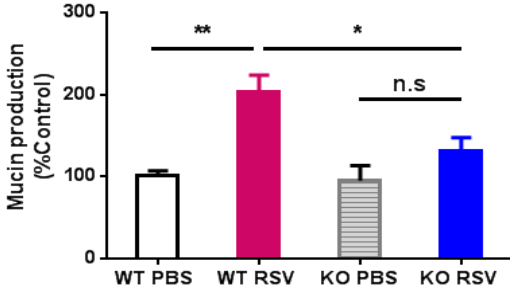
E



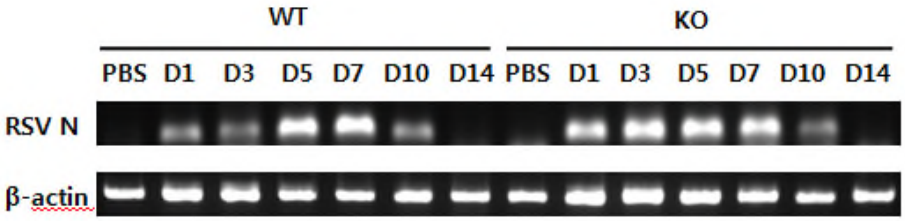
F



G



H



I

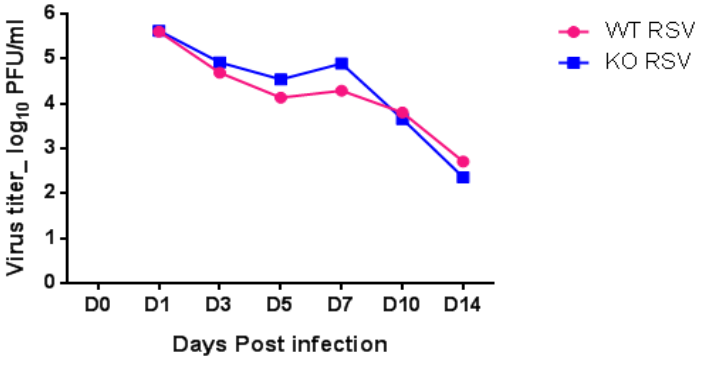


Figure 2. BRP-39 deficiency attenuated RSV-induced inflammatory changes in mice

WT and BRP-39 null mice were infected with RSV, and the resulting inflammatory changes were compared. On the day 7 after RSV infection, WT mice showed increased levels of (A) weight loss, (B) airway resistance and (C, D) total and differentiated inflammatory cells in BALF. (E) H&E staining of the lung sections demonstrated perivascular and peribronchiolar infiltration of inflammatory cells in RSV-infected WT mice. But the inflammatory responses induced by RSV infection were significantly decreased in BRP-39 KO mice. Mucus production was evaluated using PAS staining (F) and ELISA (G). RSV infection increased mucus production in WT mice, but not in BRP KO mice. (H, I) BRP-39 did not affect lung viral load as measured on days 1, 3, 5, 7, 10, and 14 after infection.

(A-D) The values were mean $\pm$ SD of at least 3 independent experiments. n.s., not significant.

For Fig.2 A and D, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared between WT PBS and WT RSV and $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ compared between WT RSV and KO RSV.

For Fig.2 B, $***p < 0.001$ compared between WT RSV and both WT PBS and KO RSV.

$*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

BRP-39 regulates Th2 inflammation induced by RSV infection

In the lungs of RSV-infected WT mice, increased numbers of CD4+ T cells, dendritic cells (DCs), and eosinophils were seen, but not in BRP-39 KO mice (Fig.3 A-C). RSV infection caused significant increases in Th2 cytokine mRNA in the lungs from WT mice. IL-4 and IL-5 measured in the lungs from WT RSV mice were increased 1.3 and 2.0 folds compared to WT PBS mice (p = 0.03 and p = 0.04, respectively). Strikingly, lung IL-13 mRNA in WT RSV mice was increased 6.2 folds compared to WT PBS mice (p = 0.001). However, in BRP-39 null mice, levels of Th2 cytokine mRNA did not change significantly under any conditions (Fig.4 A-C). In BALF, IL-13 levels were also statistically increased in WT RSV infections (45 $\pm$ 15 pg/ml for WT PBS, 350 $\pm$ 60 pg/ml for WT RSV, p = 0.04). But, in BRP-39 KO mice, IL-13 levels in BALF were not detected (Fig.4 D). Regarding other inflammatory cytokines, RSV infection induced increases in IFN-γ in both WT and BRP-39 KO mice (Fig.5 A), while levels of MCP-1, IL-10, and IL-6 were not changed by RSV infection in both WT and BRP-39 KO mice (Fig.5 B-D). Thus, BRP-39 might exaggerate Th2 inflammation in RSV-infected mice, but IFN- γ production and expression of other inflammatory cytokines were not affected.

BRP-39 regulation of M2 macrophage activation

In accordance with increased Th2 inflammation and AHR in RSV-infected mice, macrophage activation was also evaluated. Increased expression of alternatively activated macrophages (M2 macrophages) was found in BALF cells and lung (Fig.6 A and C). To determine the activity of M2 macrophages, ariginase 1 activity was measured in BALF cells (Fig. 6 B) and lung lysates (Fig.6 D). Followed by RSV infection, both WT and BRP-39 KO mice showed significantly increased levels of arginase 1 activity. The arginase 1 activity of BRP-39 KO RSV mice, on the other hand, was decreased by > 50% compared to WT RSV mice. These results indicate that BRP-39 could regulate the accumulation and activation of M2 macrophages in RSV-infected mice.

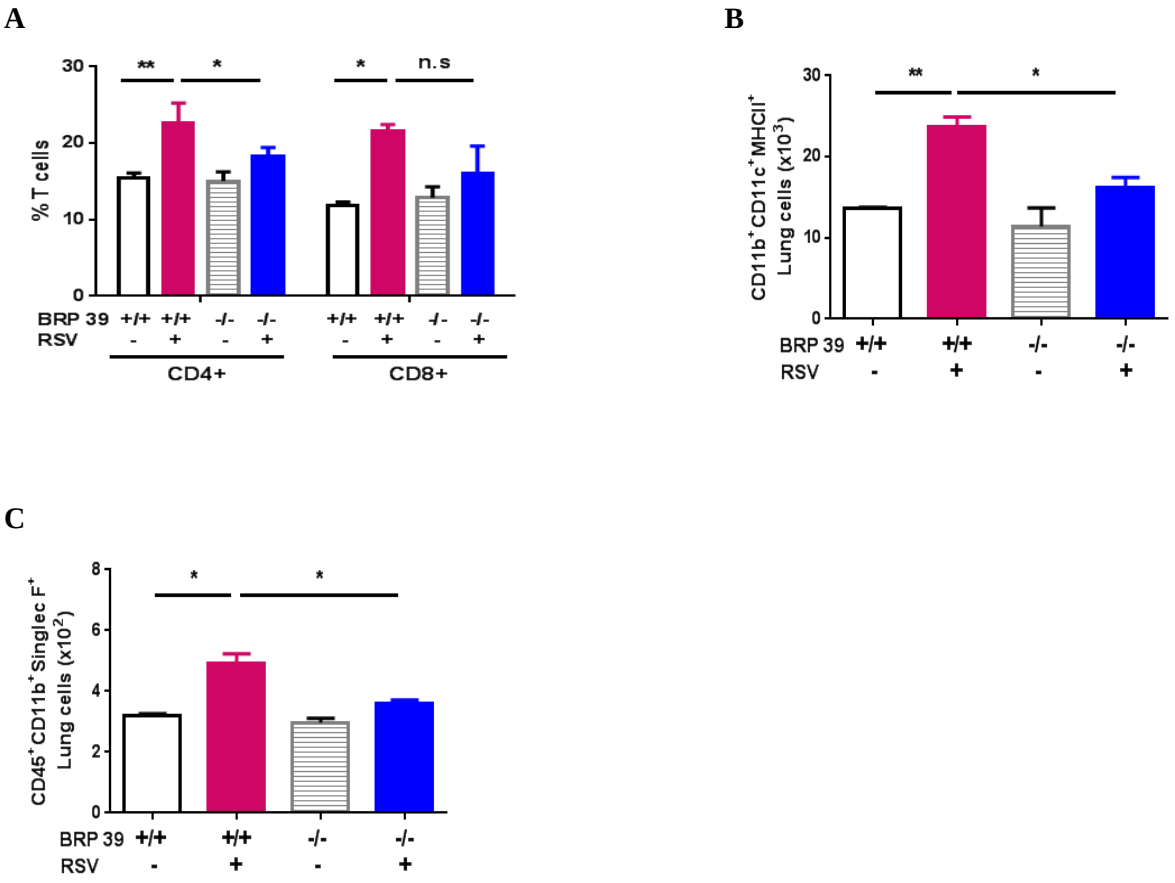


Figure 3. BRP-39 regulation in the activation of T cells, dendritic cells, and eosinophils

Lungs from each group were assessed by flow cytometry. (A) CD4 and CD8 T cells were increased in WT RSV mice, while CD4 T cells were decreased in BRP-39 KO mice. (B) Dendritic cells were detected by staining with anti-CD11b, CD11c, and MHCII. In the absence of BRP-39, DC was decreased from RSV-infected WT mice. (C) Eosinophil expression was also increased in the lungs of WT RSV-infected mice but decreased in BRP-39 KO mice. n.s., not significant.

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

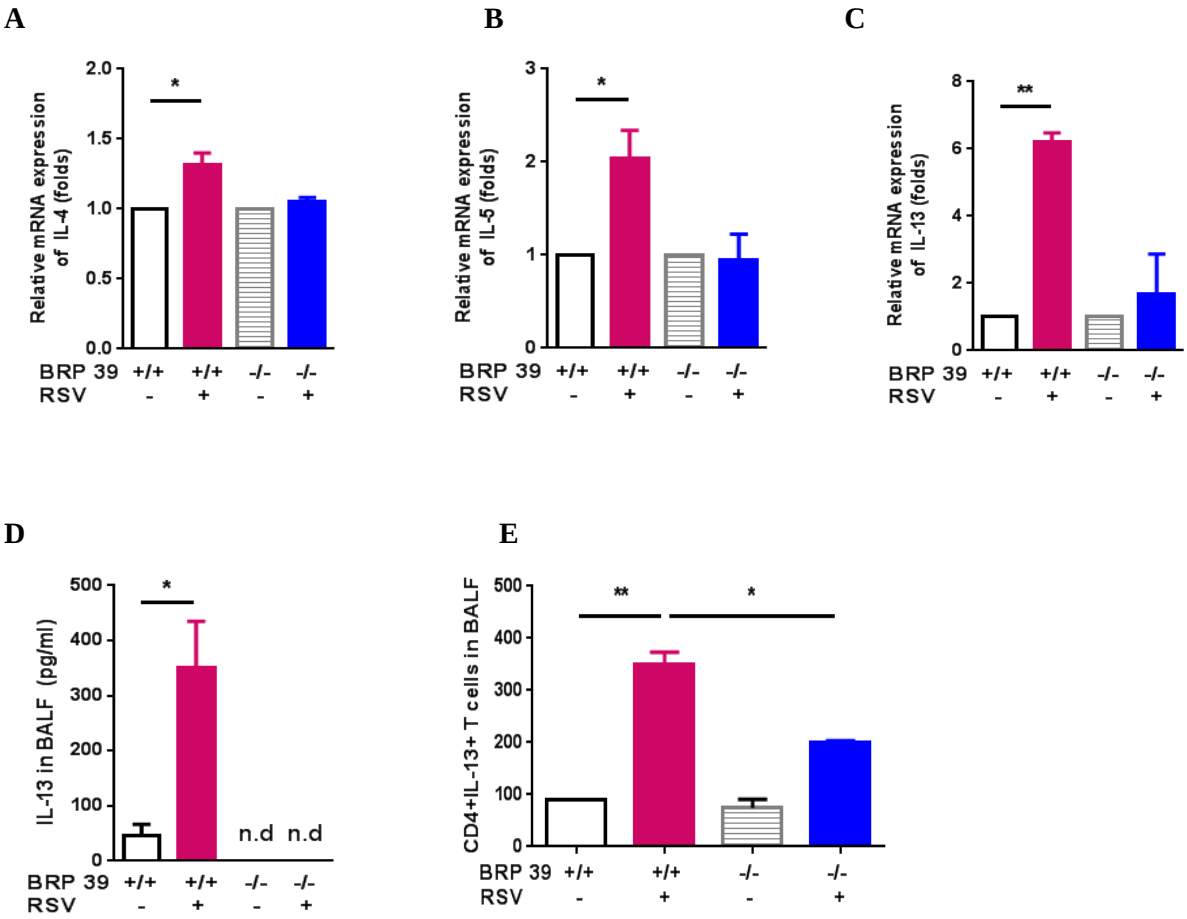


Figure 4. Role of BRP-39 in RSV-induced Th2 responses

WT and BRP-39 KO mice were inoculated with RSV and euthanized at 7 dpi. (A-C) The mRNA levels of Th2 cytokines were determined by RT-PCR, and among Th2 cytokines, IL-13 showed the largest increase in RSV-infected WT mice. In BRP-39 KO mice, Th2 cytokines were not significantly increased by RSV infection. And (D) the level of IL-13 in BALF was also significantly increased in WT RSV mice, measured by ELISA. (E) IL-13 expressing CD4 T cells in BALF cells were assessed. The values are the mean ± SD of at least 3 independent experiments. n.d., not detected.

*$p < 0.05$, **$p < 0.01$.

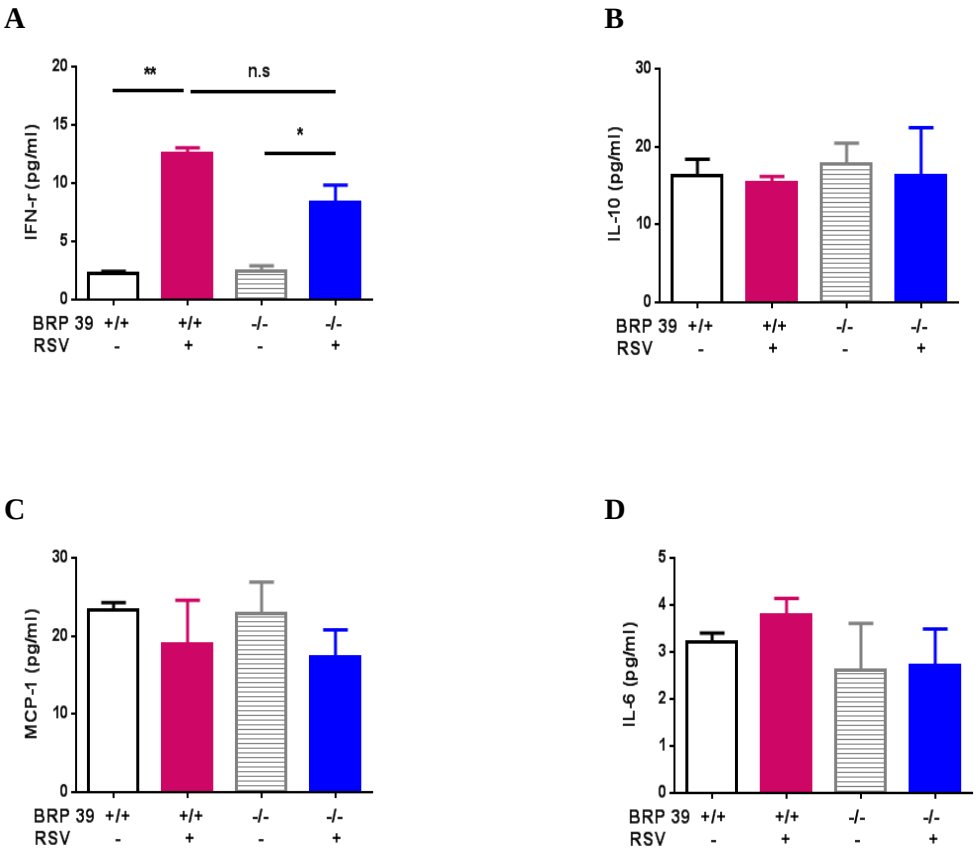


Figure 5. Inflammatory cytokines in BRP-39 null mice after RSV infection

(A) IFN-γ, (B) IL-10, (C) MCP-1, and (D) IL-6 in BALF were evaluated using cytokine bead assay (CBA). Production of IFN-γ increased similarly after RSV infection in both WT and BRP-39 KO mice. BRP-39 did not change the expression of other inflammatory cytokines. n.s., not significant.

*$p < 0.05$, **$p < 0.01$.

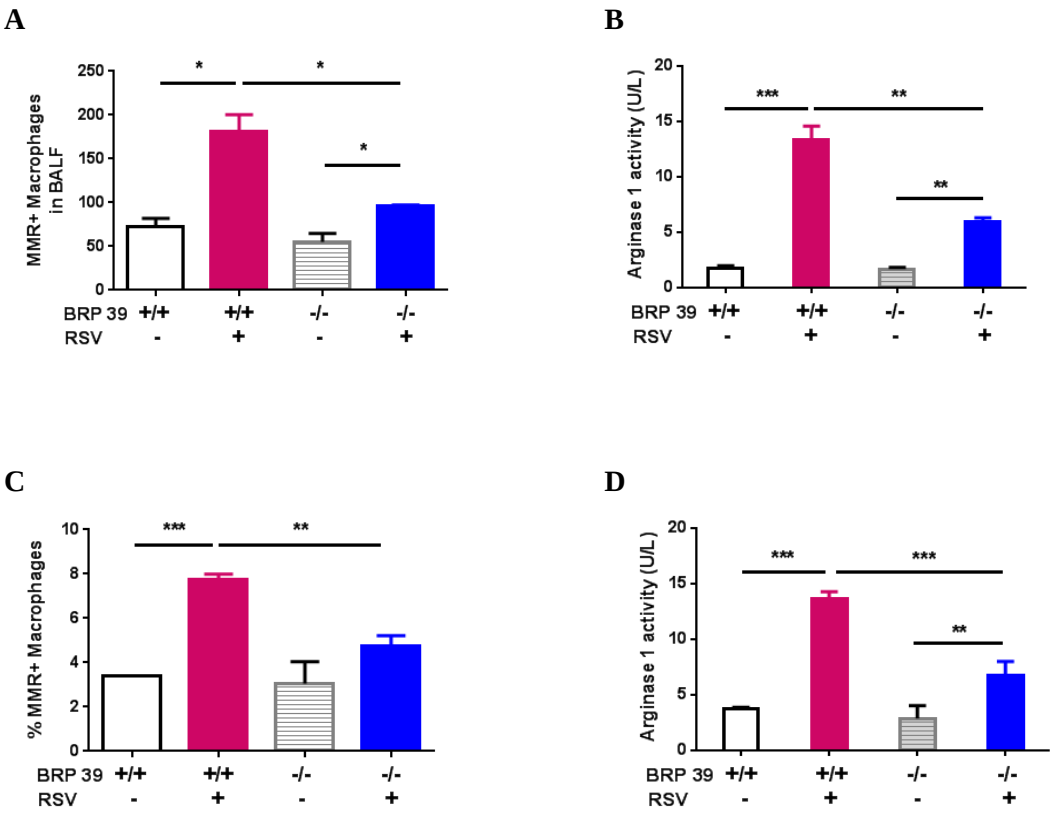


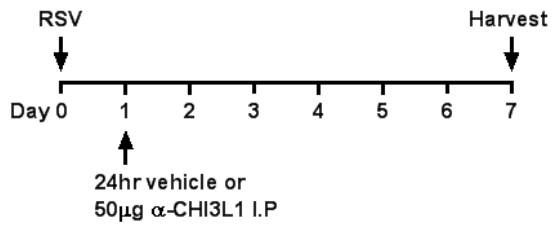
Figure 6. BRP-39 regulation of macrophage activation in RSV infection

To compare M2 macrophage activation between WT and BRP-39 KO mice, (A, C) MMR expressing alternatively activated macrophages were quantitated and ariginase 1 activity was measured in BALF cells (B) and lung lysates (D). MMR+ macrophages and the arginase 1 activity of BRP-39 KO RSV mice were decreased compared WT RSV mice. The values are the mean ± SD of at least 3 independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

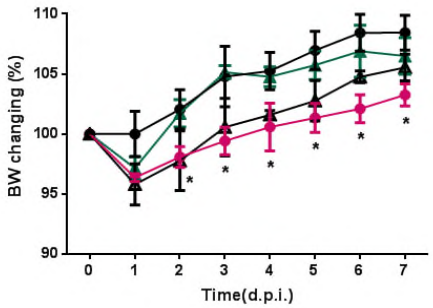
Effects of anti-CHI3L1 on RSV-induced inflammatory changes in mice

To evaluate the therapeutic effect of anti-CHI3L1 in RSV infection, RSV-infected WT mice were i.p treated with anti-mCHI3L1 (AF2649, R&D Systems). WT RSV mice were treated 24 h after RSV infection with a single 50 μg dose of anti-mCHI3L1 or with vehicle only (Fig.7 A). Inflammatory responses induced by RSV infection were not different between untreated WT RSV mice and vehicle-treated mice. Mice given anti-CHI3L1 treatment, however, showed less body weight loss (Fig.7 B) and decreased AHR (Fig.7 C) compared to WT RSV mice. Inflammatory cell recruitment in mice given anti-CHI3L1 treatment was also decreased compared with WT RSV mice (Fig.7 D and E). Increased expression of BRP-39 as a result of RSV infection was also decreased in mice treated with anti-CHI3L1 (Fig.7 F and G). Mucus production in mice given treatment was decreased compared to untreated RSV-infected WT mice (Fig.7 H and J). However, anti-CHI3L1 treatment did not directly alter viral load in the lungs. Between each group, there was no difference in viral load (Fig.7 I). With regard to RSV-induced Th2 responses, expression of Th2 cytokines was significantly increased in WT RSV mice than WT PBS mice but was decreased in mice treated with anti-CHI3L1. (Fig.8 A-F) A decrease in IL-4 and IL-13 was observed in the anti-CHI3L1 group, but the degree of the decrease in IL-13 was larger than that of IL-4. These results indicate that BRP-39 could be a potential therapeutic target for attenuating Th2-associated immunopathology during RSV infection.

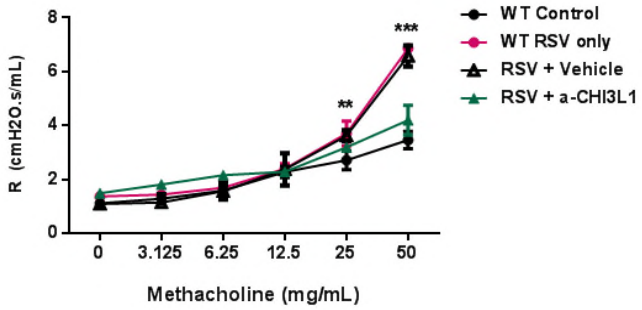
A



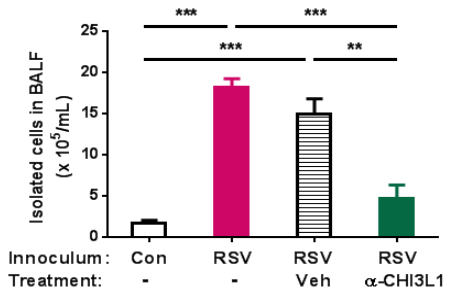
B



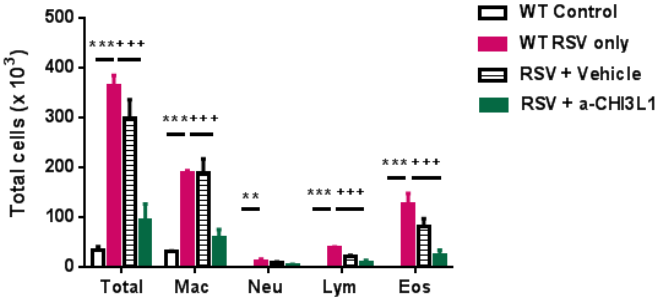
C



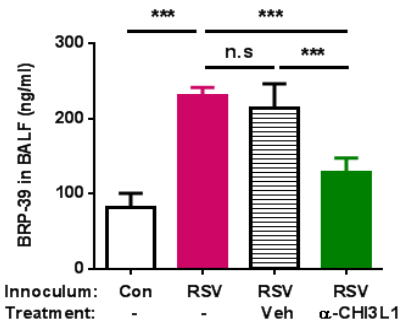
D



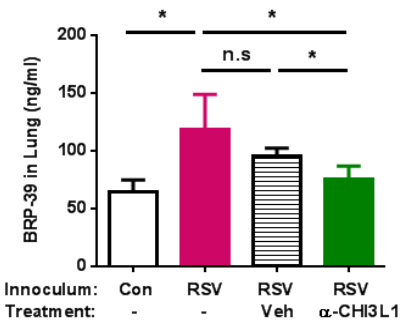
E



F



G



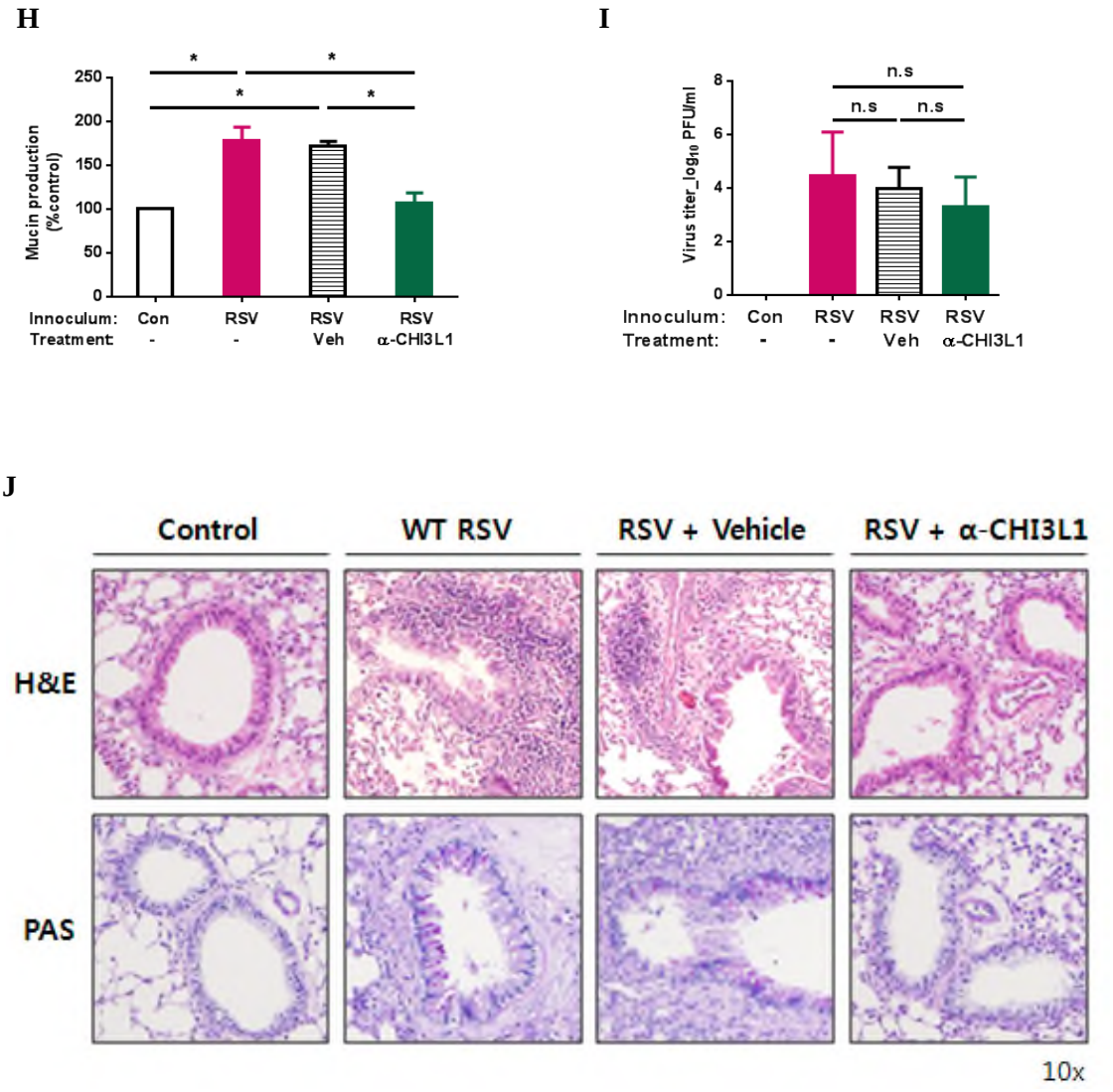


Figure 7. Effects of anti-CHI3L1 on RSV-induced airway inflammation

(A) Protocol for treatment with either anti-CHI3L1 or vehicle. (B) Mice treated with anti-CHI3L1 24 h after RSV infection showed less body weight loss than WT RSV mice. At 7 dpi, mice were evaluated for AHR (C), BAL total and differentiated cells (D, E), levels of BRP-39 (F, G) and mucus production (H). (I) There was no difference in viral load between each group. (J) H&E and PAS staining were used to evaluate airway inflammation and mucus production. Anti-CHI3L1 treatment in RSV-infected mice decreased inflammatory changes by RSV infection. All data from 3 mice per group plotted as means ± SD. Con, control; veh, vehicle; n.s., not significant.

For Fig.7 B and C, WT PBS mice compared with both WT RSV mice and vehicle-treated mice.

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

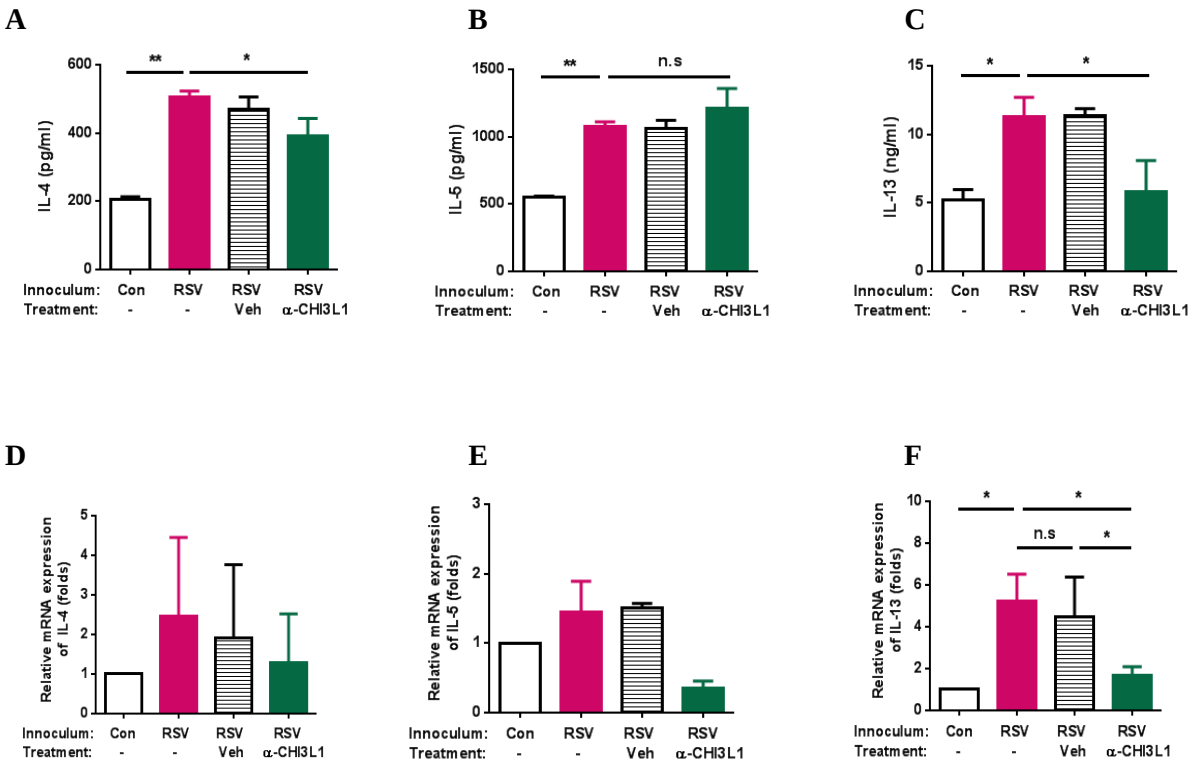


Figure 8. Effects of anti-CHI3L1 on Th2 responses in RSV-infected mice

(A-C) Levels of whole-lung Th2 cytokines measured by ELISA at day 7 after RSV infection. (D-F) mRNA expressions of Th2 cytokines in the lungs of each group were compared. Production of Th2 cytokines was decreased by treatment with anti-CHI3L1 in RSV-infected mice, and IL-13 was the most prominent cytokine to show statistically significant change (C and F). All data from 3 mice per group plotted as means ± SD. Con, control; veh, vehicle; n.s., not significant.

*$p < 0.05$, **$p < 0.01$.

YKL-40 expression in children with RSV infection

To determine whether the CHI3L1 expression is increased in patients with RSV infection, human homologue YKL-40 was measured in nasopharyngeal aspirates (NPA) collected from hospitalized children presenting with acute respiratory symptoms. 32 patients with RSV infection confirmed by PCR diagnosis and 8 patients without any confirmed pathogen were compared (Fig.9 A). The clinical characteristics of subjects are summarized in Table 1. There were no significant differences in age, gender, duration of admission, past history and laboratory values between the 2 groups. However, children with confirmed RSV infection showed higher scores for acute respiratory symptoms measured at admission (median, 5; IQR, 5 to 9.8) than the control group (median, 10; IQR 8.3 – 11, p = 0.011).

When comparing YKL-40 levels in NPA, YKL-40 levels in children with RSV infection (median, 2168 pg/mL; IQR, 998 - 2357 pg/mL) were significantly higher than in control subjects (median, 762.0 pg/mL; IQR, 213.5 - 2017 pg/mL, p = 0.0009) (Fig.9 B). And IL-13 levels in NPA (median, 3.911 pg/mL; IQR, 2.836 - 8.434 pg/mL) was also increased in the RSV infection group compared to control subjects (median, 2.440 pg/mL; IQR, 1.993 - 3.215 pg/mL, p = 0.0049) (Fig.9 C). In all subjects, YKL-40 levels were positively correlated with symptom scores (r = 0.374, p = 0.018) (Fig.9 D). In addition, YKL-40 concentrations in NPA showed a positive correlation with IL-13 levels in NPA (r = 0.383, p = 0.015) (Fig.9 E). These data suggest that the expression of CHI3L1 is also increased by RSV infection in humans and correlated with severity of symptoms. They also suggest that CHI3L1 expression might be related to IL-13 expression and IL-13-induced airway inflammation further.

Table 1. Clinical characteristics

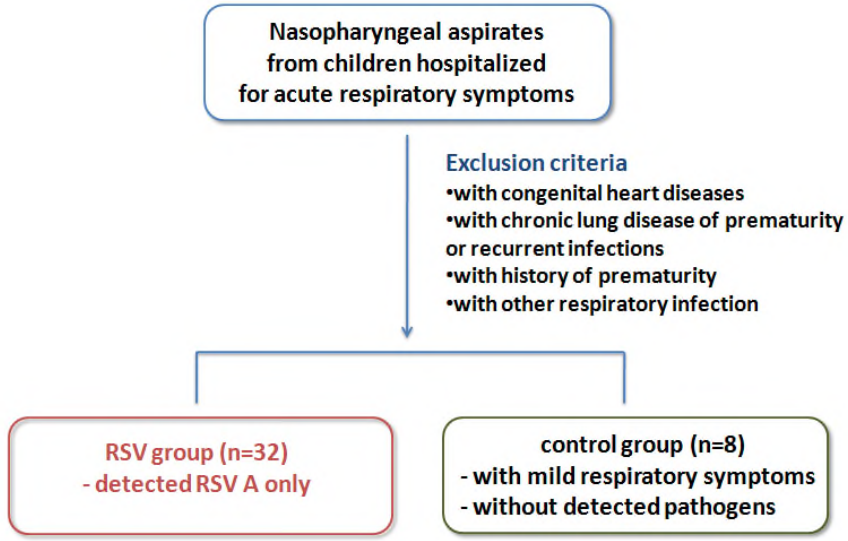
Characteristics	Control (n=8)	RSV (n=32)	*p*-value
Age, months	11.9 (3.5 – 17.8)	7.0 (3.3 – 13.5)	0.454
Male, n (%)	6 (75.0)	16 (50.0)	0.258
LOS, days	4 (3 – 6.3)	5 (4 – 6.8)	0.065
Symptom Scores	5 (5 – 9.8)	10 (8.3 – 11)	0.011*
Steroid Tx, n (%)	6 (75.0)	29 (90.6)	0.257
Past History			
Bronchiolitis Hx, n (%)	2 (25.0)	6 (18.8)	0.650
Allergic Hx, n (%)	1 (12.5)	4 (12.5)	1.000
Parental allergic Hx, n (%)	2 (25.0)	1 (3.1)	0.096
Laboratory Values			
WBC, μL	9950 (7453 – 12325)	9435 (7268 – 12338)	0.728
Hb, g/dL	11.4 ± 1.09	11.5 ± 0.9	0.704
Hct, %	33.4 ± 2.8	33.9 ± 2.3	0.796
Platelets, 10^3/μL	398 ± 134	412 ± 140	0.649
Neutrophil, μL	3560 (2605 - 6805)	3310 (1522 – 5485)	0.309
Eosinophil, μL	155 (25 – 325)	80 (35 – 253)	0.539
ESR, mm/hr	9 (2.5 – 56)	20.5 (3.3 – 43.3)	0.607
CRP, mg/L	4.2 (0.5 – 15.1)	9.5 (2.3 – 36.3)	0.359

Data are presented as number of patients (%) or median value (inter-quartile range).

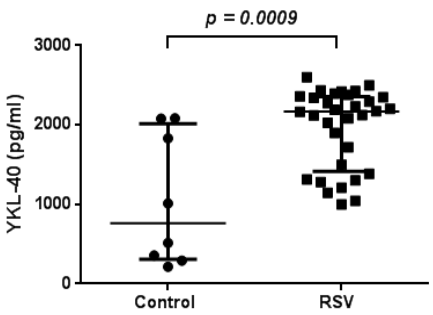
LOS, length of stay; Tx, treatment; Hx, history; WBC, white blood cell count; Hb, hemoglobin; Hct, hematocrit; ESR, erythrocyte sedimentation rate; CRP, C-reactive protein.

*$p < 0.05$.

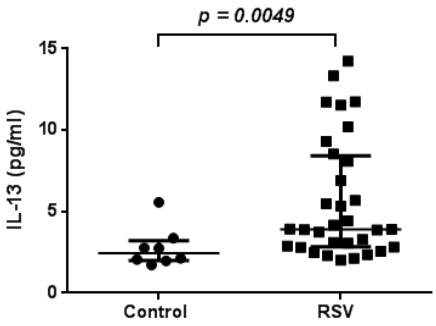
A



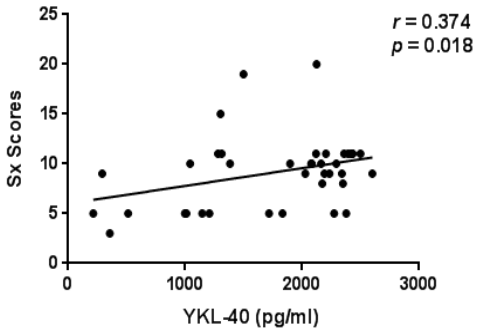
B



C



D



E

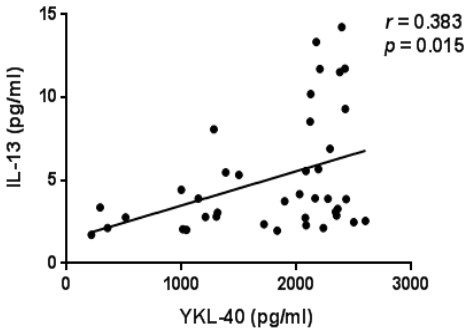


Figure 9. YKL-40 levels in human nasopharyngeal aspirates

(A) Enrollment of study population. Children with RSV infection had significantly higher levels of YKL-40 (B) and IL-13 (C) in NPA than control subjects (P = 0.0009, and P = 0.0049, respectively). In addition, YKL-40 in NPA showed a positive correlation with scores of clinical symptoms (r = 0.374, p = 0.018) (D) and with IL-13 levels in NPA (r = 0.383, p = 0.015) (E). The scatter dot plots indicate the individual value of each patient, with the median line and the error bars representing the 25^{th} and 75^{th} percentiles.

IV. Discussion

In this study, a mouse CHI3L1 protein, BRP-39, was shown to be necessary for severe lung immunopathology followed by RSV infection in a murine model. BRP-39-deficient mice had reduced weight loss, airway hyperresponsiveness, inflammatory cell infiltration into the airway, and mucus production, compared with WT mice while maintaining a similar viral load, suggesting that BRP-39 might be a potential therapeutic target for IL-13 dominant immunopathology associated with RSV infection. Moreover, in vivo neutralization of CHI3L1 using an anti-CHI3L1 antibody could make IL-13-biased airway inflammation during RSV infection less severe. In human subjects, the importance of CHI3L1/YKL-40 in RSV infection and its relationship with IL-13 were also proved.

Chitinase-like proteins are structurally similar with chitinases but lack of enzyme activity to chitin. Recent studies have demonstrated the roles of played by mammalian CLPs at sites of inflammation and tissue remodeling, not even triggering responses or attracting eosinophils and T cells to parasitic infection.[9,22] In allergic responses, BRP-39 was demonstrated to be a key regulator of Th2 inflammation, M2 macrophages differentiation, and Th2 cell and macrophages apoptosis/cell death. BRP-39 null mice showed a significant defect in antigen-induced Th2 inflammation and IL-13-induced inflammation remodeling in previous studies.[8,9] In humans, circulating level of YKL-40 was increased in patients with asthma, and correlated with the severity of disease. In addition, serum YKL-40 levels correlated positively with the level of expression of YKL-40 in the airway, the thickness of the subepithelial basement membrane, and clinical indexes of disease severity but correlated inversely with lung function.[10] YKL-40 concentrations in BALF measured in children with asthma were also higher than non-asthmatics.[23] A promoter SNP (−131C→G) in CHI3L1, the chitinase 3–like 1 gene encoding YKL-40, was also reported to be associated with elevated serum YKL-40 levels, asthma, bronchial hyperresponsiveness and measures of pulmonary function.[17] In addition, the g.-247C/T polymorphism in the CHI3L1 promoter region was shown to be associated with the risk of atopy in Korean children.[24] BRP-39/YKL-40 was also decreased in the hyperoxic state, and they were reported to be critical regulators of oxidant injury, inflammation, and epithelial apoptosis in the murine and human lung.[12] With regard to bacterial infection, CHI3L1 induced by *Streptococcus pneumoniae* was demonstrated to play a central role in promoting bacterial clearance and mediating host tolerance.[14] The mechanisms via which CHI3L1/BRP-39/YKL-40 mediates these biologic effects are poorly understood. A recent study demonstrated that CHI3L1 bound to IL-13 receptor α2 (IL-13Rα2) composing a multimeric complex with IL-13.[25] However, there is still a lack of studies investigating the relationship between CHI3L1 and respiratory viral infection induced Th2 inflammation or asthma.

RSV is an enveloped virus of the Paramyxoviridae family with a single-stranded negative-sense RNA. RSV is divided into 2 antigenic subgroups, A and B. Strains of both subtypes often co-circulate, but

generally one of the subtypes predominates.[26,27] RSV is the most significant cause of severe lower respiratory diseases in infants and young children. It has also identified as an increasing cause of morbidity and mortality in the elderly and in immune-suppressed individuals. Severe RSV infection in young children is characterized by an infiltration of inflammatory cells into the airspaces airway epithelial cell destruction and sloughing, mucus hyperproduction, peribronchiolar inflammation, and edema of the airway wall. These processes lead to a narrowing of the airway lumen, airflow obstruction, overinflation, and impaired gas exchange. Eventually, children with severe RSV infection present with crackles and wheeze with labored breathing, tachypnea, and even hypoxia.[2]

The primary target of RSV infection is airway epithelial cells, particularly those that are ciliated.[5] Airway inflammation is initially caused by the necrosis of airway epithelial cells due to the direct cytopathological effect of RSV, then the immune response to RSV may damage the airways through inflammatory processes.[28] When RSV infects the airway epithelial cells and the pulmonary macrophages, the up-regulation of cytokines and chemokines such as MCP-1, IL-6, IL-8, TNF-α, RANTES, and IL-1β could be followed. These responses promote inflammation and recruitment of neutrophils, eosinophils, and monocytes into the lungs. Although T cells are essential for the clearance of viral infection and the generation of protective immunity, cellular immunity is also paradoxically associated with increased severity of RSV infection. Classically, $CD4^+$ T cells have been associated with advanced airway inflammation in RSV infection.[29,30] RSV infection has been associated with skewing the immune system away from an antiviral Th1 response and towards a Th2 response.[31,32] This is closely to the development of wheezing and asthma. Th2 cytokines (IL-4 and IL-13) contribute to enhancing the lung damage stimulating airway hyperactivity, the chemotaxis of eosinophils and mucus production.[28,29,32] In addition, alternative alveolar macrophages (M2 macrophages) induced by IL-4 and IL-13 are also produced during RSV infection and if M2 macrophages persist, it can lead to Th2-skewed immune responses that lead to the development of asthma.[33,34] Meanwhile, recent studies reported the importance of the epithelium-associated cytokines IL-25, IL-33, and thymic stromal lymphopoietin, which can induce proliferation and activation of Group 2 innate lymphoid cells (ILC2s).[35,36] RSV infection causes a wide array of immunologic responses. However, the immunologic contributions to the pathophysiology of severe RSV infection are incompletely understood.

In addition to the significant morbidity and mortality caused by the acute infection, a large proportion of these young patients continue to have recurrent post-bronchiolitis episodes of lower airway obstruction, that may continue for years after the acute infection has resolved.[4,5] It is possible that there is a certain relationship between early-life RSV LRTI and later-onset asthma. In fact, increased prevalence of asthma/recurrent wheezing (39 vs 9%), clinical allergy (43 vs 17%) and sensitization to perennial allergens (41 vs 14%) were present at age 18 in the RSV cohort compared with controls.[7] Those reports suggested that RSV played an important causative role in the pathogenesis of recurrent wheezing, and asthma further.

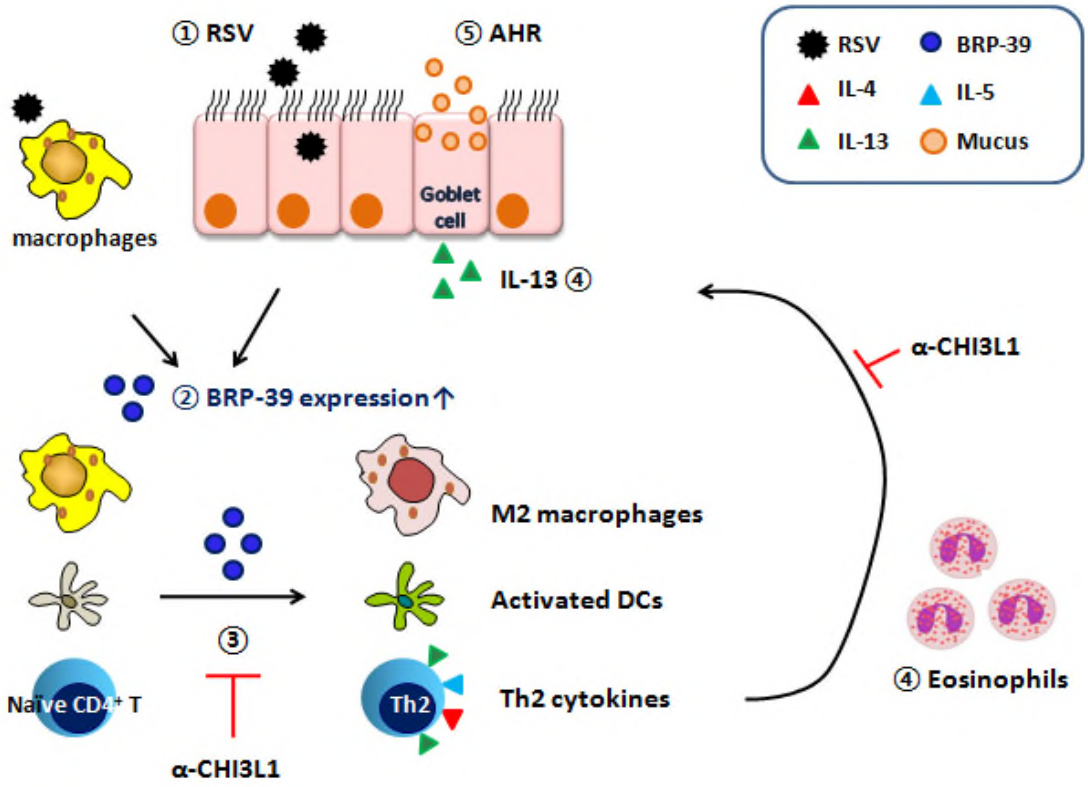
However, the link between RSV infection and the development of chronic airway dysfunction – usually diagnosed as asthma – has long been debated. It is certain about increasing risk of subsequent wheezing in children who have had RSV infection in early life, but the question remains as to whether RSV is a risk factor, or rather a marker of predisposition to asthma. Therefore, it is necessary to find another factor or molecule involved in the RSV-asthma pathway to prevent asthma development earlier.

I investigated whether there is a relationship between BRP-39 and RSV-induced LRTI using BRP-39 null mice. The production of BRP-39 was increased followed by RSV infection in the airway. And BRP-39 was produced by airway epithelial cells and alveolar macrophages. After RSV infection, WT mice showed decreased body weight, increased airway hyperresponsiveness, and inflammatory cell recruitments, but those inflammatory responses were diminished in BRP-39$^{-/-}$ mice. RSV-induced Th2 responses in the airways, especially IL-13 production, and activation of DCs and M2 macrophages were also decreased in BRP-39$^{-/-}$ mice. In this animal experiment, decreased Th2 inflammation during RSV infection in BRP-39 null mice might have resulted from decreased activation of DCs and M2 macrophages. Because DCs are considered major antigen-presenting cells in RSV infection, their maturation and ability to recognize antigen could alter the cytokine milieu through a variety of mechanisms.[26,37] In addition, M2 phenotype macrophages are reported as being critical to regulating the immune response in severe acute viral infection via IL-4/IL-13 dependent differentiation and activated M2 macrophages could contribute to IL-13 production in a positive feedback loop.[34,38] In these ways, a deficiency of BRP-39 could alter the progression of inflammation induced by RSV infection, without effect on the virus itself. Interestingly, anti-CHI3L1 administered 24h after RSV infection attenuated RSV-induced airway inflammation and the production of Th2 cytokines, IL-13 in particular. These results suggested that BRP-39 was required for RSV-induced airway inflammation, and that BRP-39 could be a therapeutic target for RSV-related LRTI.

The precise mechanism was not studied in this experiment. But i*n vitro* study reported that bronchial epithelial cells treated with YKL-40 resulted in a significant increase in IL-8 production, which was dependent on activation of MAPK (JNK and ERK) and NF-kB pathways, causing proliferation and migration of bronchial smooth muscle cells.[39] IL-8 production through the activation of NF-kB pathway is also increased during RSV infection.[40] When BEAS-2B cells, the human bronchial epithelial cells, were infected with RSV, they showed increased levels of YKL-40 and IL-8. In addition, production of YKL-40 and IL-8 was decreased in shYKL-40-treated BEAS-2B cells after RSV infection. Based on these results, I anticipated that the regulation of BRP-39/YKL-40/CHI3L1 in RSV-induced airway inflammation might be involved in the NF-kB pathway. In this study, the results focused on RSV-induced Th2 inflammation during the adaptive immune phase, because Th2 inflammation, especially IL-13, was thought to be a bridge between RSV and BRP-39. Attention to the interaction of RSV-airway epithelium or to the immune responses during the early stages of RSV infection was little. And the expression of IL-13Rα2, a receptor for CHI3L1 during effector responses was not investigated. The roles of CHI3L1 as a regulator in airway

inflammation, oxidant injury, antibacterial responses, and TGF-β1 production were dependent on IL-13Rα2.[25] Therefore, further studies focusing on innate immune responses (e.g. ILC2s) during the early phases of RSV infection in BRP-39 null mice, and on IL-13Rα2 expression and regulation in RSV infection might be needed in order to better understand the relationship between BRP-39 and RSV infection. In addition, a neonatal animal model of RSV infection using BRP-39 null mice might be also considered to determine the role of BRP-39 in asthma development, consistent with the role of early-life viral bronchiolitis in asthma inception.

V. Conclusion



In conclusion, this study demonstrated that BRP-39 could be an important regulator of RSV-induced airway inflammation. RSV infection induces increased expression of BRP-39 produced by macrophages and epithelial cells at sites of Th2 inflammation. A lack of BRP-39 could attenuate significantly weight loss, airway hyperresponsiveness, and Th2 (IL-13) responses, and activation of DC and M2 macrophages. RSV-induced airway inflammation and IL-13 production were decreased following anti-CHI3L1 treatment. Additionally, in human NPA from children with RSV-related LRTI, the expression of CHI3L1 and IL-13 were significantly increased. These data suggest that BRP-39 could play an important role in RSV-induced airway inflammation and the neutralization of BRP-39 could be a potential therapeutic for RSV-related LRTI.

REFERENCES

1. Florin TA, Plint AC, Zorc JJ. Viral bronchiolitis. The Lancet. 2017;389:211-24.
2. Meissner HC. Viral Bronchiolitis in Children. N Engl J Med. 2016;374:62-72.
3. Hall CB, Weinberg GA, Iwane MK, Blumkin AK, Edwards KM, Staat MA, et al. The burden of respiratory syncytial virus infection in young children. N Engl J Med. 2009;360:588-98.
4. Piedimonte G. Respiratory syncytial virus and asthma: speed-dating or long-term relationship? Curr Opin Pediatr. 2013;25:344-9.
5. Villenave R, Shields MD, Power UF. Respiratory syncytial virus interaction with human airway epithelium. Trends Microbiol. 2013;21:238-44.
6. Beigelman A, Bacharier LB. The role of early life viral bronchiolitis in the inception of asthma. Curr Opin Allergy Clin Immunol. 2013;13:211-6.
7. Sigurs N, Aljassim F, Kjellman B, Robinson PD, Sigurbergsson F, Bjarnason R, et al. Asthma and allergy patterns over 18 years after severe RSV bronchiolitis in the first year of life. Thorax. 2010;65:1045-52.
8. Lee CG, Hartl D, Lee GR, Koller B, Matsuura H, Da Silva CA, et al. Role of breast regression protein 39 (BRP-39)/chitinase 3-like-1 in Th2 and IL-13-induced tissue responses and apoptosis. J Exp Med. 2009;206:1149-66.
9. Lee CG, Da Silva CA, Dela Cruz CS, Ahangari F, Ma B, Kang MJ, et al. Role of chitin and chitinase/chitinase-like proteins in inflammation, tissue remodeling, and injury. Annu Rev Physiol. 2011;73:479-501.
10. Geoffrey L. Chupp, Chun Geun Lee, Nizar Jarjour, Yun Michael Shim, Carole T. Holm, Susan He, et al. A Chitinase-like Protein in the Lung and Circulation of Patients with Severe Asthma. N Engl J Med. 2007;357:2016-27.
11. Carole Ober, Zheng Tan, Ying Sun, Jennifer D. Possick, Lin Pan, Raluca Nicolae, et al. Effect of Variation in CHI3L1 on Serum YKL-40 Level, Risk of Asthma, and Lung Function. N Engl J Med 2008;358:1682-91.
12. Sohn MH, Kang MJ, Matsuura H, Bhandari V, Chen NY, Lee CG, et al. The chitinase-like proteins breast regression protein-39 and YKL-40 regulate hyperoxia-induced acute lung injury. Am J Respir Crit Care Med. 2010;182:918-28.
13. Matsuura H, Hartl D, Kang MJ, Dela Cruz CS, Koller B, Chupp GL, et al. Role of breast regression protein-39 in the pathogenesis of cigarette smoke-induced inflammation and emphysema. Am J Respir Cell Mol Biol. 2011;44:777-86.
14. Dela Cruz CS, Liu W, He CH, Jacoby A, Gornitzky A, Ma B, et al. Chitinase 3-like-1 promotes Streptococcus pneumoniae killing and augments host tolerance to lung antibacterial responses. Cell Host Microbe. 2012;12:34-46.

15. Kim S, Joo DH, Lee JB, Shim BS, Cheon IS, Jang JE, et al. Dual role of respiratory syncytial virus glycoprotein fragment as a mucosal immunogen and chemotactic adjuvant. PLoS One. 2012;7:e32226.
16. Lee HJ, Ryu J, Park SH, Seo EK, Han AR, Lee SK, et al. Suppressive effects of coixol, glyceryl trilinoleate and natural products derived from Coix Lachryma-Jobi var. ma-yuen on gene expression, production and secretion of airway MUC5AC mucin. Arch Pharm Res. 2015;38:620-7.
17. Estripeaut D, Torres JP, Somers CS, Tagliabue C, Khokhar S, Bhoj VG, et al. Respiratory syncytial virus persistence in the lungs correlates with airway hyperreactivity in the mouse model. J Infect Dis. 2008;198:1435-43.
18. Shim DH, Park YA, Kim MJ, Hong JY, Baek JY, Kim KW, et al. Pandemic influenza virus, pH1N1, induces asthmatic symptoms via activation of innate lymphoid cells. Pediatr Allergy Immunol. 2015;26:780-8.
19. Pulichino AM, Wang IM, Caron A, Mortimer J, Auger A, Boie Y, et al. Identification of transforming growth factor beta1-driven genetic programs of acute lung fibrosis. Am J Respir Cell Mol Biol. 2008;39:324-36.
20. Bohr S, Patel SJ, Vasko R, Shen K, Golberg A, Berthiaume F, et al. The Role of CHI3L1 (Chitinase-3-Like-1) in the pathogenesis of infections in burns in a mouse model. PLoS One. 2015;10:e0140440.
21. Gern JE, Martin MS, Anklam KA, Shen K, Roberg KA, Carlson-Dakes KT, et al. Relationships among specific viral pathogens, virus-induced interleukin-8, and respiratory symptoms in infancy. Pediatr Allergy Immunol 2002;13:386-93.
22. Lee CG, Elias JA. Role of breast regression protein-39/YKL-40 in asthma and allergic responses. Allergy Asthma Immunol Res. 2010;2:20-7.
23. Goldman DL, Li X, Tsirilakis K, Andrade C, Casadevall A, Vicencio AG. Increased chitinase expression and fungal-specific antibodies in the bronchoalveolar lavage fluid of asthmatic children. Clin Exp Allergy. 2012;42:523-30.
24. Sohn MH, Lee JH, Kim KW, Kim SW, Lee SH, Kim KE, et al. Genetic variation in the promoter region of chitinase 3-like 1 is associated with atopy. Am J Respir Crit Care Med. 2009;179:449-56.
25. He CH, Lee CG, Dela Cruz CS, Lee CM, Zhou Y, Ahangari F, et al. Chitinase 3-like 1 regulates cellular and tissue responses via IL-13 receptor alpha2. Cell Rep. 2013;4:830-41.
26. Borchers AT, Chang C, Gershwin ME, Gershwin LJ. Respiratory syncytial virus--a comprehensive review. Clin Rev Allergy Immunol. 2013;45:331-79.
27. Hall CB. Resrpiatory Syncytial Virus and Parainfluenza Virus. N Engl J Med. 2001;344:1917-28.
28. Vandini S, Calamelli E, Faldella G, Lanari M. Immune and inflammatory response in bronchiolitis due to respiratory syncytial virus and rhinovirus infections in infants. Paediatr Respir Rev. 2017

http://dx.doi.org/10.1016/j.prrv.2016.11.006.

29. Openshaw PJ, Chiu C. Protective and dysregulated T cell immunity in RSV infection. Curr Opin Virol. 2013;3:468-74.
30. Christiaansen AF, Knudson CJ, Weiss KA, Varga SM. The CD4 T cell response to respiratory syncytial virus infection. Immunol Res. 2014;59:109-17.
31. Becker Y. Respiratory syncytial virus (RSV) evades the human adaptive immune system by skewing the Th1/Th2 cytokine balance toward increased levels of Th2 cytokines and IgE, markers of allergy--a review. Virus Genes. 2006;33:235-52.
32. Lotz MT, Peebles RS, Jr. Mechanisms of respiratory syncytial virus modulation of airway immune responses. Curr Allergy Asthma Rep. 2012;12:380-7.
33. Shirey KA, Pletneva LM, Puche AC, Keegan AD, Prince GA, Blanco JC, et al. Control of RSV-induced lung injury by alternatively activated macrophages is IL-4R alpha-, TLR4-, and IFN-beta-dependent. Mucosal Immunol. 2010;3:291-300.
34. Liu YC, Zou XB, Chai YF, Yao YM. Macrophage polarization in inflammatory diseases. Int J Biol Sci. 2014;10:520-9.
35. Stier MT, Bloodworth MH, Toki S, Newcomb DC, Goleniewska K, Boyd KL, et al. Respiratory syncytial virus infection activates IL-13–producing group 2 innate lymphoid cells through thymic stromal lymphopoietin. Journal of Allergy and Clinical Immunology. 2016;138:814-24.e11.
36. Petersen BC, Dolgachev V, Rasky A, Lukacs NW. IL-17E (IL-25) and IL-17RB promote respiratory syncytial virus-induced pulmonary disease. J Leukoc Biol. 2014;95:809-15.
37. Ravendra Garg, Pratima Shrivastava, Sylvia van Drunen, Hurk L-vd. The role of dendritic cells in innate and adaptive immunity to respiratory syncytial virus, and implications for vaccine development. Expert Rev Vaccines. 2012;11:1441-57.
38. Byers DE, Holtzman MJ. Alternatively activated macrophages and airway disease. Chest. 2011;140:768-74.
39. Tang H, Sun Y, Shi Z, Huang H, Fang Z, Chen J, et al. YKL-40 induces IL-8 expression from bronchial epithelium via MAPK (JNK and ERK) and NF-kappaB pathways, causing bronchial smooth muscle proliferation and migration. J Immunol. 2013;190:438-46.
40. Lay MK, Gonzalez PA, Leon MA, Cespedes PF, Bueno SM, Riedel CA, et al. Advances in understanding respiratory syncytial virus infection in airway epithelial cells and consequential effects on the immune response. Microbes Infect. 2013;15:230-42.

ABSTRACT (IN KOREAN)

호흡기성 융합 바이러스 감염에 의한 기도 염증 반응에서 breast regression protein-39 (BRP-39)의 역할

<지도교수 손 명 현>

연세대학교 대학원 의학과

김 민 정

Chitinase 3-like 1 (CHI3L1)는 천식과 같은 만성 염증성 질환에서 알레르겐의 감작과 Th2 염증 반응에 관여하는 것으로 알려져 왔다. 하지만 호흡기 바이러스 감염에 의한 급성 기도 염증 반응에 있어서 그 역할에 대한 연구는 없었다. 본 연구는 마우스 모델에서 마우스 CHI3L1 인 breast regression protein-39 (BRP-39) 이 호흡기성 융합 바이러스 (respiratory syncytial virus, RSV) 감염으로 나타나는 기도 염증 반응과 어떤 연관성이 있는지 알아보고자 하였다.

본 연구에서는 야생형 마우스와 BRP-39 결핍 마우스에 각각 RSV A2 를 감염 시킨 후 감염 7 일째 염증 세포 수, 기도 과민성 반응, BRP-39 과 싸이토카인 양 등을 측정하였다. RSV 감염에 따른 T 세포와 수지상 세포, 이형 대식세포 및 호산구의 활성화 정도를 비교하였다. 폐 조직 염색 (H&E, PAS 염색)을 통해 폐 조직 내 염증 및 손상 정도와 점액 생성 정도를 비교 분석하였다. 그리고 CHI3L1 의 치료적 효과를 확인하기 위해 RSV 에 감염된 야생형 마우스에 항-CHI3L1 항체를 복강 내 주입하였다. 또한 급성 호흡기 증상으로 입원한 소아 환자 중 호흡기성 융합 바이러스 감염이 확인된 환아군과 대조군의 비인두 흡입액에서 CHI3L1 의 발현양을 측정하였다.

야생형 마우스에 RSV 감염 시킨 7 일째 폐와 폐 세척액에서 BRP-39 의 발현이 증가하였다. 야생형 마우스가 RSV 에 감염되었을 때 대조군에 비해 몸무게가 감소하였고 메타콜린 유발 검사상 기도 과민성 반응이 증가하였음이 확인되었다. 또한 감염군의 폐 세척액 내 염증 세포 수가 증가하였고 특히 호산구의 발현이 증가한 것을 확인하였다. 하지만 BRP-39 결핍 마우스의 경우 RSV 에 감염되었음에도 야생형 마우스가 감염되었을 때보다는 폐 염증반응이 약화된 것을 확인할 수 있었다. RSV 감염으로 야생형 마우스 폐에서는 CD4+ T 세포와 수지상세포, 호산구 및 이형 대식세포가 활성화되었으나 BRP-39 결핍 마우스에서는 활성화 정도가 낮았고 Th2 염증반응 관련 사이토카인의 발현 역시 야생형 마우스 감염군에서 증가한 반면 BRP-39 결핍 마우스에서는 증가 폭이 낮았다. 특히 가장 변화가 큰 사이토카인은 IL-13 이었다.

BRP-39 결핍 마우스에서는 RSV 감염으로 인해 나타나는 기도 과민성 반응 및 기도 염증 반응이 약화된 결과를 바탕으로 항-CHI3L1 항체의 RSV 감염에 대한 치료 효과를 확인하였다. 야생형 마우스에 RSV 를 감염시키고 24 시간 후 항-CHI3L1 항체를 투여하였고 감염 후 7 일째 기도 과민성 반응 및 염증반응을 비교 분석하였다. 치료군의 경우 대조군에 비해 기도 과민성 및 폐 세척액 내 염증 세포수 증가 정도, BRP-39 발현양, Th2 사이토카인 증가 정도 모두 감소하는 것을 확인할 수 있었다. 또한 급성 호흡기 증상을 가진 환아들의 비인두 흡입액을 비교하였을 때 RSV 군이 대조군에 비해 YKL-40 와 IL-13 발현양이 증가하였고 YKL-40 발현양은 환아의 증상 점수와 IL-13 발현양과 양의 상관관계를 보이는 것으로 나타났다.

이러한 결과를 토대로 BRP-39 이 RSV 감염에 의한 기도 염증 반응에 관여하는 것을 확인하였다. 또한 BRP-39 을 표적화할 경우 Th2 연관 면역반응을 약화시킴으로써 RSV 감염으로 인한 하기도 질환 치료가 가능할 것으로 보인다.

핵심되는 말: 모세기관지염, 호흡기성 융합 바이러스, Chitinase 3-like 1 protein, Th2 면역