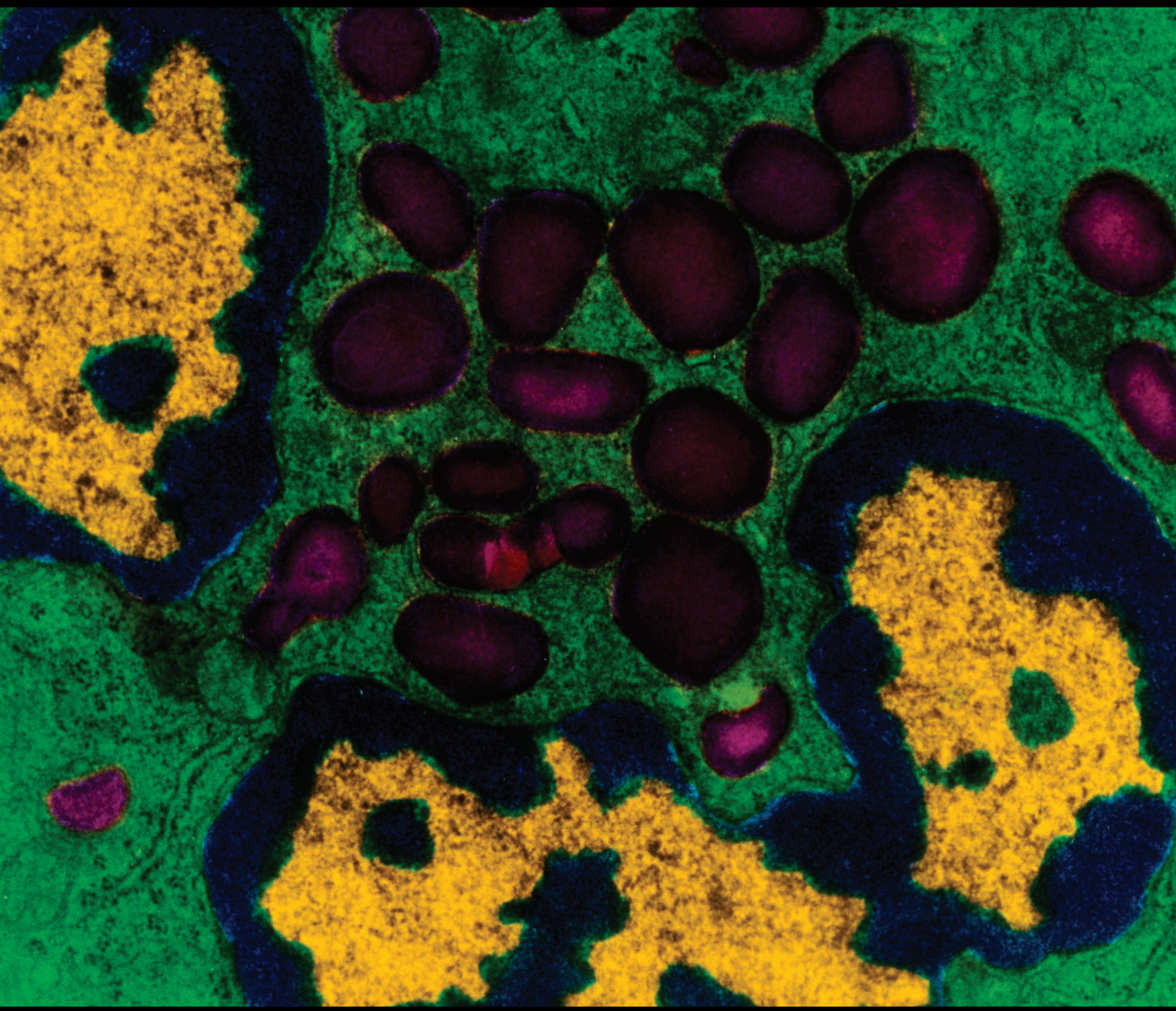
Mediators of Inflammation

Mediators of Allergic Asthma and Rhinosinusitis

Lead Guest Editor: Younghyo Kim
Guest Editors: Tsuguhisa Nakayama and Da-Tian Bau



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Editorial Board

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https://doi.org/10.1155/2017/7405245

Editorial

Mediators of Allergic Asthma and Rhinosinusitis

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Received 11 September 2017; Accepted 12 September 2017; Published 4 October 2017

The pathophysiologic mechanism of allergic asthma and rhinosinusitis is so complicated and involves thousands of inflammatory mediators. They are world-wide health problems, with its steadily increasing prevalence and its negative impact on quality of life. In spite of many studies throughout the world, there are still much more pathophysiologic mechanisms to be uncovered and the current mainstream of medication still remains symptom control including bronchodilators such as $\beta 2$ agonists, inhaled and systemic corticosteroid, antihistamines, and leukotriene modifiers, all only with transient symptomatic relief. Therefore, we should focus on a novel mechanism and mediators so that we could better understand and cure allergic asthma and rhinosinusitis.

S. Palikhe et al. evaluated a role of the ABCC4 gene polymorphism in airway inflammation of asthmatics. They investigated the potential associations between ABCC4 gene polymorphisms and asthma phenotype. In total, 270 asthma patients and 120 normal healthy controls were enrolled for a genetic association study. As a result, asthmatic patients carrying the G allele at −1508A>G had significantly higher serum levels of periostin, myeloperoxidase, and urinary levels of 15-hydroxyeicosatetraenoic acid and sphingosine-1-phosphate compared with noncarrier asthma patients. Luciferase activity was significantly enhanced in human epithelial A549 cells harboring a construct containing the −1508G allele. Therefore, they concluded that a functional polymorphism in the ABCC4 promoter, −1508A>G, may increase extracellular 15-hydroxyeicosatetraenoic acid, sphingosine-1-phosphate, and periostin levels, contributing to airway inflammation in asthmatics.

D. W. Kim et al. aimed to investigate the role of IL-25 in allergic rhinitis patients sensitized to house dust mite. IL-25 expression in the nasal mucosa from control, nonallergic rhinitis patients, and patients with allergic rhinitis sensitized to house dust mite was assessed using immunohistochemistry (IHC), double IHC, and quantitative reverse transcription PCR. Correlations between IL-25 and other inflammatory markers were also explored.

They observed significantly elevated concentrations of IL-25 in the human nasal epithelial cell samples with the highest doses of mite extracts. Nasal tissues from allergic patients sensitized to mites showed significantly higher IL-25 expression, compared to those from the control or nonallergic patients. They also found that the expression of IL-25 in nasal tissues from allergic patients was positively associated with Th2 immunity markers, such as ECP and GATA3. Therefore, they concluded that IL-25 expression increased with high-dose house dust mite stimulation and it was associated with Th2 immunity markers.

A. Jia et al. evaluated the regulatory role of MBD2 in regulating Th17 cell differentiation and experimental severe asthma by affecting IRF4 expression. As so little is known about that epigenetic regulation of MBD2 in both immunological pathogenesis of experimental severe asthma and $CD4^+$ T cell differentiation, they first aimed to establish a neutrophil-predominant severe asthma model, characterized by airway hyperresponsiveness (AHR), BALF neutrophil

granulocyte (NEU) increase, higher NEU and IL-17 protein levels, and more Th17 cell differentiation. In the model, MBD2 and IRF4 protein expression increased in the lung and spleen cells. Under overexpression or silencing of the MBD2 and IRF4 genes, the differentiation of Th17 cells and IL-17 secretion showed positive changes. IRF4 protein expression showed a positive change with overexpression or silencing of the MBD2 gene, whereas there was no significant difference in the expression of MBD2 under overexpression or silencing of the IRF4 gene. These data provide novel insights into epigenetic regulation of severe asthma.

H.-M. Lee et al. evaluated the role of a chemical chaperone of endoplasmic reticulum by inhibiting epithelial-mesenchymal transition induced by TGF-β1 in airway epithelium via the c-Src pathway. They investigated the role of endoplasmic reticulum (ER) stress and c-Src in TGF-β1-induced epithelial-mesenchymal transition (EMT). A549 cells, primary nasal epithelial cells (PNECs), and inferior nasal turbinate organ cultures were exposed to 4-phenylbutylic acid (4PBA) or PP2, and then stimulated with TGF-β1. They found that E-cadherin, vimentin, fibronectin, and α-SMA expression were increased in nasal polyps compared to inferior turbinates. TGF-β1 increased expression of EMT markers such as E-cadherin, fibronectin, vimentin, and α-SMA and ER stress markers (XBP-1s and GRP78), an effect that was blocked by PBA or PP2 treatment. 4-PBA and PP2 also blocked the effect of TGF-β1 on the migration of A549 cells and suppressed TGF-β1-induced expression of EMT markers in PNECs and organ cultures of inferior turbinate.

J. Encarnación-Medina et al. studied the association between selective ATP-binding cassette subfamily C gene expression and proinflammatory mediators released by 2 BEAS-2B after $PM_{2.5}$, budesonide, and cotreated exposures. They assessed ABCC1–4 gene expression changes and proinflammatory cytokine (IL-6, IL-8) release in human bronchial epithelial cells (BEAS-2B). A real-time PCR assay revealed that ABCC1 was upregulated in BEAS-2B exposed after 6 to 7 hr to $PM_{2.5}$ extract or budesonide but downregulated after 6 hr of the cotreated exposure. ABCC3 was downregulated after 6 hr of budesonide and upregulated after 6 hr of the cotreated exposure. ABCC4 was upregulated after 5 hr of $PM_{2.5}$ extract, budesonide, and the cotreated exposure. So they concluded that cotreatment showed an opposite effect on exposed BEAS-2B as compared with budesonide.

H. Park et al. studied the potential biomarkers for NSAID-exacerbated respiratory disease. NSAID-exacerbated respiratory disease (NERD) is an endotype characterized by asthma, chronic rhinosinusitis (CRS) with nasal polyps, and hypersensitivity to aspirin/cyclooxygenase-1 inhibitors. NERD is more associated with severe asthma than other asthma phenotypes. In this review, they summarized the known potential biomarkers of NERD that are distinct from those of aspirin-tolerant asthma and they also provided an overview of the different NERD subgroups.

Finally, Y. Choi et al. evaluated the role of neutrophil extracellular DNA traps in inducing autoantigen production by airway epithelial cells. They aimed to prove that neutrophil extracellular DNA traps (NETs), cytotoxic molecules released from neutrophils, are a key player in the stimulation of airway epithelial cells (AECs) to produce autoantigens. This study showed that NETs significantly increased the intracellular expression of tissue transglutaminase (tTG), but did not affect that of CK18 in AECs. NETs induced the extracellular release of both tTG and CK18 in a concentration-dependent manner. Moreover, NETs directly degraded intracellular α-enolase into small fragments. However, antibodies against neutrophil elastase (NE) or myeloperoxidase (MPO) attenuated the effects of NETs on AECs. Furthermore, each NET isolated from healthy controls (HC), nonsevere asthma (NSA), and SA had different characteristics.

I hope that by reading this special issue, you will get not only the latest insights into mediators related to the pathophysiology of allergic asthma and rhinosinusitis but also an opportunity to get a good idea of motivating your recent research.

Acknowledgments

This work was supported by an Inha University Hospital Research grant.

Young Hyo Kim
Tsuguhisa Nakayama
Da-Tian Bau

Hindawi
Mediators of Inflammation
Volume 2017, Article ID 5675029, 7 pages
https://doi.org/10.1155/2017/5675029

Research Article

Neutrophil Extracellular DNA Traps Induce Autoantigen Production by Airway Epithelial Cells

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Received 30 May 2017; Revised 20 July 2017; Accepted 1 August 2017; Published 30 August 2017

Academic Editor: Younghyo Kim

The hypothesis of autoimmune involvement in asthma has received much recent interest. Autoantibodies, such as anti-cytokeratin (CK) 18, anti-CK19, and anti-α-enolase antibodies, react with self-antigens and are found at high levels in the sera of patients with severe asthma (SA). However, the mechanisms underlying autoantibody production in SA have not been fully determined. The present study was conducted to demonstrate that neutrophil extracellular DNA traps (NETs), cytotoxic molecules released from neutrophils, are a key player in the stimulation of airway epithelial cells (AECs) to produce autoantigens. This study showed that NETs significantly increased the intracellular expression of tissue transglutaminase (tTG) but did not affect that of CK18 in AECs. NETs induced the extracellular release of both tTG and CK18 in a concentration-dependent manner. Moreover, NETs directly degraded intracellular α-enolase into small fragments. However, antibodies against neutrophil elastase (NE) or myeloperoxidase (MPO) attenuated the effects of NETs on AECs. Furthermore, each NET isolated from healthy controls (HC), nonsevere asthma (NSA), and SA had different characteristics. Taken together, these findings suggest that AECs exposed to NETs may exhibit higher autoantigen production, especially in SA. Therefore, targeting of NETs may represent a new therapy for neutrophilic asthma with a high level of autoantigens.

1. Introduction

Asthma is a chronic inflammatory disease of the airways. Approximately 5%–10% of patients with asthma exhibit severe symptoms that are not easily controlled by regular medication [1–3]. Severe neutrophilic asthma is a major phenotype of severe asthma (SA), in which neutrophils significantly contribute to the exacerbation of symptoms and airway remodeling [4]. However, the role of neutrophils in pathophysiological mechanisms responsible for SA has not been fully determined.

Recent studies have demonstrated that neutrophils participate in autoimmune disease [5, 6]; furthermore, autoimmune mechanisms, such as the deposition of autoantibodies in specific tissues, are known to play a role in asthma [7]. Our group previously found circulating autoantibodies, such as anti-cytokeratin (CK) 18, anti-CK19, and anti-α-enolase antibodies, against proteins expressed by airway epithelial cells (AECs) in patients with SA [8, 9]. Moreover, antibodies against tissue transglutaminase (tTG) were detected in patients with toluene diisocyanate-induced occupational asthma, which is generally associated with a neutrophilic phenotype [10]. Although autoimmune responses are associated with the pathogenesis of asthma [11], the mechanisms by which these autoantigens are generated in SA remain poorly understood.

Neutrophils, which are the most abundant leukocytes in humans, produce cytotoxic granule proteins [12]. Recently, it has been suggested that activated neutrophils undergo a novel form of cell death during which a meshwork of chromatin with bound granule proteins, known as neutrophil extracellular DNA traps (NETs), is released [13, 14]. A

previous study demonstrated high levels of NETs in patients with SA and showed that NETs stimulate the production of proinflammatory cytokines by AECs [15]. In addition, NETs have been suggested to elicit the production of autoantibodies in various autoimmune diseases [16]. However, the mechanisms by which high levels of NETs induce autoantigen production in SA have not been demonstrated to date.

Diverse proteins have been identified as airway epithelial autoantigens associated with SA [8]. To investigate the effects of NETs on AECs to produce autoantigens, the present study attempted to evaluate protein expression, especially that of CK18, tTG, and α-enolase, in these cells.

2. Materials and Methods

2.1. Study Subjects. The study was approved by the Institutional Review Board of Ajou University Hospital (AJIRB-GEN-GEN-09-140). All patients provided written informed consent at the time of recruitment. We enrolled 5 patients with SA who were diagnosed as a result of recurrent episodes of wheezing, dyspnea, cough, and evidence of either airway hyperresponsiveness to methacholine or reversible airway obstruction improved by treatment with a short-acting β_2-agonist [17]. To investigate differences in NETs individually, 3 healthy controls (HC), individuals with nonsevere asthma (NSA), and individuals with SA were recruited, respectively.

2.2. Isolation of Neutrophils from Peripheral Blood. At the time of diagnosis, venous blood from patients with asthma was collected into BD Vacutainer tubes containing acid citrate dextrose solution (BD Biosciences, Franklin Lakes, NJ, USA) and processed within 2 h of collection. Blood was layered on Lymphoprep solution (Axis-Shield, Oslo, Norway), followed by centrifugation at 879 ×g at 20°C for 25 min, without any brake. The layer containing granulocytes was separated and placed in Hank's balanced salt solution (HBSS) buffer, with 2 mM ethylenediaminetetraacetic acid (EDTA) and 2% dextran, for 20 min at 26–30°C. The neutrophil-rich layer was collected and washed once with HBSS buffer containing 2 mM EDTA. Red blood cells (RBCs) were eliminated by hypotonic lysis. Peripheral blood neutrophils were maintained in RPMI-1640 medium with 2% fetal bovine serum (FBS), penicillin (100 IU/mL), and streptomycin (50 μg/mL).

2.3. Induction of Neutrophil Extracellular DNA Traps. Peripheral blood neutrophils were stimulated and isolated as described previously [18]. Isolated neutrophils were treated with 100 nM phorbol myristate acetate (PMA) for 3 h. Each well was washed twice with RPMI to eliminate PMA and NET-dissociated molecules. To confirm the removal of residual PMA, which could affect target cells, from isolated NETs, the supernatants from the third wash (Sup) were collected and used as controls to treat target cells. RPMI (1 mL) containing micrococcal nuclease (MNase) (1 U/μL) was then added to each well to digest NETs at 37°C for 20 min. NETs were collected by eliminating cell or cell debris. Isolated NETs were quantified by measuring the DNA concentration using the PicoGreen assay (Invitrogen, Paisley, UK) according to the manufacturer's instructions.

2.4. Detection of Neutrophil Extracellular DNA Traps. Peripheral blood neutrophils (2×10^5) were seeded on L-lysine-coated slides (Polysciences, Warrington, PA, USA). Neutrophils were stained with anti-NE antibody and DAPI to detect NET formation. Cells were examined under a Zeiss LSM710 confocal microscope with a 63 × oil objective lens (Carl Zeiss, Oberkochen, Germany). The images were analyzed using ZEN 2009 software (Carl Zeiss).

2.5. Cell Culture. A549 cells (American Type Culture Collection, Manassas, VA, USA) were cultured in RPMI 1640 Medium supplemented with 10% FBS, penicillin (100 IU/mL), and streptomycin (50 μg/mL). Cells were grown at 37°C in humidified air with 5% CO_2. To investigate autoantigen production, cells were treated with isolated NETs with final DNA concentrations of 1 μg/mL and 5 μg/mL. To investigate the effects of neutrophil elastase (NE) and myeloperoxidase (MPO), cells were treated with NETs that had been preincubated with antibodies against NE (Santa Cruz, Dallas, TX, USA) or MPO (Cell Signaling, Minneapolis, MN, USA).

2.6. Immunoblot Analysis. CK18, tTG, and α-enolase expressions in cell lysates and culture supernatants were evaluated by Western blot analysis. Consequently, relative expression of each protein to actin was evaluated. Anti-CK18 antibody (Cell Signaling), anti-tTG (Cell Signaling) antibody, anti-α-enolase antibody (Santa Cruz), and anti-actin antibody (Santa Cruz) were used.

2.7. Statistical Analysis. Data were analyzed by one-way ANOVA with Bonferroni's post hoc test. Statistical analyses were performed with SPSS software, version 22.0 (SPSS Inc., Chicago, IL, USA). $P < 0.05$ was considered to indicate statistical significance. GRAPHPAD PRISM 5.0 software (GraphPad Inc., San Diego, CA, USA) was used for graphs, with values presented as the mean ± standard deviation (SD) of at least three independent experiments.

3. Results

3.1. Clinical Characteristics of the Study Subjects. Five patients with SA (GINA guidelines step 4-5) were enrolled for isolation of NETs. Three males and two females were recruited; the mean age of the patients was 28.60 ± 6.66 years. The baseline % forced expiratory volume in 1 sec (FEV_1) was 99.02 ± 14.18%. All females, but none of the males, were atopic.

3.2. Activated Peripheral Blood Neutrophils Release Neutrophil Extracellular DNA Traps. Peripheral blood neutrophils stimulated with phorbol myristate acetate (PMA) produced not only web-like extracellular DNA but also cytotoxic granule proteins such as neutrophil elastase (NE). Blue DAPI staining indicates the nucleus (in particular, DNA) and red colored staining with anti-NE antibody indicates NE. Neutrophils stained with both dyes

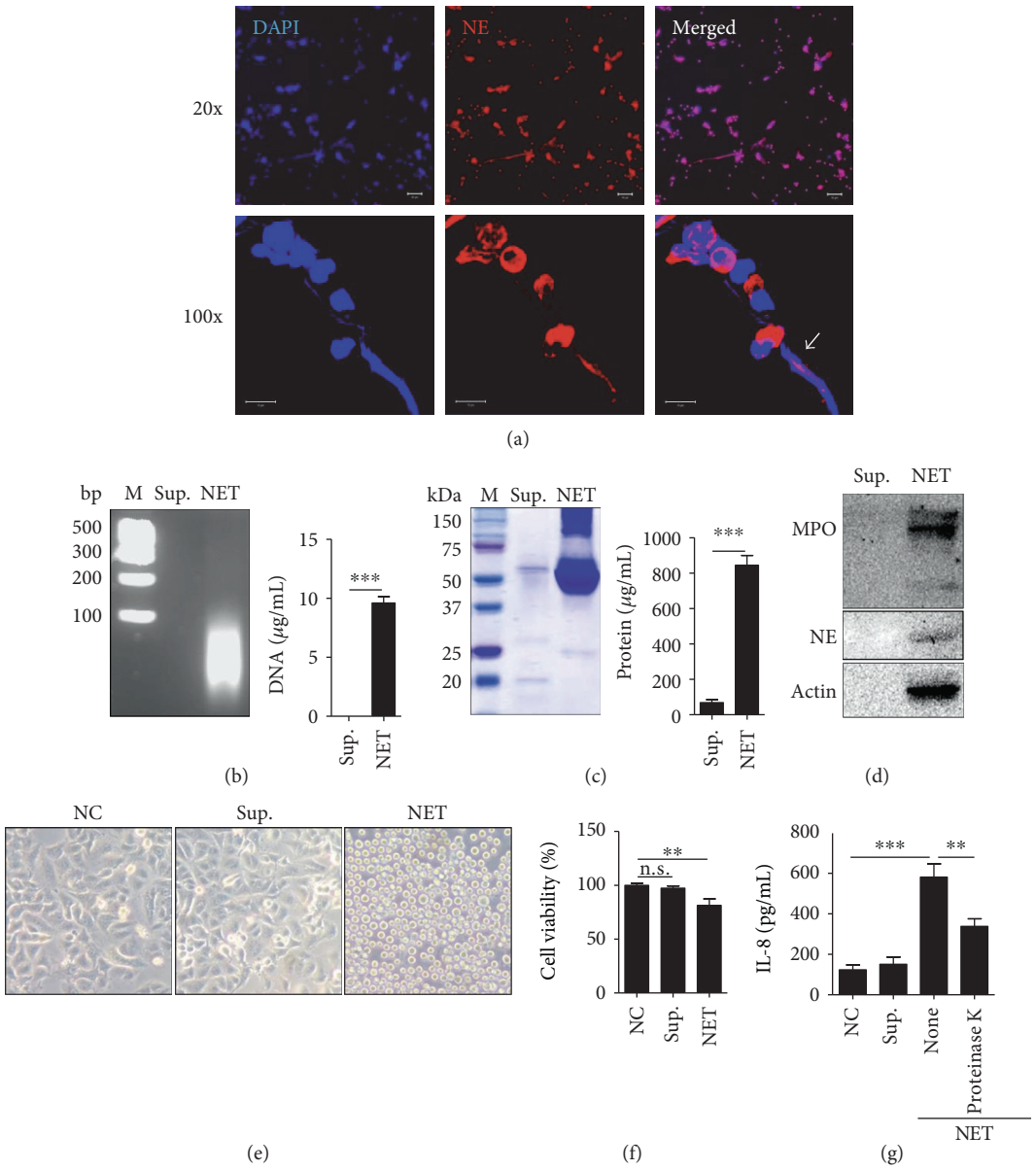

Figure 1: Characterization of NETs isolated from peripheral blood neutrophils of SA. (a) Detection of NET production (a white arrow); scale bar, 10 μm. (b) DNA bands (left panel) and concentration (right panel). (c) Protein profile (left panel) and concentration (right panel). (d) Western blot analysis of granule proteins. (e) Change in A549 cell morphology following NET treatment. (f) Cell viability assessed by Cell Counting Kit-8 (CCK8) assay. (g) Proinflammatory effects of NETs on A549 cells. $^{**}P < 0.01$, $^{***}P < 0.001$. n.s., not significant.

are activated cells that undergo cell death following NET production (Figure 1(a)).

3.3. Neutrophil Extracellular DNA Traps Contain Specific Extracellular DNA and Granule Proteins. To investigate extracellular DNA released by neutrophils, NETs were loaded on 0.8% agarose gel. NETs treated with micrococcal nuclease (MNase) showed specific DNA bands of under 100 bp in size (Figure 1(b), left panel). The DNA concentration was approximately 10 μg/mL (Figure 1(b), right panel). Protein profile analysis performed by Coomassie Brilliant Blue staining indicated that proteins in NETs were of a specific size (between 50 and 75 kDa) (Figure 1(c), left panel). The protein concentration was approximately 800 μg/mL (Figure 1(c), right panel). Western blot analysis of NETs showed that granule proteins colocalize with DNA (Figure 1(d)).

3.4. Neutrophil Extracellular DNA Traps Exert Cytotoxic Effects on AECs to Induce Inflammation. To demonstrate the cytotoxic effects of NETs on AECs, cell morphology was observed following NET treatment. Initially, AECs that were elongated and spindle-shaped were observed to gently attach to culture plates. However, the cells were found to acquire a round shape and detach from the culture plates after NET treatment (Figure 1(e)). Cell

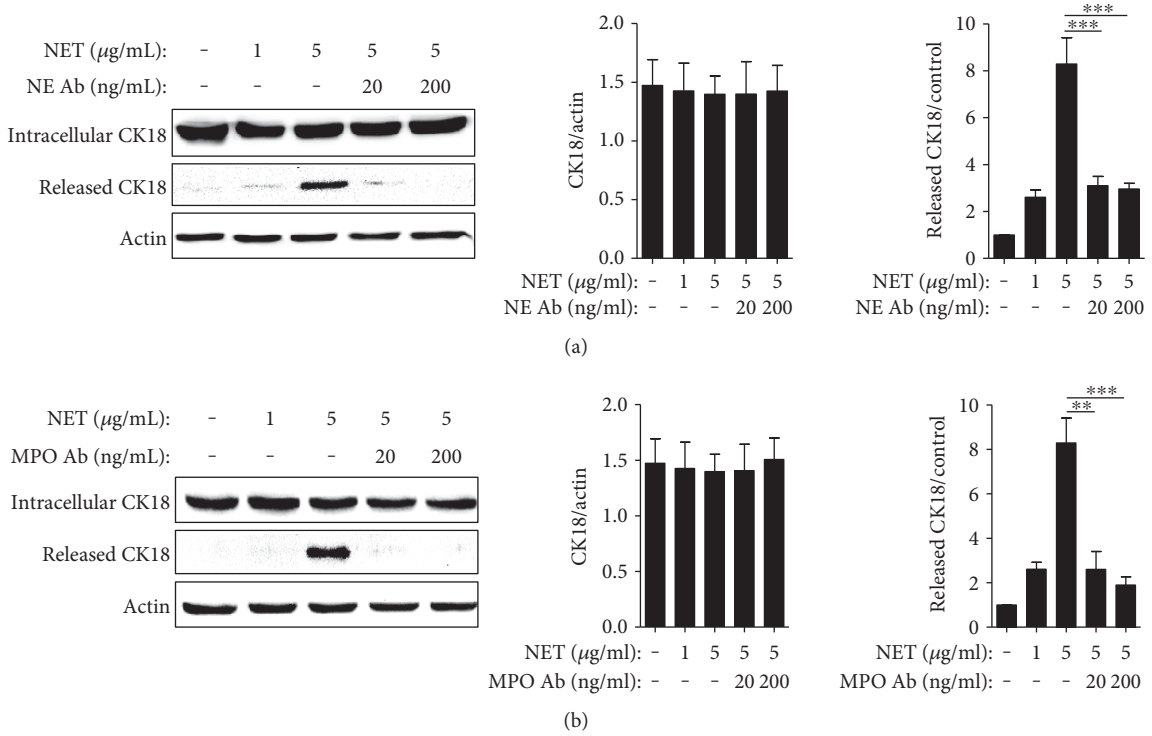

Figure 2: NETs induced CK18 expression and extracellular release from A549 cells. Effects of NETs on A549 cells incubated with/without NE (a) or MPO (b) antibody. Significance is represented by $^{**}P < 0.01$ and $^{***}P < 0.001$.

viability was also measured; NETs at a final concentration of 5 μg/mL DNA induced more than 30% cell death in the total cell population (Figure 1(f)). When AECs were treated with NETs, the cells produced significantly high levels of IL-8. However, NETs preincubated with proteinase K elicited a lower degree of production of proinflammatory cytokines by AECs (Figure 1(g)).

3.5. Ability of Neutrophil Extracellular DNA Traps to Induce CK18 Production from AECs. To determine whether NETs could enhance autoantigen production, CK18 expression in cell lysates and culture supernatants from AECs was evaluated by Western blot analysis. NETs were found to significantly upregulate the release of CK18 into the culture supernatant in a concentration-dependent manner. However, intracellular expression of CK18 was not affected by NETs. NET preincubated with antibodies against NE or MPO showed weaker effects on AECs (Figures 2(a) and 2(b)). We confirmed that NETs, at the concentrations tested, did not contain a detectable amount (if any) of CK18.

3.6. Expression of tTG in AECs Is Mediated by Neutrophil Extracellular DNA Traps. Another autoantigen, tTG, was detected in both cell lysates and culture supernatants. In contrast to the expression of CK18, NETs dramatically increased the intracellular expression of tTG. In addition, NETs also concentration-dependently induced the release of CK18 into the culture supernatant. Similar to the CK18 expression data, NETs preincubated with antibodies against NE or MPO had attenuated effects on AECs in terms of eliciting intracellular tTG expression and extracellular tTG release (Figures 3(a) and 3(b)).

3.7. Neutrophil Extracellular DNA Traps Degrade Intracellular α-Enolase into Small Fragments. The expression of α-enolase in AECs following NET treatment was investigated. NETs degraded intracellular α-enolase (55 kDa) into a 43-kDa fragment at a concentration of 1 μg/mL DNA, or 43-kDa and 36-kDa fragments at 3 μg/mL of DNA, and 36-kDa and 32-kDa fragments at 5 μg/mL DNA. However, neither α-enolase nor its fragments were detected in the cell culture supernatants (Figure 4).

3.8. Different Characteristics of Neutrophil Extracellular DNA Traps Isolated from HC, NSA, and SA. HC, NSA, and SA patients were enrolled, respectively, to identify differences in each NET of the study subjects (in Supplementary Table available online at https://doi.org/10.1155/2017/5675029). NETs extracted from SA had higher concentration of DNA compared to those from HC and NSA (Figure 5(a)). These NETs also contained more proteins (Figure 5(b)). In addition, the composition of granule proteins in each NET was different (Figure 5(c)). Furthermore, every NET showed significant effects on protein expression in AECs (Figure 5(d)).

4. Discussion

Neutrophil activity has been implicated in SA; however, the precise role of neutrophils remains unclear [4]. A recent

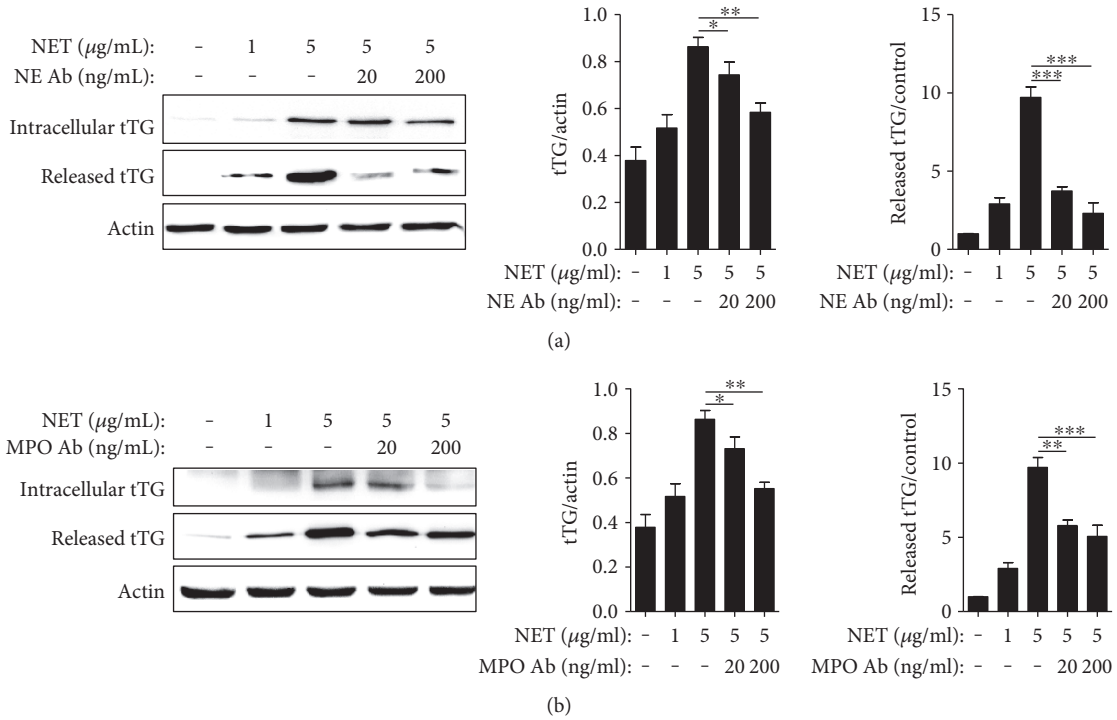

Figure 3: NETs induced tTG expression and extracellular release from A549 cells. Effects of NETs on A549 cells incubated with or without NE (a) or MPO (b) antibody. Significance is represented by $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

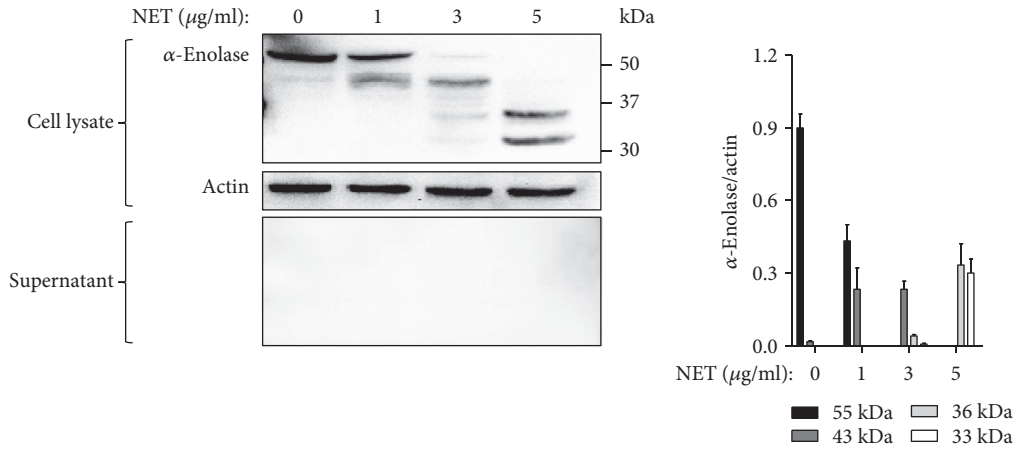

Figure 4: α-Enolase in A549 cells was degraded into small fragments by NET treatment. α-Enolase expression in cell lysates and culture supernatants was evaluated by Western blot.

study demonstrated that activated neutrophils induce NETs in patients with SA, thus activating eosinophils and AECs and enhancing airway inflammation [15]. This study proposes another role of neutrophils in SA: the production of NETs, which could increase autoantigen production by AECs. Autoimmune responses to such autoantigens may represent a pathogenic mechanism underlying the induction of airway inflammation.

The cytotoxic effects of NETs may contribute to the pathogenesis of asthma [19]. NE and MPO are the two main granule proteins localized within NETs that are implicated in airway epithelium and cell damage [20, 21]. MPO has been believed to play a more critical role in this process [18]. However, in the current study, blocking the exposure of these two granular proteins by preincubation with antibodies against NE or MPO resulted in inhibitory effects on AECs, thereby demonstrating that both proteins play an equally important role. Moreover, NETs preincubated with proteinase K showed reduced toxicity. The present study suggests that airway inflammation in asthma may be induced by both extracellular DNA and granule proteins in NETs.

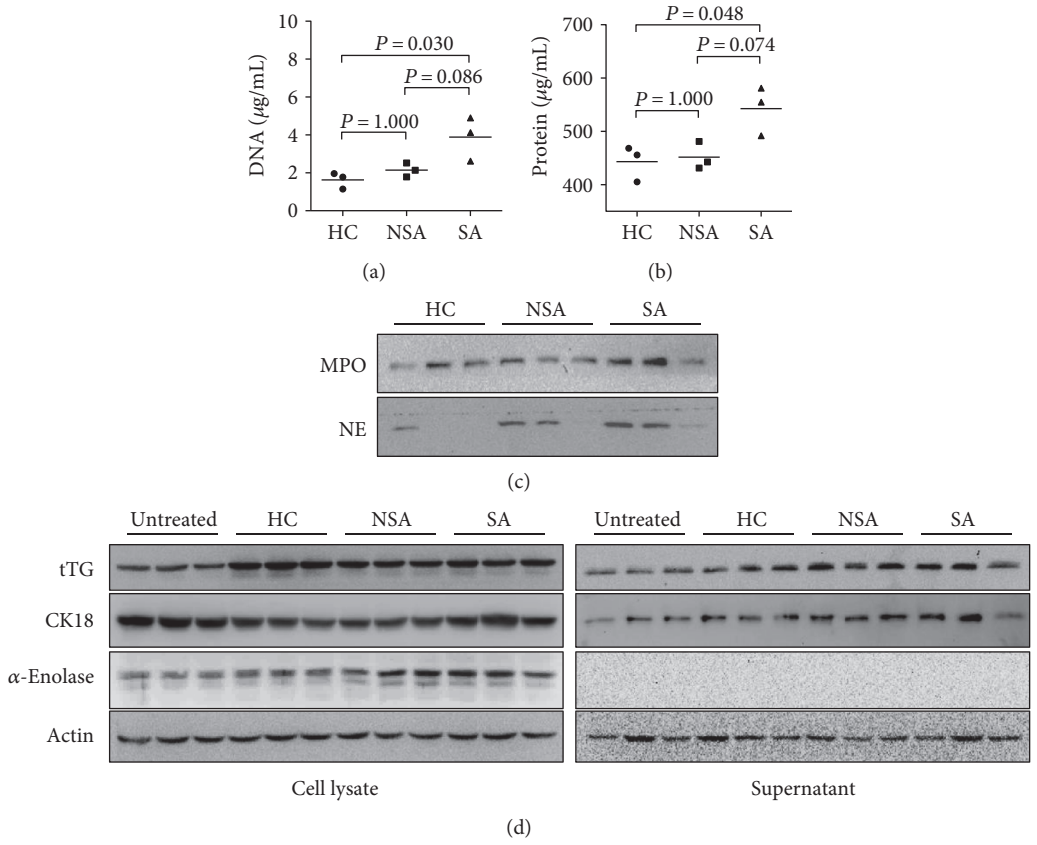

Figure 5: Comparison of NETs isolated from HC, NSA, and SA. (a) DNA concentration measured by PicoGreen assay. (b) Protein concentration evaluated by BCA assay. (c) Western blot analysis of NE and MPO in NETs. (d) Change in protein expression of AECs by NET treatment.

NETs are also known to play a role in autoimmune disease [16]; the induction of such responses is considered to contribute to asthma. Autoantibodies have been suggested to directly or indirectly (via T cell interactions) induce cytotoxicity, thereby enhancing airway inflammation in SA [22]. Our group previously detected several autoantibodies, such as anti-CK18, anti-CK19, anti-α-enolase, and tTG antibodies, in the sera of patients with SA [10, 23–25]. However, the mechanisms underlying autoantibody production in SA are not clear. The present study showed that NET-treated AECs significantly increased the expression of CK18 and tTG. Moreover, NETs degraded intracellular α-enolase into several small fragments, which may have been comprised of autoantigens, to elicit autoantibody production. The current study proposes a new pathway for enhancing autoantibody production in SA, through the production or modification of autoantigens by NETs.

Neutrophils from patients with SA and NSA release different amounts of NETs; however, a significantly higher release was noted in SA patients [15]. The previous study did not perform a detailed characterization of NETs individually. This study showed different compositions of each NET, even within the same group. However, all NETs isolated from neutrophils affected protein expression in AECs. As neutrophils from SA produced a large number of NETs, the degree of neutrophil activation could be one possibility that enhances autoantigen production and increases asthma severity.

The present study has limitations: First, a positive correlation between autoantigen and autoantibody production *in vivo*, which would have directly explained the increased levels of autoantibodies, was not demonstrated. Secondly, although NETs exert cytotoxic effects, resulting in the production and modification of intracellular autoantigens, it is not clear why the expression of each autoantigen is different in AECs. This may be attributed to the complexity of the mechanisms by which NETs affect signaling molecules in the cells. Thirdly, the potential contribution of other immune cells to autoimmune responses involving NETs should be additionally clarified.

5. Conclusions

In conclusion, activated neutrophils produce NETs, which could contribute to airway epithelial damage, proinflammatory cytokine induction, and autoantigen production. Therefore, inhibition of NETs may be a novel therapeutic approach to asthma presenting a neutrophilic phenotype.

Conflicts of Interest

The authors declare no competing financial interest.

Authors' Contributions

Youngwoo Choi and Le Duy Pham designed and performed the experiments; Dong-Hyun Lee performed the experiments; Ga-Young Ban and Ji-Ho Lee collected the patient samples; Seung-Hyun Kim joined in designing the experiments; and Hae-Sim Park supervised all processes. Youngwoo Choi and Le Duy Pham contributed equally to this work.

Acknowledgments

This study was supported by a grant from the Korean Health Technology R&D Project, Ministry of Health and Welfare, Republic of Korea (H14C2628).

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Hindawi
Mediators of Inflammation
Volume 2017, Article ID 3908049, 8 pages
https://doi.org/10.1155/2017/3908049

Research Article

IL-25 Could Be Involved in the Development of Allergic Rhinitis Sensitized to House Dust Mite

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Received 16 April 2017; Revised 3 June 2017; Accepted 18 June 2017; Published 23 August 2017

Academic Editor: Da-Tian Bau

Background and Purpose. When house dust mite (HDM), a common allergen, comes into the mucosal membrane, it may stimulate innate immunity. However, the precise role of interleukin- (IL-) 25 in the development of HDM-induced nasal allergic inflammation is still unclear. Therefore, we investigated the role of IL-25 in allergic rhinitis (AR) patients sensitized to HDM. *Methods*. To confirm the production of IL-25 in human nasal epithelial cells (HNECs), we stimulated HNECs. IL-25 expression in the nasal mucosa from control, non-AR (NAR) patients, and HDM-sensitized AR patients was assessed using immunohistochemistry, and quantitative reverse transcription PCR. Correlations between IL-25 and other inflammatory markers were explored. *Results*. An in vitro study showed significantly elevated concentrations of IL-25 in the HNEC samples with highest doses of HDM. Nasal tissues from AR patients sensitized to HDM showed significantly higher IL-25 expression, compared to those from the control or NAR patients. Moreover, the expression of IL-25 in nasal tissues from AR patients sensitized to HDM was positively associated with Th2 markers, such as ECP and GATA3. *Conclusions*. IL-25 expression increased with high-dose HDM stimulation and was related to Th2 markers. Therefore, IL-25 neutralization might offer a new strategy for treating patients with HDM-sensitized AR.

1. Introduction

Allergic rhinitis (AR) is a Th2 immune-mediated hypersensitivity in the nasal mucosa characterized by nasal obstruction, rhinorrhea, sneezing, and itching [1]. It is accompanied by an accumulation of eosinophils and mast cells in the nasal mucosa, as well as increased serum levels of antigen-specific IgE [2]. The nasal epithelium, which is the first site of exposure to inhaled antigens, may play an essential role in innate immunity to AR. Recent studies have demonstrated that epithelial cell-derived cytokines, including thymic stromal lymphopoietin (TSLP), interleukin- (IL-) 25, and IL-33, are critical regulators of innate and adaptive immune responses associated with Th2 cytokine-mediated inflammation at nasal mucosal tissues [3–5]. Of these, several studies have described the process by which IL-25 (an IL-17 cytokine family member) can enhance the production of Th2 cell expansion and Th2-type cytokines such as IL-4 and IL-5 [6, 7]. Also, an elevated expression of IL-25 was observed in tissues of patients with asthma, atopic dermatitis, and chronic rhinosinusitis, indicating a possible link between the functions of IL-25 and the exacerbation of allergic disorders [8–11].

House dust mite (HDM; *Dermatophagoides* sp.) is one of the major inhalant allergens that produce patients with

perennial AR. Some studies have estimated that 10–20% of the population of any given country is allergic to HDM [12, 13]. As is known, exposure to HDM induces specific antibody production and nasal inflammation by various inflammatory cells, including mast cells, eosinophils, and nasal epithelial cells [14, 15]. Previous animal studies have supported this and described cases of severe nasal symptoms and nasal mucosa remodeling that were observed in the mouse model with HDM-induced AR, but not noted in that with pollen-induced AR [16, 17]. Other animal studies have shown that IL-25 is not crucial for the development of the HDM-induced allergic mice model [18, 19].

To date, regardless of these discrepancies, the role of IL-25 in nasal mucosa of patients with HDM-sensitized AR is still unclear. Therefore, the objective of this study is to investigate the expression of IL-25 in nasal mucosa obtained from patients with HDM-sensitized AR. We also examined the relationship between IL-25 and various inflammatory markers in patients with HDM-sensitized AR.

2. Materials and Methods

2.1. Subjects. The sinonasal tissue from the inferior turbinate mucosa was obtained through septoplasty from normal control (control; $n = 8$), allergic rhinitis patients (AR; $n = 14$), and nonallergic rhinitis patients (non-AR; $n = 10$). All of the patients provided informed written consents. The internal review board of Dankook University Hospital (number 2012-11-008) approved the study. The exclusion criteria were as follows: (1) the patients were younger than 18 years of age; (2) the patients had prior treatment with antibiotics, systemic or topical corticosteroids, or other immune-modulating drugs during 4 weeks before surgery; and (3) the patients had other chronic sinusitis including rhinosinusitis, antrochoanal polyps, allergic fungal sinusitis, cystic fibrosis, or immotile ciliary disease. Rhinitis was defined as a minimum of 2 nasal complaints (itching, nasal obstruction, rhinorrhea, and sneezing) for more than one year. Patients with a strong positive response to a skin prick test (SPT) were classified as allergic rhinitis patients, and patients with negative SPT response were classified as nonallergic rhinitis patients. A strong positive reaction in the SPT was defined as an allergen to histamine (A/H) ratio of wheel size ≥ 2. The control tissues were obtained from patients without any nasal inflammatory diseases with negative SPT responses during septoplasty.

2.2. Immunohistochemistry and Quantitative RT-PCR. Immunohistochemical staining was performed with polink-2, polymerized horseradish peroxidase (HRP), and a broad DAB-Detection System (Golden Bridge International Labs, WA, USA). Briefly, after deparaffinization, the sections were incubated with 3% hydrogen peroxidase to inhibit endogenous peroxidases. Heat-induced epitope retrieval was then performed by microwaving samples in a ten mmol/L citrate buffer (pH 6.0). The sections were incubated for 60 minutes (min) at room temperature in a primary antibody. The primary antibodies were rabbit anti-human IL-25 (1 : 500; Abcam, Cambridge, UK). The sections were incubated in broad-antibody enhancer and polymer-HRP for the rabbit and mouse antibodies. The sections were then stained with the DAB Detection System. Finally, the slides were counterstained with hematoxylin. The numbers of the positive cells in the epithelium, glands, and submucosa were counted in the five densest visual fields (×400) by two independent observers, and the average values were determined. To identify the cellular sources of IL-25, sequential stainings were employed using polymer-HRP and alkaline phosphatase (AP) kits to detect mouse and rabbit primary antibodies for human tissue with Permanent Red and Emerald (Polink DS-MR-Hu C2 Kit; Golden Bridge International Labs). The mouse antimast cell tryptase (1 : 500; Abcam) was mixed with the rabbit anti-human IL-25 (1 : 500; Abcam), applied to the tissue, and then incubated for 30–60 min. The polymer mixtures were made by adding the AP polymer anti-mouse IgG and polymer-HRP anti-rabbit IgG at a 1 : 1 ratio and applied to cover each section. Unless noted otherwise, the manufacturer's instructions were carefully attended to.

In addition, the mRNA expressions of various inflammatory markers in nasal mucosa tissues were determined using quantitative real-time PCR. Total RNA was extracted from the tissue samples by using a TRI reagent (Invitrogen, Carlsbad, CA, USA). One microgram total RNA was reverse transcribed to cDNA using a cDNA synthesis kit (*amfiRivert Platinum* cDNA Synthesis Master Mix, GenDEPOT). Quantitative real-time PCR was carried out using the LightCycler® 480 Probes Master (Roche, Mannheim, Germany). For analysis of IL-25 (Hs03044841_m1), IL-33 (Hs00369211_m1), TSLP (Hs00263639_m1), IFN-γ (Hs00989291_m1), and GAPDH (Hs02758991_g1), predeveloped assay reagent kits of primers and probes were purchased from TaqMan Assays (Life Technologies Korea, Seoul, Korea). In addition, a quantitative real-time PCR assay was performed with appropriate primers that specifically amplified T-bet, GATA3, RORC, ECP, and TGF-β1. The primers were as follows: T-bet, 5′-GTCAATTCCTTGGGGGAGAT-3′ for the forward primer and 5′-TCATGCTGACTGCTCGAAAC-3′ for the reverse primer; GATA3, 5′-ACCACAACC ACACTCTGGA GGA-3′ for the forward primer and 5′-TCGGTTTC TGGTCTGGATGCCT-3′ for the reverse primer; RORC, 5′-GCTGTGATCTTGCCCAGAACC-3′ for the forward primer and 5′-CTGCCCATCATTGCTGTTAATCC-3′ for the reverse primer; ECP, 5′-TCGGAGTAGATTCCGG GTG-3′ for the forward primer and 5′-GAACCACAGGA TACCGTGGAG-3′ for the reverse primer; TGF-β1, 5′-TG AACCGGCCTTTCCTGCTTCTCATG-3′ for the forward primer and 5′-GCGGAAGTCAATGTACAGCTGCCGC-3′ for the reverse primer; and GAPDH, 5′-CATGGGTG TGAACCATGAGAA-3′ for the forward primer and 5′-GGTCATGAGTCCTTCCACGAT-3′ for the reverse primer. GAPDH was measured as a housekeeping gene for normalization. Relative gene expression was calculated using the comparative $2^{-\Delta\Delta CT}$ method.

2.3. Cell Culture and Treatments. Healthy adult volunteers were recruited for a nasal brushing of the inferior turbinate to obtain human nasal epithelial cells (HNECs). The samples were placed in a 15 mL conical tube containing 8 mL of DMEM and transported on ice. The samples were then

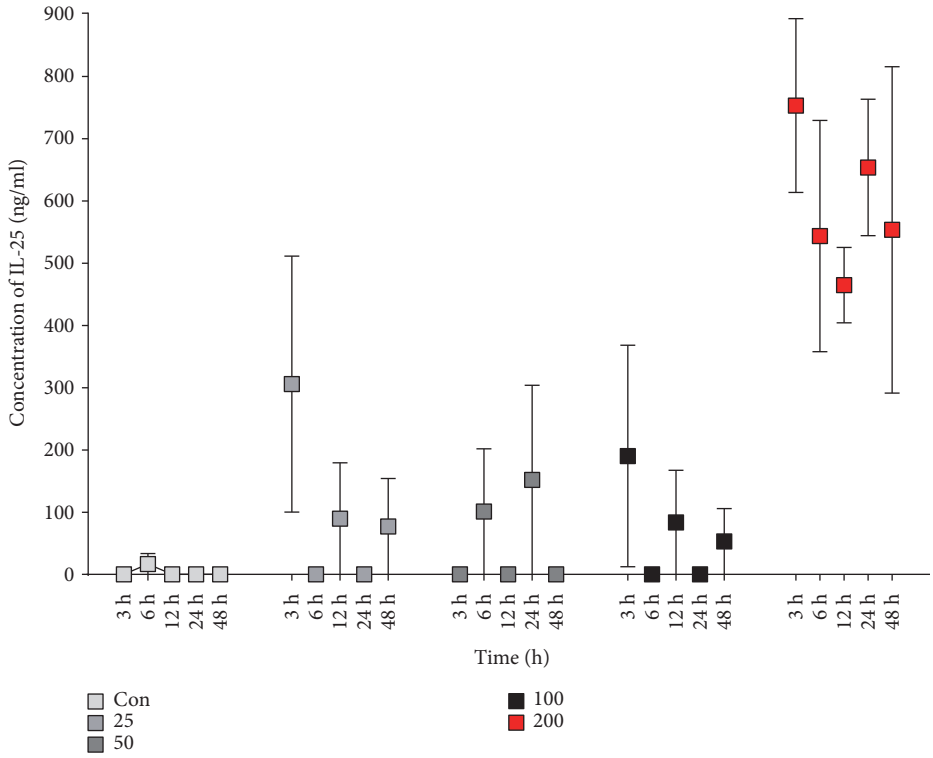

Figure 1: House dust mite-induced IL-25 production in human nasal epithelial cells (HNECs). We used different doses of house dust mite to stimulate the HNECs, including 0, 25, 50, 100, and 200 μg/mL.

filtered through cell strainers with a pore size of 70 μm and then washed twice with DMEM. After centrifugation, the supernatants were discarded, and the pellets were resuspended in serum-free bronchial epithelial growth medium (BEGM, Lonza Walkersville Inc., Walkersville, MD, USA) supplemented with Single Quots. The cell suspensions were transferred to precoated culture dishes at a concentration of 1×10^6 cells/mL. After incubating them in a tissue culture incubator for 24 hours, the nonadherent cells were removed, and the adherent cells were maintained in BEGM supplemented with Single Quots at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The culture medium was replaced daily. Subculture was performed when the cells reached 80–90% confluency. Briefly, the culture media was aspirated, and the cells were washed twice with serum-free DMEM. Then, one mL of 0.25% trypsin was added to each dish, and the dishes were incubated at 37°C until the cells became detached. The cells were dislodged by repeatedly pipetting up and down the trypsin solution. The detached cells were then transferred to 15 mL conical tubes with BEGM supplemented with Single Quots. When the cells reached 80–90% confluency, the culture medium was replaced with Single Quots free BEGM for 24 hours to maintain a low basal level of cytokine expression. In this experiment, we used first passaged cells. Before stimulation, the HNECs were cultured in BEBM without hydrocortisone for 24 hours. After 24 hours of starvation, the HNECs were stimulated with *Dermatophagoides farinae* (25, 50, 100, and 200 μg/mL) for 3, 6, 12, 24, and 48 hours.

2.4. Statistical Analysis. Statistical analyses were performed with SPSS 18.0 (SPSS Inc., Chicago, Ill). Statistical analyses were performed by using the Kruskal-Wallis and Mann–Whitney U tests with a 2-tailed test for unpaired comparisons. The Spearman test was used to determine correlations. The significance level was set at α value of 0.05 ($^*P < 0.05$, $^{**}P < 0.010$, and $^{***}P < 0.001$).

3. Results

3.1. Induction of IL-25 Expression in Cultured HNECs In Vitro. To investigate whether HDM induced allergic condition and IL-25 expression in HNECs, we cultured HNECs in air-liquid fashion. We stimulated the HNECs with various concentrations of HDM (25, 50, 100, and 200 μg/mL). In our in vitro study, we found that only the highest concentration of HDM (200 μg/mL) significantly increased IL-25 secretions (Figure 1). The levels of IL-25 in the supernatants of cultured HNECs with 200 μg/mL of HDM were significantly higher than in those with lower concentrations of HDM. This observation suggests that IL-25 levels increase in patients with HDM-sensitized AR.

3.2. Expression of Interleukin-25 in Patients with Allergic Rhinitis. To investigate the expression of IL-25 in the nasal mucosa of patients with HDM-sensitized AR, we performed the real-time quantitative PCR. The expression of IL-25 mRNA was significantly higher in the human nasal mucosa of HDM-sensitized AR compared to that of the control and

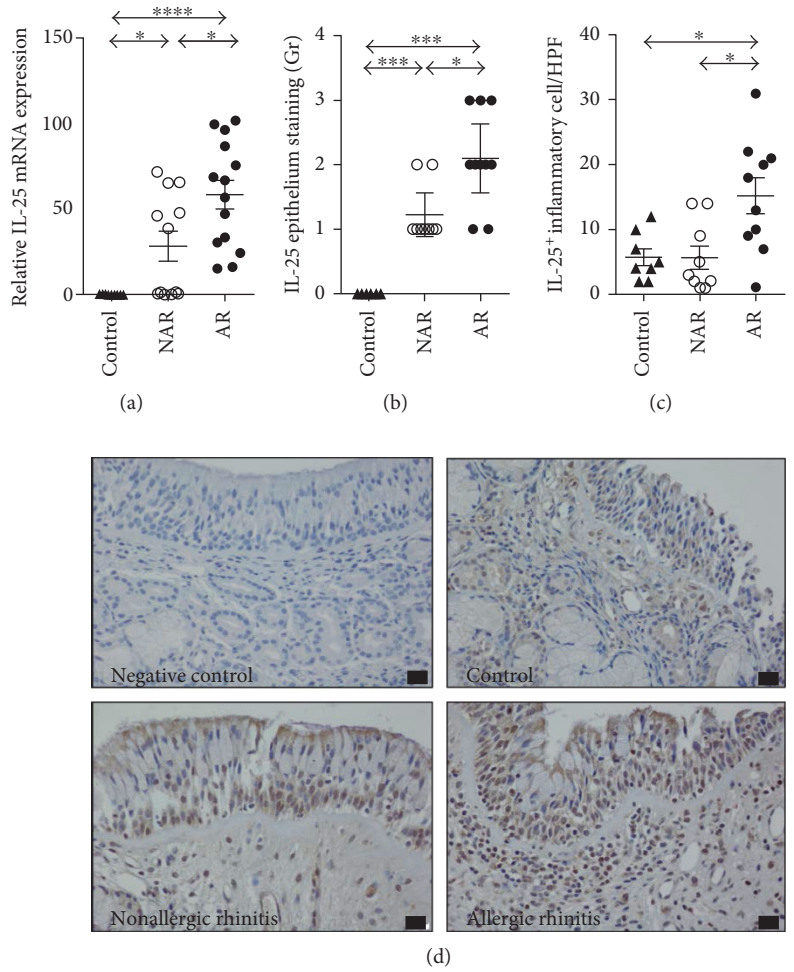

Figure 2: Expression of IL-25 in nasal tissues from control, nonallergic rhinitis patients, and house dust mite-sensitized allergic rhinitis patients. RT-PCR analysis and IHC detection of IL-25 were performed. (a) IL-25 mRNA expression, (b) IL-25 epithelium staining, and (c) IL-25-positive-inflamatory cells in the nasal tissues from each group. (d) Representative images for IL-25 immunohistochemical staining in nasal tissues ($^{*}P < 0.05$, $^{**}P < 0.010$, $^{***}P < 0.001$, and $^{****}P < 0.0001$).

non-AR patients (Figure 2(a)). Immunohistochemistry (IHC) showed that the expression of IL-25 was higher in the epithelial cells of patients with HDM-sensitized AR than in those of the control subjects and patients with non-AR (Figures 2(b) and 2(d)). In addition, the IL-25-positive inflammatory cells were significantly increased in patients with HDM-sensitized AR, compared to the control subjects and patients with non-AR (Figures 2(c) and 2(d)). Meanwhile, the expression level of IL-33 mRNA was significantly higher in patients with non-AR than in patients with HDM-sensitized AR (Supplementary Fig. 1A available online at https://doi.org/10.1155/2017/3908049). In addition, the expression level of TSLP mRNA was significantly higher in the nasal tissues of HDM-sensitized AR and NAR patients than in those of the control (Supplementary Fig. 1B).

Next, we used double IHC staining to identify IL-25 positive cells in the subepithelial layer. Double-positive IL-25 and tryptase cells were frequently detected in patients with HDM-sensitized AR (Figure 3(a)). In addition, we found a meaningful relationship between IL-25-immunoreactive cells and total IgE levels in patients with HDM-sensitized AR ($r = 0.4169$), although there was no such correlation in patients with non-AR (Figure 3(b)).

3.3. Correlation between Interleukin-25 mRNA Expression and Inflammatory Markers in Patients with Allergic Rhinitis. To investigate the implication of upregulated IL-25 in patients with HDM-sensitized AR, we examined whether IL-25 expression correlated with other inflammatory markers, such as ECP, GATA3 (a major transcriptional factor in Th2 responses), FOXP3 (a major transcriptional factor in Treg responses), RORC (a major transcriptional factor in Th17 responses), INF-γ, and TGF-β1. The present study showed that the expression of mRNA for ECP ($r = 0.9053$ and $P < 0.0001$), GATA3 ($r = 0.5699$ and $P = 0.0359$), and FOXP3 ($r = 0.8242$ and $P = 0.0005$) were positively correlated with IL-25 mRNA expression (Figures 4(a), 4(b), and 4(c)), whereas the expression level of IL-25 mRNA bore no correlation with that of RORC (Figure 4(d)). However, IL-25 mRNA expression was negatively associated with INF-γ ($r = -0.8505$ and $P = 0.0002$) and TGF-β1 ($r = -0.7802$ and $P = 0.0015$) mRNA expression (Figures 4(e) and 4(f)).

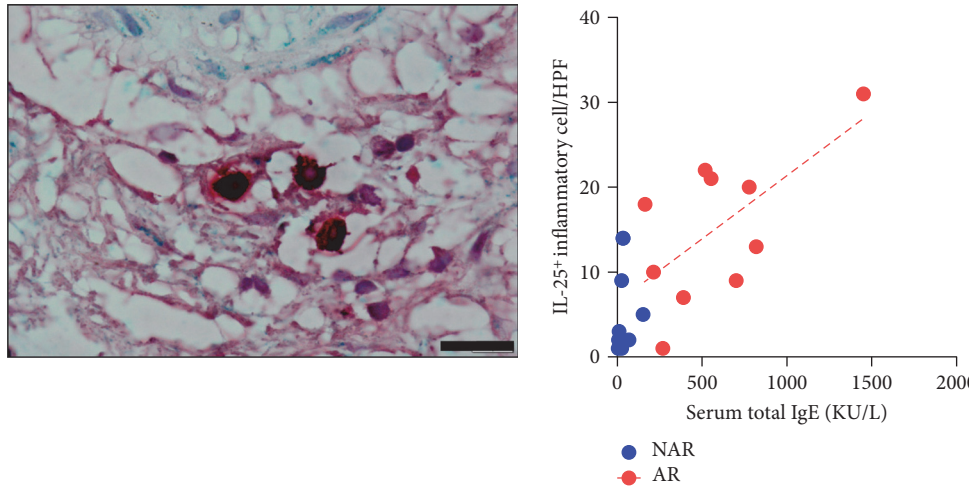

Figure 3: Cellular origin of IL-25 in nasal tissues from house dust mite-sensitized allergic rhinitis: (a) double immunohistochemical staining for IL-25 and mast cells and (b) correlation between the number of IL-25 inflammatory cells and serum total IgE.

4. Discussion

The prevalence of AR is increasing, affecting about 18.5% of the Korean population for all ages based on the Korean National Health and Nutrition Survey [20]. In addition, compared with healthy subjects, asthma, nasal polyps, chronic rhinosinusitis, and olfactory dysfunction are more prevalent in patients with AR [21]. Thus, early diagnosis and appropriate treatment of AR are crucial. However, despite a substantial understanding of the clinical characteristics in patients with HDM-sensitized AR, the initial cellular and molecular events that cause susceptible subjects to acquire HDM-induced AR are still unclear.

Recent studies have found the innate immune response to exacerbate inflammation in the nasal airway mucosa [22]. Epithelial cell-derived cytokines, including TSLP, IL-25, and IL-33 produced by airway epithelial cells are important Th2-augmenting cytokines that affect eosinophilic homeostasis and airway inflammation [3–5]. This shows that innate cytokines, such as TSLP, IL-25, and IL-33, are involved in the development of allergic disease by acting as a link between the innate and adaptive airways. HDM allergens could potentially lead to the release of epithelial cell-derived cytokines because sensitization of HDM allergens has resulted in injury to the nasal epithelial cells [23]. Previously, some studies have demonstrated that in allergic reactions, the mugwort pollen allergen was mainly characterized by IgE binding and T-cell proliferation [24, 25], whereas the HDM allergen mediated direct nonspecific damage and allergic reactions in the respiratory epithelium [26, 27]. Specifically, among epithelium-associated cytokines, IL-25 exacerbates allergic inflammation by epithelial cell hyperplasia, mucus secretion, airway hyperresponsiveness, and production of specific Th2 cytokines [28, 29].

To date, several animal studies described that innate cytokines are crucial for the development of acute allergic airway inflammation. However, there were little studies about the relationship between innate cytokines and chronic allergic airway inflammation. To our knowledge, this study is the first to investigate the relationships between IL-25 and chronic allergic airway inflammation, using human nasal tissues. In the present study, we found that in an in vitro assay, HNECs stimulated with high concentrations of HDM produced an increased expression of IL-25. In addition, the expression of IL-25 mRNA level was increased in the nasal mucosa of patients with HDM-sensitized AR, and the number of IL-25-positive-epithelial cells and IL-25-positive-inflammatory cells was significantly higher in the nasal mucosa of patients with HDM-sensitized AR. However, the expression of IL-33 was significantly lower in AR patients than in NAR patients, whereas there was no significant difference in TSLP expression between AR and NAR patients. Consistent with our findings, another study recently demonstrated that IL-25 induced an increased IL-13 expression in the peripheral blood mononuclear cells of HDM-AR patients compared to those of mugwort-AR patients [30]. It means that IL-25 may play a more important role in chronic allergic airway inflammation, such as AR induced by HDM than TSP or IL-33. Therefore, to support our conclusion, we need to investigate the role of IL-25 in the development of HDM-induced human allergic nasal inflammation further.

Interestingly, in the analysis of the IL-25 and inflammatory markers, we observed a meaningful relationship between the IL-25-positive inflammatory cells and total IgE. Moreover, the IL-25 mRNA level was significantly correlated with inflammatory markers such as ECP and GATA3 for the Th2 immune response. These findings suggest that IL-25 may play a major role as one of the mediators for the development of Th2 immune response in patients with HDM-induced AR. Although other prior studies on the HDM-induced allergic mice model have demonstrated that IL-25 is unnecessary for Th2 priming and the subsequent effector responses to the HDM allergens [18, 19], we believe this discrepancy can be explained by their reliance on acute allergic mice models, as opposed to our study's use of human nasal tissues—a type of chronic model for HDM-induced AR.

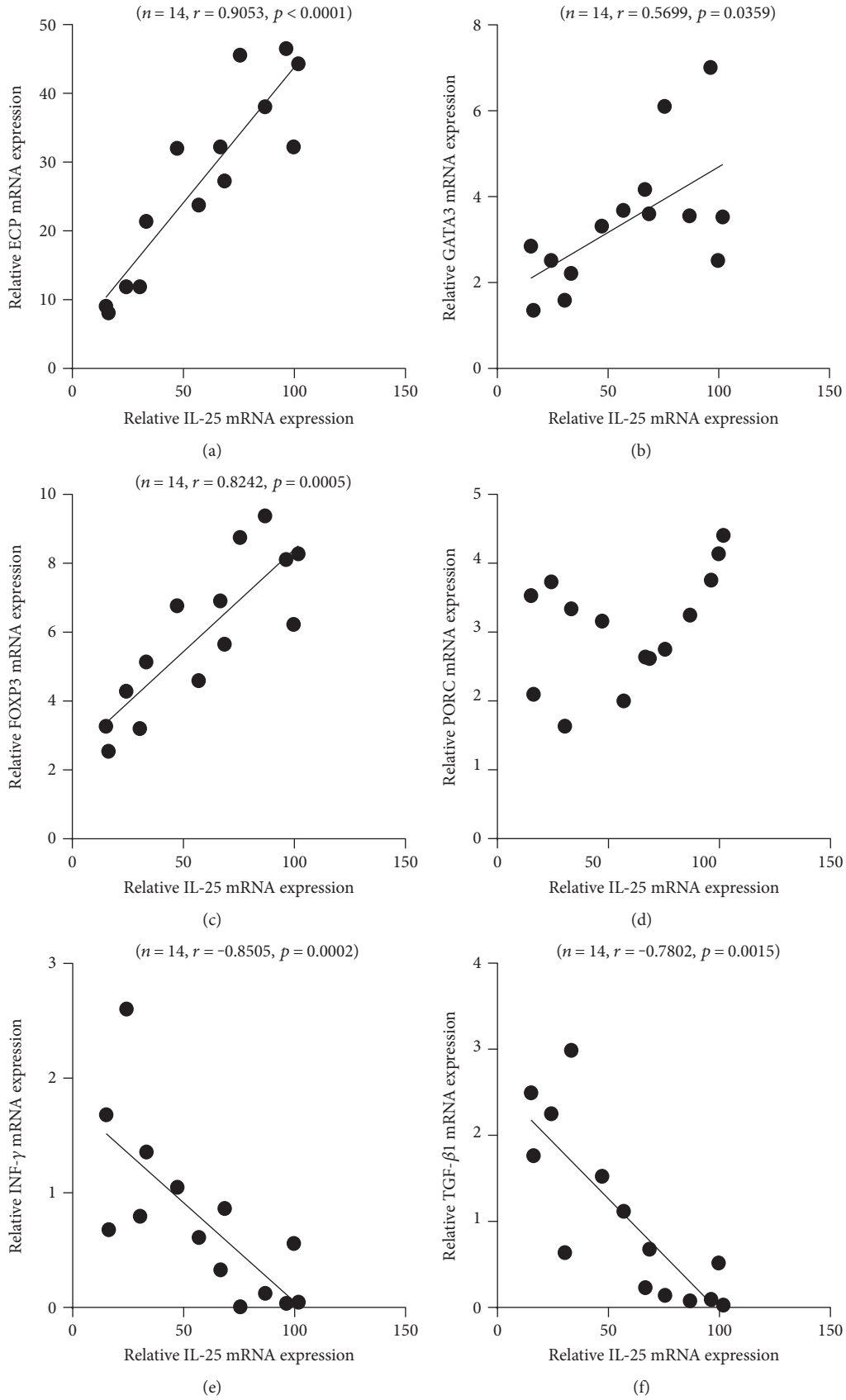

Figure 4: Correlation between IL-25 mRNA expression and inflammatory markers in nasal tissues from house dust mite-sensitized allergic rhinitis: (a) ECP, (b) GATA3, (c) FOXP3, (d) RORC, (e) INF-γ, and (f) TGF-β1.

Therefore, to more elucidate the role of IL-25 in AR, we need further studies such as IL-25 blocking antibody or IL-25 knockout mice studies, using animal models for chronic allergic airway inflammation.

5. Conclusions

In the present study, we have confirmed the increased production of IL-25 in cultured HNECs, when stimulated with high concentrations of HDM. Particularly interesting are the increased expression of IL-25 in nasal tissues from patients sensitized with HDM-induced AR and the positive correlation between the IL-25 and Th2 markers observed in the present study. These findings suggest that IL-25 may involve in the development of Th2 immune response in HDM-induced AR. Therefore, IL-25 neutralization might be a potential approach for the treatment of patients with HDM-sensitized AR.

Conflicts of Interest

The authors declare that there are no competing interests regarding the publication of this paper.

Authors' Contributions

Dae Woo Kim and Dong-Kyu Kim contributed equally to this work.

Acknowledgments

This study was supported by Grant no. 0320140420 (2014-1309) from the SNUH Research Fund (to Dae Woo Kim) and by a grant from the Korean Health Technology R&D Project, Ministry of Health & Welfare, Republic of Korea (HI14C2161, to Ji-Hun Mo).

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Hindawi
Mediators of Inflammation
Volume 2017, Article ID 6827194, 12 pages
https://doi.org/10.1155/2017/6827194

Research Article

Selective ATP-Binding Cassette Subfamily C Gene Expression and Proinflammatory Mediators Released by BEAS-2B after $PM_{2.5}$, Budesonide, and Cotreated Exposures

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Received 13 May 2017; Accepted 2 July 2017; Published 16 August 2017

Academic Editor: Tsuguhisa Nakayama

ATP-binding cassette subfamily C (ABCC) genes code for phase III metabolism proteins that translocate xenobiotic (e.g., particulate matter 2.5 ($PM_{2.5}$)) and drug metabolites outside the cells. IL-6 secretion is related with the activation of the ABCC transporters. This study assesses ABCC1–4 gene expression changes and proinflammatory cytokine (IL-6, IL-8) release in human bronchial epithelial cells (BEAS-2B) exposed to $PM_{2.5}$ organic extract, budesonide (BUD, used to control inflammation in asthmatic patients), and a cotreatment (Co-T: $PM_{2.5}$ and BUD). A real-time PCR assay shows that ABCC1 was upregulated in BEAS-2B exposed after 6 and 7 hr to $PM_{2.5}$ extract or BUD but downregulated after 6 hr of the Co-T. ABCC3 was downregulated after 6 hr of BUD and upregulated after 6 hr of the Co-T exposures. ABCC4 was upregulated after 5 hr of $PM_{2.5}$ extract, BUD, and the Co-T exposures. The cytokine assay revealed an increase in IL-6 release by BEAS-2B exposed after 5 hr to $PM_{2.5}$ extract, BUD, and the Co-T. At 7 hr, the Co-T decreases IL-6 release and IL-8 at 6 hr. In conclusion, the cotreatment showed an opposite effect on exposed BEAS-2B as compared with BUD. The results suggest an interference of the BUD therapeutic potential by $PM_{2.5}$.

1. Introduction

Airborne particulate matter 2.5 ($PM_{2.5}$) is within the most regulated pollutant worldwide due to its intrinsic physicochemical properties that make it hazardous to the respiratory and cardiovascular system [1]. $PM_{2.5}$ is composed of inorganic compounds (metals and minerals) and organic pollutants (bacterial endotoxins, fungal spores, pollen fragments, polycyclic aromatic hydrocarbons (PAH), and carbonaceous elements) [2–7]. Due to the small diameter of these particles (2.5 μm), once they are inhaled through the respiratory system, they are easily transported into the arterial circulation [4, 8, 9]. The inhaled components can narrow the airways and induce inflammatory responses that can aggravate any existing respiratory conditions such as asthma or chronic obstructive pulmonary disease (COPD) [5, 10–14]. $PM_{2.5}$ has been widely studied [4, 10, 15] and is extremely regulated in the United States by the Environmental Protection Agency (USEPA); however, the metabolism of this xenobiotic in lung cells [16–18] and its effect in the local immune response have not been fully elucidated.

Previous studies have shown that the antioxidant response to $PM_{2.5}$ in bronchial epithelial cells (e.g., BEAS-2B) is activated through nuclear factor-erythroid 2-related factor 2 (Nrf2) which leads to the activation of mRNAs for heme oxygenase (HMOX1) and glutathione-S-transferase (GSTP1) that code for enzymes responsible for activating the second phase of the metabolism [4, 19, 20]. In addition to the cell

detoxification mechanism, bronchial epithelial cells are responsible for modulating the local immune response against foreign agents like $PM_{2.5}$ [21, 22]. As part of the local immune response of BEAS-2B against PM exposure, toll-like receptor- (TLR-) 2 and 4 activation followed by secretion of interleukins 6 (IL-6) and 8 (IL-8) has been reported [3, 13, 23, 24]. Nfr2 transcription factor activation as well as the IL-6 secretion are related with the activation of the ATP-binding cassette subfamily C (ABCC) transporters [25]. Since we have previously demonstrated that Nrf2 and IL-6 are induced in BEAS-2B upon $PM_{2.5}$ exposure [4, 26, 27], we expect to find an upregulation of the ABCC1–4 genes upon the $PM_{2.5}$ exposure. To the best of our knowledge, there is no information about the ABCC1–4 gene expression in BEAS-2B exposed to $PM_{2.5}$, which involves the third phase of the xenobiotic metabolism.

The third phase of the cell metabolism is depending on the ATP-binding cassette subfamily C genes encoding the multidrug-resistant proteins (MRPs). The ABCC1–4 genes encode membrane proteins involved in various physiological events and function as efflux pumps of metabolic waste products (e.g., glutathione (GSH), glucuronide, and sulfate conjugates) [28–31]. Overexpression of these transporters can be responsible for drug inefficacy, which occurs when the cell pumps out the drug without any selectivity. Activation of ABCC1–4 has been associated with the presence of corticosteroids, and its derivate products have also been identified as potential regulators or precursors of ABCC1–4 genes [28, 32, 33]. Thus, we also expect regulation to be affected after budesonide (BUD) corticosteroid treatment in BEAS-2B. Moreover, a cotreatment (Co-T) of $PM_{2.5}$ and corticosteroids was performed to study the efficiency of the corticosteroids upon $PM_{2.5}$ exposure using the BEAS-2B.

Bronchial epithelial cells are one of the first targets for environmental and inhaled drug metabolism due to its location in the respiratory tract. These cells play an important role in the xenobiotic and drug fate that includes the clearance of these molecules through a set of enzymes (phase II) and ABCC1–4 gene activation. Our results will allow us to provide a background about the ABCC gene regulation that may happen once $PM_{2.5}$, corticosteroids, or both interact in bronchial epithelial cells that might decide the fate of these molecules. There is very limited research on the effects of $PM_{2.5}$ or BUD on the ABCC1–4 transporters and even less on the effects of the combined exposure of these treatments. Our experimental approach included the use of the most prescribed synthetic corticosteroid, BUD [34]. It is important to highlight that no therapeutic drug has been proven to be effective against air pollution-induced asthma or COPD. BUD is known to reduce ozone-induced inflammation, but does not protect against decreased lung function [35, 36]. However, the primary role of corticosteroids such as BUD consists in downregulating inflammatory cytokines to reduce the level of concomitant inflammation. As previously mentioned, it has been demonstrated that $PM_{2.5}$ induces IL-6 and IL-8 in human bronchial epithelial cells [2–5, 10, 37]. Therefore, the aim of this study was to assess the response induced by $PM_{2.5}$ and BUD in BEAS-2B by measuring the levels of IL-6 and IL-8 through a specific time course at 5, 6, and 7 hr. These data will allow us to indirectly determine the drug effect. Our results provide additional information on the cytotoxic effects of $PM_{2.5}$ and BUD to BEAS-2B. In addition, we aimed to elucidate the effects of these treatments on the expression of ABCC1–4 genes at 5, 6, and 7 hours after exposure. Our results provide a guide towards the elaboration of a biochemical framework to explain the role of $PM_{2.5}$, BUD, and the Co-T on MRP regulation.

2. Materials and Methods

2.1. Site and Sample Selection. $PM_{2.5}$ samples were collected in circular Teflon filters by the Puerto Rico Environmental Quality Board, which maintains a net of monitoring stations at various strategic points into the island to monitor the air quality. The designated station is in Guayama, Puerto Rico. Data from Fajardo Puerto Rico, a reference location, was used for comparison of $PM_{2.5}$ toxicity of the two sites.

2.2. Sampler Collection Equipment and $PM_{2.5}$ Extractions. Teflon filters were set for 24 hr for the entire period of January 01, 2008 to December 31, 2008 in a $PM_{2.5}$ sampler air collector (R&P model 2025), previously programmed with a standard method developed by the USEPA specifications. The filters were exchanged after the 24 hr completion. Once the collection was completed, the filters were labeled with a number referring to the collection day and place and identified as $PM_{2.5}$. Teflon filters were stably weighed, following the USEPA methodology (Environmental Protection Agency, 1998). All glassware was washed using a modified cleanup procedure that includes the acidic treatment [38]. The collected $PM_{2.5}$ filters were extracted during 15 minutes with 180 ml of hexane/acetone 1 : 1 (Fisher) using a microwave-assisted extraction system (MAE) (Ethos plus Microwave Labstation). Sample digestion was performed according to Alvarez-Avilés et al. [39]. Briefly, after the digestion with MAE, the large amount of solvent was removed with a gentle steam of nitrogen across the surface of the conic vials, using a tank of compressor gas. The protocol had the following constant parameters: 80°C and 1000 W and time points: $T_1 = 10$ min and $T_2 = 5$ min. The analytical weight of the samples was performed following gravimetric method. The organic extracts were resuspended in dimethyl sulfoxide (DMSO, Molecular Biology, Fisher) to a concentration of 100 mg/ml as a final stock solution. An organic extract composite corresponding to July 2008 filters was prepared and then stored at −20°C. The $PM_{2.5}$ extract is the limited reagent of the study; therefore, the experiments were designed only to answering the proposed questions.

2.3. Cell Culture. Human bronchial epithelial cells (BEAS-2B, ATCC® #CRL-9609™) were cultured and maintained in keratinocyte growth medium 2 (KGM-2, Walkersville, MD). The cells were incubated in a humidified atmosphere of 5% CO_2 at 37°C. Before each biological assay, the cells were seeded at a density of 5×10^4 cells/well into 96-well plates and incubated for 24 hr.

2.4. Cell Viability Assay. The neutral red bioassay (Sigma, St. Louis, MO) was performed to obtain the lethal dose (LD_{50})

for 50% mortality of the different agents. The cell treatments included $PM_{2.5}$ (25, 50, 75, and 100 μg/ml), GSH, Sigma, MO (5, 10, 25, 75, 100, 250, and 500 μg/ml), and the suspension BUD (0.01, 0.03, 0.05, and 0.1 μg/ml). The Co-T concentration was obtained from the nontoxic concentrations of $PM_{2.5}$ (25 μg/ml) and Bud (0.05 μg/ml). After 24 hr, the supernatants with the treatments were removed. The neutral red dye was added for 3 hr. After removing the dye, the cells were fixed with a 0.5% formaldehyde/1% calcium chloride solution and rinsed with 1x phosphate-buffered saline (PBS) to eliminate unfixed cells, excess dye, and formaldehyde residues. The BEAS-2B were then lysed using a 1% acetic acid/50% ethanol solution. Cell viability was determined with spectrophotometry at 540 nm using an Ultramark microplate reader (Bio-Rad, Richmond, CA, USA). Triton-X treatment (25 μg/ml) was used as a positive control. Values less than 80% cell viability were considered cytotoxic. The different solvents including media, DMSO, and H_2O were used as negative controls.

2.5. Real-Time Polymerase Chain Reaction (PCR). Gene expression assay validation was performed using TaqMan® (Applied Biosystems, CA). A calibration curve was constructed using the GSH exposure (50 μg/ml); GSH is a positive inductor of the target gene, ATP-binding cassette C subfamily (ABCC1–4) [38–41]. Cells were exposed to $PM_{2.5}$ extract (25 μg/ml), BUD (0.05 μg/ml), and the Co-T at different time points (5, 6, and 7 hr). Total RNA was extracted using TRIZOL reagent (Invitrogen, CA). The high-capacity complementary DNA (cDNA) reverse transcription kit (Applied Biosystems, CA) was used to synthesize cDNA. Quantitative florescent amplification of cDNA of ABCC1 (Hs01561502_m1), ABCC3 (Hs00978473_ml), and ABCC4 (Hs00988717_m1) was performed using TaqMan Gene Expression Assays (Applied Biosystems, CA). The real-time polymerase chain reaction (RTPCR) was conducted in a StepOne Real-Time PCR System (Applied Biosystems, CA). β-Actin (Hs03023943_ml) was used as a housekeeping gene to normalize the target genes.

2.6. Cytokine Assay. The cytokines were obtained from cell supernatant collected after 5, 6, and 7 hr of treatment with $PM_{2.5}$ extract, BUD, and the Co-T and transferred to a 96-well plate. A simultaneous and quantitative detection of the proinflammatory mediators IL-6, IL-8, IL-10, and IL-13 was performed using a multiplex bead assay (Multianalyte Profiling Kit from R&D Systems, Minneapolis, MN) and a Luminex 100 (Luminex Corp., Austin, TX, USA) instrument according to the manufacturer's instructions. Lipopolysaccharide (LPS) at 10 μg/ml was used as a positive control in the assay.

2.7. Statistical Analyses. To assess the differences between individual groups, the unpaired Student's t-test was employed. The criterion for statistical significance was set at $^{***}p < 0.001$, $^{**}p < 0.01$, and $^{*}p < 0.05$. Statistical analyses were performed using the GraphPad InStat 3 software. Analyses were based on three independent experiments.

3. Results

3.1. Cell Viability Assay. The toxicity of GSH, $PM_{2.5}$ extracts, BUD, and the Co-T was evaluated in BEAS-2B. From these experiments, we selected the nontoxic concentrations to be used for gene expression assays and measurement of cytokine levels. A linear relationship between GSH concentration and cell viability was obtained with an estimated lethal dose (LD_{50}) of 253 μg/ml in BEAS-2B. The highest toxicity was observed at 250 and 500 μg/ml GSH, reducing cell viability to 56.22% and 13.13%, respectively ($p < 0.001$) (Figure 1(a)). These results were used to establish a nontoxic concentration (25 μg/ml) for the positive control of ABCC gene induction. Dose-response experiments for the $PM_{2.5}$ extracts indicate that concentrations above 25 μg/ml are significantly toxic to cells (Figure 1(b)). Our results show that 50, 75, and 100 μg/ml $PM_{2.5}$ extracts reduce cell viability to almost 69.36%, 49.32%, and 33.60%, respectively ($p < 0.01$). An inverse relationship between $PM_{2.5}$ extract concentration and cell viability was observed. From these results, the LD_{50} for $PM_{2.5}$ extract was calculated to be 76.7 μg/ml. Concentrations of BUD above 0.1 μg/ml caused toxic effects on BEAS-2B. (Figure 1(c)). A dose-response curve was obtained showing an inverse relationship between cell viability and BUD increasing concentration. Although higher concentrations were not included, an LD_{50} of 0.17 μg/ml was extrapolated. The nontoxic concentrations of 25 μg/ml for $PM_{2.5}$ extract and 0.05 μg/ml for BUD were selected for the subsequent experiments as well as to create the Co-T concentration. The cotreatment did not induce any cell toxicity at the concentration tested (Figure 1(d)).

3.2. Partial Time Course of ATP-Binding Cassette Genes (ABCC 1, 2, 3, and 4) Expression with Treatments. A partial time course of the ABCC1–4 gene expression in BEAS-2B exposed to GSH, $PM_{2.5}$ extract, BUD, and the Co-T was performed to determine any alterations on their regulation due to these exposures. The ABCC2 was not induced by any of these treatments. $PM_{2.5}$ extract induced an upregulation of ABCC1 and ABCC4 gene expression at various time points in BEAS-2B (Figures 2(a) and 2(c)). Significant inductions of ABCC1 were found when comparing DMSO-treated cells with $PM_{2.5}$ extract-treated cells after 6 and 7 hr of exposure ($p < 0.05$). There is a direct time response relationship of ABCC1 expression and $PM_{2.5}$ extract exposure in BEAS-2B (Figure 2(a)). A suppression of the ABCC1 gene was observed after 5 hr of $PM_{2.5}$ extract exposure when compared with the DMSO control ($p < 0.05$). The ABCC3 expression was also evaluated at 5, 6, and 7 hr after the $PM_{2.5}$ extract exposure; however, no significant differences were detected when comparing with control cells (Figure 2(b)). As for the ABCC4, the peak induction of gene expression was found at 7 hr although no statistical significance was observed. The second highest expression was seen at 5 hr, which was statistically significant (Figure 2(c)).

Overall, BUD treatment induced the expression of ABCC1 and ABCC4 at the time points studied (Figures 1(a) and 1(c)). Specifically, ABCC1's highest induction was observed at 7 hr of exposure ($p < 0.01$) (Figure 2(a)). BUD

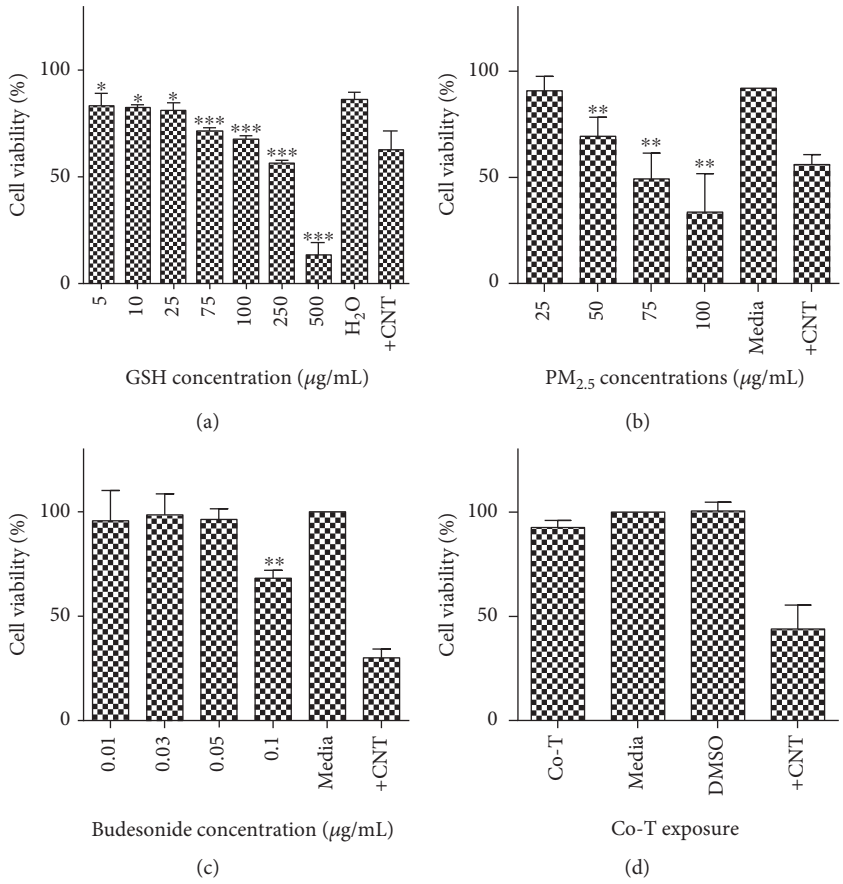


Figure 1: Cell viability assays for GSH, $PM_{2.5}$ extracts, budesonide (BUD), and the Co-T. BEAS-2B were treated for 24 hr with each treatment. (a) The 25 μg/ml dose of GSH was selected as the highest nontoxic concentration and used as the positive control for ABCC1–4 gene expression experiments. (b) The highest nontoxic concentration of $PM_{2.5}$ extracts selected for further experiments was 25 μg/ml. (c) The highest nontoxic concentration for the BUD exposure was 0.05 μg/ml. (d) Using the nontoxic concentrations of $PM_{2.5}$ and BUD, a Co-T was established. Bars represent the mean cell viability of three independent experiments ($N = 3$). Triton-X (25 μg/ml) was used as positive control (+CNT). Asterisks denote statistical significance: $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$.

suppressed the expression of ABCC3 at 6 hr (Figure 2(b)). ABCC3 expression was unaltered at 5 hr of exposure; however, its highest expression was seen at 7 hr of BUD treatment although it was not significantly different when compared with that of control cells (Figure 2(b)). The highest induction of ABCC4 was seen at 6 hr of BUD exposure ($p < 0.05$) (Figure 2(c)).

The Co-T did not significantly alter ABCC1 expression at 5 or 7 hr of exposure (Figure 2(a)). However, a significant reduction of ABCC1 expression was observed after 6 hr (Figure 2(a)). Both $PM_{2.5}$ extract and BUD independently increased ABCC1 expression significantly at 6 hr, but the Co-T had an opposite effect. The gene expression of ABCC3 peaked at 6 hr ($p < 0.01$), but no differences were found at neither 5 nor 7 hr of the Co-T (Figure 2(b)). The ABCC4 showed a significant increase of expression after 5 hr of the Co-T ($p < 0.01$) (Figure 2(c)). This induction of ABCC4 at 5 hr is analogous to the results obtained by the $PM_{2.5}$ extract and BUD exposure (Figure 2(c)).

3.3. Partial Time Course of Cytokine Measurements after Treatments. After exposure to $PM_{2.5}$ extract, IL-6 and IL-8 concentrations were assessed. Previous studies report induction of IL-6 and IL-8 at short periods of time after $PM_{2.5}$ exposure [4]. Therefore, we selected the time points of 5, 6, and 7 hours as in the gene expression experiments. Our results show that IL-6 concentration decreases in a time-dependent manner after exposure to $PM_{2.5}$ extract, BUD, and the Co-T (Figure 3(a)). The highest concentration of IL-6 was obtained at 5 hr and was the highest of any treatment. The cotreatment significantly reduced IL-6 secretion when compared to the effect of any of the individual treatments (Figure 3(a)). This inhibitory effect was also seen for IL-8 in cotreated cells at 6 hr when compared to $PM_{2.5}$ extract and BUD treatments alone at the same exposure time. Concentrations of IL-13 and IL-10 with all the treatments described above after 24 hr in BEAS-2B were also evaluated; however, no significant changes in cytokine expression were observed. Slight increases in IL-8 at different time points were observed with the various treatments, but these were not significantly different from controls (Figure 3(b)). The most prominent finding in IL-8 was observed with the cotreatment at 6 hr where a significant reduction was reported.

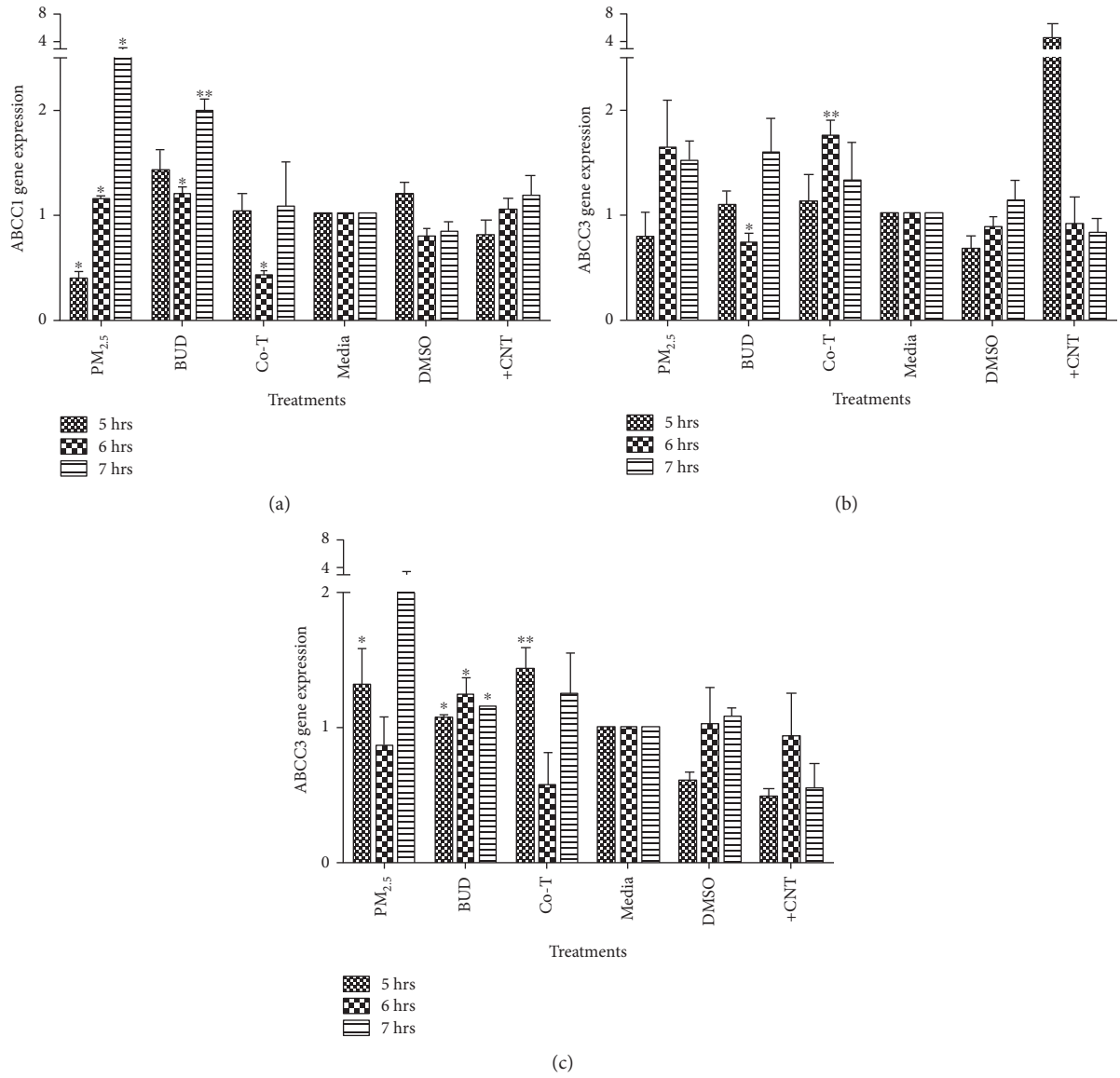
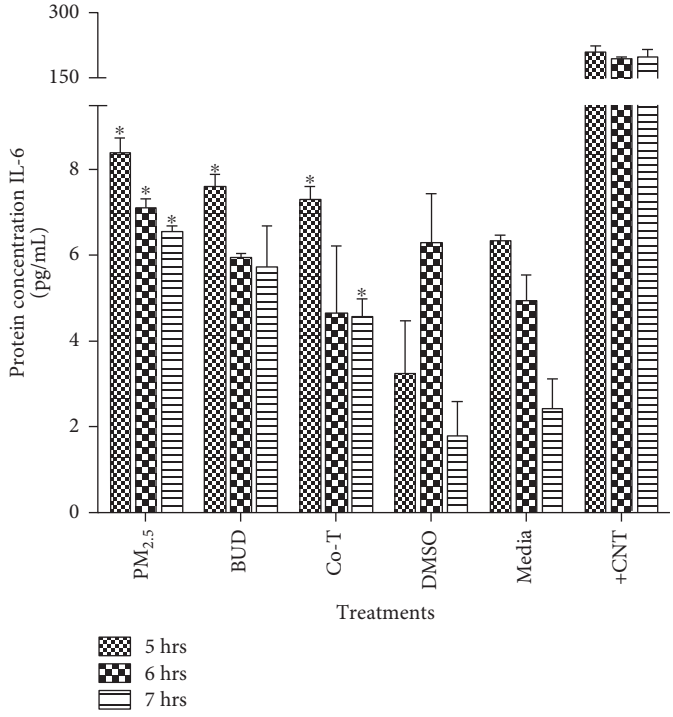


Figure 2: Time course of ABCC1, ABCC3, and ABCC4 mRNA in BEAS-2B with the different treatments: ABCC1–4 mRNA response to $PM_{2.5}$ extract (25 μg/ml), budesonide (BUD) (0.05 μg/ml), and to the cotreatment at various time points (5, 6, and 7 hr) of exposure. Bars represent mean cell viability ± SEM of three independent experiments ($N = 3$); $^{**}p < 0.01$, $^{*}p < 0.05$. Asterisks over the bar indicate comparison of the treatments with the solvent (media or DMSO). GSH 25 μg/ml was used as positive control.

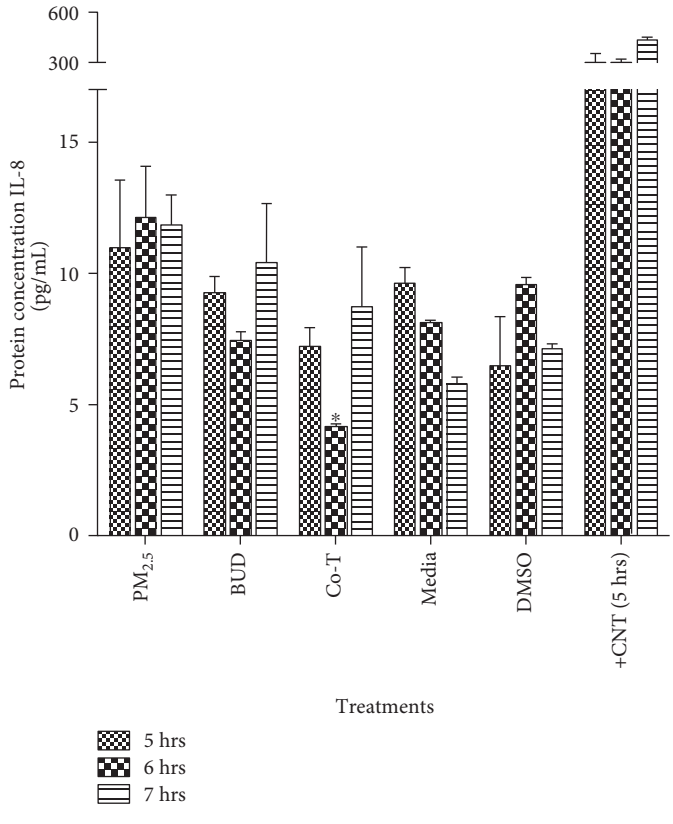
4. Discussion

Exposure to $PM_{2.5}$ causes exacerbation of several conditions of the respiratory system and cardiovascular diseases [5, 14, 40]. The results from the toxicity assays of $PM_{2.5}$ extract showed nontoxic effects in BEAS-2B at 25 μg/ml, as previously reported by Rodriguez-Cotto et al. and Akhtar et al. using lung cell lines [1, 5]. These studies also report that concentrations above 75 μg/ml are toxic to lung cells, like our findings. A difference between this study and the one by Rodriguez-Cotto et al. was observed after their LD_{50} was taken into consideration [1, 5]. The toxicity of Guayama $PM_{2.5}$ extract was significantly higher ($LD_{50} = 76.7$ μg/ml) than that of Fajardo's ($LD_{50} = 122$ μg/ml) [5]. The main reason for this is that Guayama is more likely an urban industrialized site while Fajardo is a rural site. Since the physicochemical properties of $PM_{2.5}$ depend on its size and the source of origin, it was not surprising to find this trend between the different areas [1, 41]. In addition, the topographic and seasonal changes are different among sites, such as the African dust phenomenon that affect both sites in a different manner [5, 10, 42].

The BUD glucocorticoid treatment was employed to evaluate its effect on ABCC gene expression alone and in the Co-T. Within the recommendable doses of BUD reported in the literature is 0.1 μg/ml to use in bronchial epithelial cells [43, 44]. However, we found this dose to be toxic for BEAS-2B; hence, the highest nontoxic concentration was determined to be 0.05 μg/ml. The Co-T was nontoxic to BEAS-2B. Taking that into account, the possibility of a



(a)



(b)

Figure 3: Induction of IL-6 and IL-8 in BEAS-2B exposed to $PM_{2.5}$ extract, budesonide (BUD), and the Co-T. Cytokines were measured using a multiplex bead system and Luminex instrument, after 24 hr of exposure. Bars represent mean protein concentration ± SEM of three independent experiments ($N = 3$); $^*p < 0.05$. Asterisk over the bar indicated the comparison of a treatment with DMSO. LPS (10 μg/ml) was used as positive control.

synergistic effect (between $PM_{2.5}$ and BUD) enhancing cell proliferation or death was ruled out. The cells presented a normal proliferation after the Co-T exposure. This outcome allowed us to conduct the gene expression studies with the assurance that the cellular environment was reliable and not altered by apoptosis or related mechanisms.

$PM_{2.5}$ exposure provokes an antioxidant rather than an inflammatory response. $PM_{2.5}$ has been found to induce the release of immune mediators in BEAS-2B, as previously mentioned [4, 10, 45, 46]. It has been proven that this antioxidant response takes place because of the metals in the matrix of $PM_{2.5}$ that provoke the induction of reactive oxygen species (ROS). Therefore, after a $PM_{2.5}$ exposure, Nrf2 activates an upregulation of HMOX1 and GSTP1 genes that are essential to enhance the metabolic and antioxidant defense [4]. Nrf2 is responsible for detoxification and xenobiotic removal due to its role in activating the gene transcription of antioxidant and phase II detoxification enzymes, followed by phase III efflux transporters [45, 46]. It is important to highlight that the role of Nfr2 as a transcription factor inducing ABCC proteins has been studied in many fields [26, 47–49]. Accumulations of superoxides generate oxidative stress while the Nrf2 is activating in the cell cytoplasm. Nrf2 translocates into the cell nucleus thereby activating the antioxidant response elements (AREs), which encode the ABCC1–4 genes. Moreover, studies using small interfering RNA (siRNA) have shown a direct dependence among MRPs and Nrf2 during oxidative stress conditions [26, 50, 51]. Therefore, no doubt exists regarding the positive association between Nrf2 and MRPs in different scenarios where oxidative stress is the common variable.

These mechanisms have been observed in BEAS-2B, and our data support that the ABCC1 transporter may have an important role in $PM_{2.5}$ metabolism. The cell antioxidant and protective responses include pumping out xenobiotics through the ABCC transporters, as previously mentioned. The ABCC transporters are recognized for their essential role in transporting glutathione s-conjugates, which is, thus, their importance on gene expression during oxidative stress [48, 52, 53]. Since all the xenobiotics were contained in the $PM_{2.5}$, we hypothesized an increase in ABCC1, ABCC2, ABCC3, and ABCC4 gene regulation after $PM_{2.5}$ exposure in BEAS-2B. Our results show a significant upregulation only for ABCC1 and ABCC4. This is the first report, to our knowledge, using BEAS-2B that demonstrated an ABCC1 upregulation due to the $PM_{2.5}$ exposure.

ABCC1 downregulation and immune response suppression have been related with cigarette smoke extracts using lung cells and in animal studies [54, 55]. In addition, it has been reported that smokers with COPD have been found with a deficiency in MRP1 which is the resulting product from the ABCC1 translation [56, 57]. These findings are related with the cell response that we observed at 5 hr of $PM_{2.5}$ exposure.

Studies in H69 lung cancer cells demonstrated that Nrf2 activated the MRP1 as a defense mechanism to promote cell survivor [58]; however, ABCC2 gene expression was not found in this cell line. The ABCC2 gene expression in lung cells has been debated [59]; here, we report nonsignificant induction in BEAS-2B. ABCC3 expression in lung cells has been also debated and reported differently within the same tissue including the lung tissue [60]. Nonsignificant results were found at any $PM_{2.5}$ exposure time with ABCC3 gene. It is also known that ABCC3 is a close homologous of ABCC1 and found in some reports to be mutually excluded [61]. The ABCC3 transporter has a higher preference for glucuronide conjugates rather than for glutathione conjugates. Thus, the $PM_{2.5}$ clearance depends on the antioxidant response and is logic to find ABCC1 upregulation instead of ABCC3. $PM_{2.5}$ exposures also provoke an upregulation of ABCC4 at 5 hr. The ABCC4 protein is recognized as the versatile transporter within the ABCC family, because of its remarkable ability to transport a diversity of substrates. These ABCC4 substrates may include endogenous and xenobiotic organic anionic compounds, cyclic nucleotides, eicosanoids, urate, and conjugated steroids among others [62]. Moreover, our results suggest a possible role after the $PM_{2.5}$ exposure in BEAS-2B. It is important to highlight that ABCC4 possesses a pathogenic role in the progression of pulmonary arterial hypertension (PAH) in humans [63]. More than one hypothesis was tested to screen for ABCC gene regulation by using the BUD and Co-T. The regulation of ABCC transporters with steroid drugs is more important since these drugs are commonly used to tackle inflammatory processes. The need to facilitate the transport of drug metabolites to and out of the cell is critical during treatment. Therefore, how these ABCC transporters are regulated in lung epithelial cells under BUD treatment is of great value. The BUD treatment upregulates the ABCC1 expression in BEAS-2B. In Calu-1 (lung cancer cells), this behavior is not noted [64]. Low concentration of BUD applied in cancer therapy inhibits the expression of vascular endothelial growth factor and MRP1 [65]. However, in the normal lung cell line 16HBE14o, BUD has been reported to upregulate ABCC1, supporting our research findings [66]. ABCC3 was not upregulated after BUD treatment. A significant downregulation of ABCC3 was detected at 6 hr, and then at 7 hr, its expression was stable. ABCC4 was also upregulated by BUD since MRP4 has been identified as a steroid transporter [59]. In asthma and COPD patient overexpresses, ABCC4 suggests that the steroid causes effects on its upregulation. We provide evidence that BUD generally increases ABCC1 and ABCC4 mRNA levels in epithelial lung cells during the first 6 and 7 hr of exposure. It is important to understand the dynamics of simultaneous exposure to particle pollution and inhaled corticosteroids since these are concurrently present during respiratory treatment. The effects of these two variables on ABCC regulation are an essential issue that needs to be addressed. ABCC1 was downregulated at 6 hr of Co-T exposure, opposing ABCC3 which was upregulated. The ABCC4 expression was upregulated by BUD treatment as well as $PM_{2.5}$; thus, the cell recognizes the necessity and importance of its transport as a response to treatment.

Induction of cytokines by $PM_{2.5}$ in lung cells has been previously reported [2–5, 10]. Airborne particulate matter contains a mixture of many organic and inorganic compounds, which induce a series of biochemical pathways and epigenetic changes that alter immune gene expression at

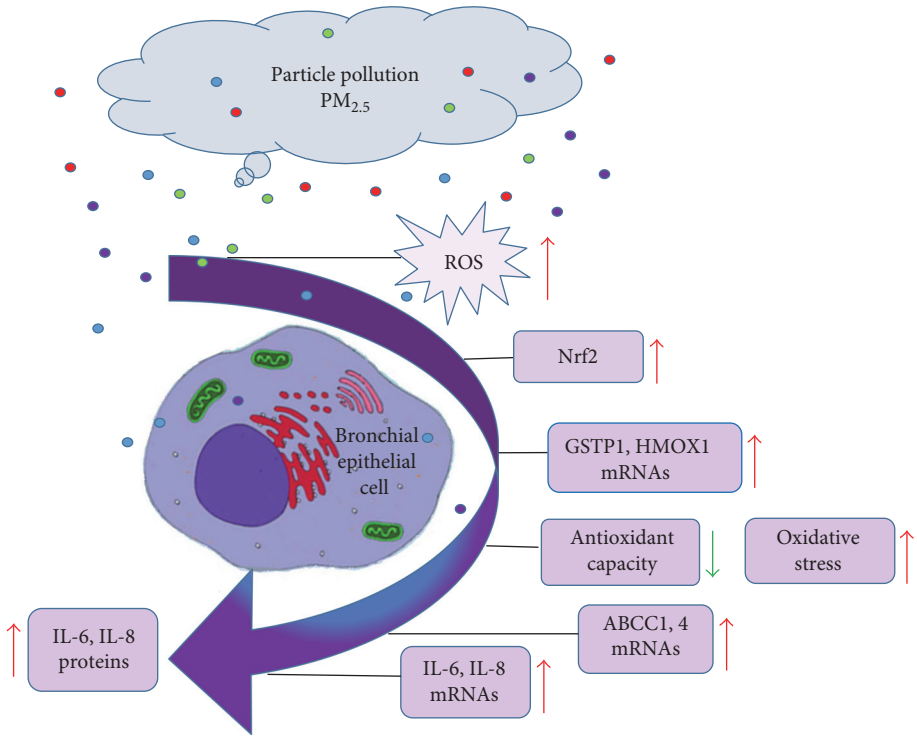


Figure 4: BEAS-2B respond to $PM_{2.5}$ organic extracts. Local particle pollution has the capacity to generate ROS triggering the activation of Nrf2 and inducing the synthesis of antioxidant mRNAs: HMOX1 (heme oxygenase 1) and GSTP1 (glutathione-S-transferase). The antioxidant capacity is reduced provoking oxidative stress and the synthesis of ABCC1 and 4 and IL-6 and IL-8 mRNAs and their respective proteins.

different levels as a defense response to environmental insult [18, 37, 67, 68]. It has been reported that PM can induce IL-6 and IL-8 secretion in BEAS-2B by ROS and through the activation of NF-κB or Nrf2 transcription factors [3, 4, 13, 48]. There is not much information in the literature to strongly support these findings by a cellular mechanism. Despite that, IL-6 and IL-8 have been detected after $PM_{2.5}$ exposure while the NF-KB has been undetected [4]. IL-8 was not detected with $PM_{2.5}$ at any of the time points. However, previous studies with $PM_{2.5}$ organic extracts in BEAS-2B showed induction of IL-8 at 6 and 8 hr, supporting the release at a longer time. Contrary, the mRNA activation of IL-6 has been reported after 6 to 7 hr of $PM_{2.5}$ exposure as well as what we reported in our time course [4]. Nrf2 directly regulates the mRNA of IL-8 in different types of cells [19]. IL-6 enhance the TH2 immune response mediated by lung epithelial cells and smooth muscle cells after the allergenic insult [69, 70]. The IL-6 overexpression was considered a byproduct of an ongoing inflammation, but recently has been recognized as a primary secreted cytokine in the epithelial cells [69]. IL-6 is also documented as one of the potential targets for the management and follow-up of chronic lung disease pathologies (e.g., asthma and COPD) [69, 71]. Since we found IL-6 in BEAS-2B, we can conclude that this response starts at an early stage of $PM_{2.5}$ exposure in normal lung cells. Thus, our work supports that $PM_{2.5}$ could lead to a major pathologic problem in the respiratory system.

Glucocorticoids are known potent regulators of inflammation and have been used pharmacologically against inflammatory, immune, and lymphoproliferative diseases for more than 50 years [34, 72]. However, glucocorticoids possess a broad variety and range of anti-inflammatory actions that are still not fully understood [72, 73]. We expected that $PM_{2.5}$ will activate IL-6 and IL-8 in BEAS-2B as a proinflammatory response, and BUD was expected to decrease cytokine levels. Thus, we expected a decrease of the cytokines with the Co-T. Nonsignificant differences were detected in IL-8 between the cells treated with BUD and control. We found induction of IL-6 secretion in BEAS-2B by BUD rather than inhibition at 5 hr. IL-6 has pleiotropic function within different organs including the lungs [74–76]. Mechanistic studies demonstrated an IL-6 induction in airway smooth muscle cells after corticosteroid exposure [75]. This effect was only observed at 5 hr; after 6 to 7 hr, the IL-6 protein concentration decayed with no statistical significance. The Co-T had a significant suppression effect on IL-8, and this might be caused by the suppressive properties of the corticosteroids since $PM_{2.5}$ did not stimulate IL-8 secretion by itself [77]. However, more experiments are needed to evaluate whether the Co-T has any influence in the IL-8 or IL-6 signaling pathways once the BEAS-2B are treated with $PM_{2.5}$.

5. Conclusions

$PM_{2.5}$ activates the antioxidant mechanisms and the induction of ABCC1 and ABCC4 mRNAs in BEAS-2B (Figure 4). Since after the 24 hr of exposure with $PM_{2.5}$ (25 μg/ml), the cells were more than 80% viable, tempting to suggest that this

finding is part of the management of xenobiotics metabolism in BEAS-2B. The Co-T exposure points out the need to perform more experiments to understand the signaling regulation in the lung cells to discriminate among ABCC1–4 gene transcription. However, most of the respiratory and cardiovascular diseases related to $PM_{2.5}$ exposures or allergens have been associated with an ABCC and cytokine dysregulation [15, 55, 56, 59, 66]. This is the first report to our knowledge that studies the mRNA expression of ABCC1, 3, and 4 genes exposed to Co-T. Most of the work done with transporters has considered exposure to diesel particles and not to ambient PM. Future experiments must consider searching for posttranscriptional modifications to elucidate the mechanism that regulates Nrf2 in the ABCC gene transcription under $PM_{2.5}$, BUD, and Co-T as well as study the activation of the MRP transporters. The cytokine experiments demonstrate an elevated expression of IL-6 at 7 hr with the $PM_{2.5}$ that slightly decreases after the Co-T exposure. This fact could be indicating that the BUD could not perform its pharmacological task completely in the presence of $PM_{2.5}$. Moreover, different mechanisms of action have been proposed and debating in the literature to explain the therapeutic and metabolic pathways associated with corticosteroids pharmacology [78, 79]. Although the inhibitory effects of corticosteroid therapy on the reproduction of osteoblast cells have been well elucidated [80], it is important to understand the fate of these corticosteroids after they are applied as therapy. Understanding the broad spectrum of molecule interaction between $PM_{2.5}$ and the bronchial epithelial cell response will provide additional evidence to comprehend the $PM_{2.5}$ role in the inflammatory process. It will also provide new avenues for innovative therapeutic approaches to benefit people over the world that are exposed to air pollutants.

Disclosure

The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Conflicts of Interest

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The study was supported by grants from the National Center for Research Resources (2G12RR003051) and National Institute on Minority Health and Health Disparities (8G12-MD007600) and from the National Institutes of Health. The authors want to acknowledge Dr. Carmen M. Ortiz Sánchez for the manuscript review.

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Hindawi
Mediators of Inflammation
Volume 2017, Article ID 8160148, 8 pages
https://doi.org/10.1155/2017/8160148

Review Article

Potential Biomarkers for NSAID-Exacerbated Respiratory Disease

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Received 16 May 2017; Accepted 26 July 2017; Published 9 August 2017

Academic Editor: Younghyo Kim

Asthma is a common chronic disease with several variant phenotypes and endotypes. NSAID-exacerbated respiratory disease (NERD) is one such endotype characterized by asthma, chronic rhinosinusitis (CRS) with nasal polyps, and hypersensitivity to aspirin/cyclooxygenase-1 inhibitors. NERD is more associated with severe asthma than other asthma phenotypes. Regarding diagnosis, aspirin challenge tests via the oral or bronchial route are a standard diagnostic method; reliable *in vitro* diagnostic tests are not available. Recent studies have reported various biomarkers of phenotype, diagnosis, and prognosis. In this review, we summarized the known potential biomarkers of NERD that are distinct from those of aspirin-tolerant asthma. We also provided an overview of the different NERD subgroups.

1. Introduction

NSAID-exacerbated respiratory disease (NERD) is characterized by adult-onset chronic rhinosinusitis (CRS) with nasal polyps, intense eosinophilic infiltration in the upper and lower airway mucosa, and severe symptoms of exacerbation in response to aspirin/cyclooxygenase- (COX-) 1 inhibitors [1]. A previous systematic review had reported a NERD prevalence of 7% among typical adult asthmatic patients and twice among patients with severe asthma [2]. NERD is therefore considered a risk factor for severe asthma [3, 4]. Among patients with CRS and nasal polyps, the prevalence of NERD was 8.7% and 9.7%, respectively [2]. NERD is associated with severe CRS with nasal polyps, recurrence after sinus surgery, and airway remodeling [5–7], suggesting that NERD causes severe asthma with CRS/nasal polyps.

NERD has a unique pathophysiology, with increased levels of lipid mediators, activated eosinophils, and mast cells, even without COX-1 inhibitor treatment. Thus, in most studies defining asthma endotypes, NERD has been identified as an independent endotype [8, 9]. However, all patients with NERD are not accompanied by severe asthma, and their clinical course is also known to be variable [10]. Confirmative diagnosis of NERD is based on provocation tests with aspirin. Oral aspirin challenge is considered the gold standard diagnostic method; however, its use is often limited by the risk of severe reactions during the test. The bronchial aspirin challenge is safer and consumes less time; however, it is limited by its low sensitivity [11]. In addition, oral or bronchial aspirin challenge test has limitations that cannot be used to predict the treatment or prognosis of NERD. Therefore, *in vitro* tests should be developed for diagnosing and monitoring NERD.

In this review, we summarized three groups of known noninvasive biomarkers that can distinguish NERD from aspirin-tolerant asthma (ATA): lipid mediators, inflammatory cells and cytokines, and genetic markers. In addition, we reviewed the subtypes of NERD and the related biomarkers for developing precision medicine in the future.

2. NERD as an Endotype of Asthma

Recently, many studies were conducted to distinguish asthma phenotypes and endotypes that affect diagnosis, treatment choice, and prognosis. A phenotype refers to "clinically observable characteristics," and it is distinguished by clinical features, pathophysiological factors, response to treatment, prognosis, and so on [12]. An endotype is a subtype of a disease that is functionally and pathologically defined by a molecular mechanism or a treatment response [13].

Table 1: Lipid mediators.

	Mediators and parameters	Biologic sample	Detection method	Baseline		Response after ASA provocation	
				Compared to ATA	Reference	Compared to ATA	Reference
COX pathway	LTE4	Urine	Immunoassay	↑	[25, 28–34]	↑	[25, 28, 30, 31, 34]
			Mass spectrometry	↑	[35, 36]	↑	[36]
		Saliva	Immunoassay	↑	[25]	↑	[27]
		Induced sputum	Immunoassay	↑	[25]		
			Mass spectrometry	↑	[26]	↑	[26]
		Blood and urine	Untargeted metabolomic analysis	↑	[37]		
	PGD2	Induced sputum	Immunoassay	↑	[32]		
			Mass spectrometry	↑	[26]		
	PGD2 metabolite	Spot urine	Immunoassay			↑	[31, 33]
			Mass spectrometry	↑	[41]	↑	[41]
		Blood	Mass spectrometry	↑	[40]	↑	[40, 42]
	PGE2	Spot urine	Immunoassay	↓	[33]		
Others	Sphingolipid metabolite	Blood	Mass spectrometry	↑	[48]	↓	[48]
		Spot urine	Mass spectrometry	↑	[48]		

ATA: aspirin-tolerant asthma; LO: lipoxygenase; LT: leukotriene; COX: cyclooxygenase; PG: prostaglandin.

Although there is no widely accepted method for endotyping, most studies have classified NERD as an endotype of asthma [9, 12]. NERD is known to be a late-onset asthma, as the first symptoms usually start at the age of 20 ~ 40 years; females are more affected, and it is not influenced by family history or geographic region [14]. Rhinitis is usually the first observed symptom followed by asthma, sensitivity to aspirin, and nasal polyps [15]. Patients with NERD presented with moderate to severe asthma (with frequent exacerbation) have poor lung function and require more frequent intubation and systemic steroid bursts [6].

The pathophysiological features of NERD include lipid mediator imbalance and intense eosinophilic inflammation. Proinflammatory cysteinyl leukotrienes (cysLTs) and prostaglandin (PG) D2 (PGD2) are known to be markedly upregulated in NERD, whereas PGE2 has been found to be constitutively decreased [16–18]. Patients with NERD have a higher number of mast cells and eosinophils infiltrating the upper and lower respiratory mucosa, even without exposure to COX-1 inhibitors and changes in tissue eicosanoid metabolism [19–21]. In NERD, cytokines and chemokines show a trend of Th2 immune response [22, 23].

3. Biomarkers of NERD

3.1. Lipid Mediators. The most reproducible and informative biomarker to distinguish NERD from ATA is a high-level urinary LTE4 (Table 1). LTE4 is the substance last metabolized in cysLTs. LTC4 and LTD4 are easily metabolized in the following stages, while LTE4 is released into the urine in a stable manner; it is therefore suitable for use as a biomarker [24]. The LTE4 levels in induced sputum and saliva are higher in NERD than in ATA [25–27]. However, urinary LTE4, which indirectly reflects the activity of cysLTs in the lungs, has been used to distinguish NERD from ATA in many studies [25, 28–36]. In addition, the nature of the urine specimen makes it easier to standardize the level of LTE4, and it has the advantage of noninvasiveness. The value of urinary LTE4 is increased, in the baseline as well as under aspirin or COX-1 inhibitor provocation, in NERD compared to ATA. Thus, baseline urinary LTE4 can be used as a biomarker to distinguish NERD from ATA. This phenomenon is present in both random urine and 24 h urine; recent studies on 24 h urine have reported an area under the curve (AUC) of 0.87 [35]. In addition, it was confirmed that the metabolites of urinary LTE4 were significantly different in NERD and ATA, even in studies that used metabolomics [37]. Urinary LTE4 can be used not only to distinguish between NERD and ATA but also to indicate the prognosis and treatment response. Urinary LTE4 is associated with a decrease in FEV1 during aspirin challenge in patients with NERD [38]. It has been reported that urinary LTE4 is significantly higher in patients with NERD who failed in aspirin desensitization than in patients who achieved aspirin desensitization successfully [36]. Although urinary LTE4 is also increased in allergic asthma, eosinophilic asthma, and severe asthma without NERD, it can be used as a biomarker in patients with NERD, as it shows a remarkable increase in NERD, compared to ATA; it can therefore be used for predicting treatment response and prognosis.

PGD2 and PGE2, which are counteracted by the products of cyclooxygenase, are known to be closely related to the pathogenesis of NERD, but their use as biomarkers is still limited. PGD2 is mainly secreted from mast cells and eosinophils and is known to act as a proinflammatory and bronchoconstrictive mediator through CRTH2 [39]. Baseline PGD2 has been observed to be significantly increased in induced sputum [26]. PGD2 metabolites in urine and blood are also

Table 2: Cellular and cytokine markers.

	Mediators and parameters	Biologic sample	Detection method	Baseline		Response after aspirin provocation	
				Compared to ATA	Reference	Compared to ATA	Reference
Cell	Eosinophil	NALF	Morphological count of stained slide			↑	[52]
	Platelet-adherent leukocyte	blood	Flow cytometry	↑	[65]		
	Soluble platelet surface marker	blood	Immunoassay	↑	[64]		
Others	ECP	NALF	Immunoassay			↑	[27]
	Periostin	Blood	Immunoassay	↑	[57]		

ATA: aspirin-tolerant asthma; NALF: nasal lavage fluid; ECP: eosinophil cationic protein.

increased after aspirin provocation [40–42]. However, these results differ among studies, and the range of overlap is wide; therefore, the use of PGD2 as a biomarker of NERD to distinguish it from ATA is limited. A previous study had reported that urinary PGD2 metabolites reflect the difference between tolerant and intolerant groups during aspirin desensitization in patients with NERD [36]. Further studies are required to validate the use of PGD2 as a biomarker for predicting the treatment response and prognosis of NERD. PGE2 is considered a key mediator in the pathogenesis of NERD. Unlike cysLTs or PGD2, it is known to have anti-inflammatory and bronchoprotective effects in airway inflammation. Inhaled PGE2 prevents bronchoconstriction and cysLT production in NERD [43]. Most urinary PGE2 metabolites are derived from COX-2, and several studies have demonstrated that airway tissues in patients with NERD showed impaired expression of COX-2 [44, 45]. Apart from one study that suggested decreased baseline PGE2 levels in NERD [33], most studies showed no significant differences in the levels of PGE2 or its metabolites between NERD and ATA groups, indicating that further investigations are needed to evaluate its use as a potential therapeutic target.

The lipid mediators, not the arachidonic acid metabolites, have also been studied as biomarkers of NERD, especially sphingolipid metabolites. Sphingolipid metabolites mediate cell growth, cell differentiation, cell death, and autophagy, and the dysregulation of sphingolipid metabolism could induce airway inflammation and bronchial hyperreactivity [46, 47]. Baseline levels of serum sphingosine-1-phosphate (S1P) and urine sphingosine were significantly increased in patients with NERD, and a significant correlation with a decrease in FEV1 has been observed after aspirin challenge [48]. Sphingolipid metabolites may be possible biomarkers for NERD, although further studies are needed to validate their use.

3.2. Inflammatory Cells and Cytokines. The cellular pathogenic mechanism in NERD involves an intense eosinophilic inflammation, in which Th2 immunity orchestrates the phenotype of eosinophilic asthma (Table 2). Based on these findings, various studies have reported the eosinophil count, eosinophil-related mediators, and Th2 cytokines as biomarkers of NERD. Sputum and blood eosinophil counts are biomarkers for asthma phenotype of airway eosinophilic inflammation [49]. NERD is characterized by phenotypes represented by adult-onset eosinophilic asthma. Furthermore, local eosinophilia has been observed in the nasal polyp tissues or the bronchial lavage fluid of patients with NERD as well as blood eosinophilia [21, 50–52]. Therefore, sputum and blood eosinophil counts are difficult to use as direct diagnostic biomarkers of NERD, but they are important as biomarkers in distinguishing eosinophilic inflammation, one of the pathogenesis of NERD. Sputum and blood eosinophil counts are biomarkers that are also useful in predicting asthma severity and response to therapy [53, 54]. Therefore, sputum and blood eosinophil counts are biomarkers that can be used to evaluate the severity of NERD and the response to therapy. Among all the mediators of Th2 immunity, the biomarker most associated with NERD is periostin. Periostin is an extracellular matrix protein that is known to regulate inflammation/remodeling of the asthmatic airway [55]. Periostin is known to be a surrogate marker of Th2 immunity [56]. In a study on 277 adult asthmatic patients, we showed that serum periostin was a useful biomarker of NERD and that it could be used as an index of blood/sputum eosinophilia and asthma severity [57]. This study showed that it is useful as a biomarker to predict NERD ($p = 0.006$) even after multivariate regression analysis, which is more efficient than predictions of severe asthma phenotype ($p = 0.04$). This suggests that periostin is a potential independent biomarker of NERD diagnosis. Various other Th2 cytokines and chemokines, including IL-4, IL-5, IL-13, IL-33, TSLP, GM-CSF, and eotaxin have been studied, and some studies have shown statistically significant differences in their levels [58, 59]. In addition, cytokines such as IL-6, IL-8, and IFN-r have also been associated with AERD [60, 61], although further studies will be needed to validate their clinical significances.

Platelet activation is associated with leukocytes, which promote the secretion of proinflammatory lipid mediators such as cysLTs in NERD. Platelet activation induces the expression of cell adhesion molecules on the extracellular surface, which bind to the leukocytes through P-selectin (CD62P)–P-selectin glycoprotein ligand 1, GPIIb/IIIa-Mac-1, and CD40 ligand (CD40L)–CD40 [62, 63]. Recent studies have reported an increased percentage of platelet-adherent leukocytes and platelet activation markers such as sP-selectin and sCD40L in the blood of patients with NERD. These phenomena contribute to the overproduction of

Table 3: Potential genetic markers.

	Gene	Polymorphisms	Patients	Ethnic group	Mechanism	Reference
Arachidonic acid metabolism	CYSLTR1	_634 C>T, _475 A>C, _336 A>G	NERD: 105, ATA: 110, NC: 125	Korean	CysLTR1 expression	[74]
	CYSLTR2	_819T > G, 2078C > T, 2534A > G	NERD: 134, ATA: 66, NC: 152	Korean	CysLTR2 expression, LTC4S gene interaction	[75]
	EP2	uS5, uS5b, uS7	NERD: 198, ATA: 282, NC: 274	Japanese	Decrease transcription level of EP2, PGE2 braking	[76]
	PTGER	PTGER2: _616 C>G, _166 G>A PTGER3: _1709 T>A, PTGER4: _1254 A>G	NERD: 108, ATA: 93, NC: 140	Korean	PGE2, TXA2 receptor polymorphism	[77]
	TBXA2R	−4684C>, 795T>C				
	PTGER	PTGER3: rs7543182, rs959	NERD: 243, ATA: 918	Korean	PGE2 receptor polymorphism	[78]
Eosinophil-associated gene	CRTH2	_446T > C	NERD: 107, ATA: 115, NC: 133	Korean	Decrease CRTH2 expression and increase eotaxin-2 production	[79]
	CCR3	_520T > C	NERD: 94, ATA: 152	Korean	Higher mRNA expression of CCR3	[80]
HLA	HLA-DPB1	DPB1*0301	59 NERD, 57 ATA, 48 NC	Polish		[66]
	HLA-DPB1	DPB1*0301	76 NERD, 73 ATA, 91 NC	Korean		[68]
	HLA-DPB1	rs3128965	264 NERD, 387 ATA, 238 NC	Korean		[70]
	HLA-DPB1	rs1042151	117 NERD, 685 ATA	Korean		[69]

NERD: NSAID-exacerbated respiratory disease; ATA: aspirin-tolerant asthma; CysLTR: cysteinyl leukotriene receptor, LT: leukotriene; PG: prostaglandin; TX: thromboxane; CRTH: chemoattractant receptor homologue expressed by type 2 helper T cells; CCR: chemokine receptor; HLA: human leukocyte antigen; DPP: dipeptidyl peptidase.

cysLTs [64, 65]. Thus, activated platelet surface markers are possible biomarkers for NERD, although further studies are needed to validate their use.

3.3. Genetic Markers. Various genetic polymorphisms have been reported through genetic association studies of targeted genes (lipid mediators and inflammatory responses) associated with the pathogenesis of NERD. In addition, a number of genome-wide association studies (GWAS) and epigenetic studies have reported potential genetic markers that distinguish NERD from ATA (Table 3).

First, the prevalence of HLA-DPB1*0301 was significantly higher in patients with NERD in a Polish population; the same results were obtained in a study on a Korean population as well [66–68]. The patients carrying this marker had higher prevalence of CRS/nasal polyps than those who had no marker. Furthermore, GWASs demonstrated significant association of two SNPs of HLA-DPB1 (rs1042151 and rs3128965) and susceptibility to NERD [69, 70], suggesting HLA-DPB1 may be a strong genetic marker for predicting the NERD phenotype.

Genetic polymorphisms related to arachidonic acid metabolism and their receptor have been reported in candidate gene association studies. Leukotriene C4 synthase (LTC4S) is an important enzyme involved in the production of cysLTs. The gene that encodes LTC4S has been extensively studied for variations; however, it has been found to vary widely, depending on ethnic groups. In the study in the Polish population, which was the first study, it was possible to distinguish between NERD and ATA [71]. However, a recent meta-analysis did not show any significant results in NERD; the only significant results were obtained in the ATA and Caucasian subgroups [72]. In a Korean study, ALOX5 (5-LO enzyme gene) ht1 [G-C-G-A] was found to be significantly higher in NERD than in ATA in the 4SNP (−1708G>A, 21C>T, 270G>A, and 1728G>A) [73]. Two groups of receptors, cysLT receptors (CysLTR1, CysLTR2) and PGE2 (EP1, EP2, EP3, and EP4) receptor polymorphisms, have been demonstrated differences in polymorphism between NERD and ATA [74–78].

Several genetic markers associated with eosinophil activation have been reported. CRTH2 in response to PGD2, and CCR3 in response to eotaxin and RANTES, could induce eosinophil activation and recruitment. Polymorphisms in CRTH2 (−466T>C) and CCR3 (−520T>C) were associated with NERD [79, 80] and higher levels of eotaxin 2, indicating that the two SNPs of CRTH2 and CCR may be potential genetic markers that represent eosinophil activation in the upper and lower airway inflammation in NERD. Mast cells were also found with genetic polymorphisms that distinguish

between NERD and ATA. The genotype frequency of *FCER1G*-237A>G was significantly different in patients with NERD, and patients with ATA and *FCERIA*-344C>T and FcεR1β−109T>C polymorphisms were associated with staphylococcal enterotoxin-specific IgE antibodies [81, 82].

So far, most genetic markers have been reported based on pathogenesis only. No genetic markers have been identified repeatedly in different patient groups, except HLA-DPB1. Further studies on diverse populations are required.

4. NERD Subtypes and Their Biomarkers

Although NERD is an endotype of asthma, it has been found in diverse phenotypes. Not all patients with NERD exhibit CRS and nasal polyps. The severity and response to treatment varies among patients. Recently, two studies of clustering in NERD cohorts were reported. The first study clustered 201 patients with NERD using a latent class analysis including clinical data from questionnaires, spirometry, atopy traits, blood eosinophilia, and urinary LTE4 concentrations as observable variables and found 4 classes [51]: class 1 patients showed moderate asthma course; class 2 showed mild asthma course; and class 3 and 4 patients showed severe asthma course. Blood eosinophilia and high urinary LTE4 were shown to be biomarkers that helped in class differentiation, especially for class 1. We performed a two-step cluster analysis using 3 clinical criteria: atopy, CRS, and urticaria to identify phenotypic clusters. We found 4 subtypes: subtype 1 (NERD with CRS/atopy and no urticaria), subtype 2 (NERD with CRS and no urticaria/atopy), subtype 3 (NERD without CRS/urticaria), and subtype 4 (NERD with urticaria). Subtypes 1 and 2 showed more severe clinical courses with higher blood/sputum eosinophilia and frequent asthma exacerbation requiring systemic steroid burst [83]. Higher levels of urinary LTE4 were observed in subtypes 1 and 3. These findings suggested that the level of LTE4 was not only a strong biomarker of NERD; it could also be applied to specific subtypes and endotypes of NERD. Classifying NERD into subtypes using biomarkers such as urinary LTE4 will help in better management of NERD.

5. Conclusion

The most useful biomarker of NERD is urinary LTE4. Urinary LTE4 level can be used for distinguishing the phenotype (including subtypes) and for predicting the response to desensitization and prognosis. Sputum/blood eosinophil counts are also biomarkers that can be used to identify the endotype of NERD and to monitor the course of treatment. Serum periostin levels and HLA-DPB1 (*0301 and genetic polymorphisms) are suggested as useful biomarkers for predicting NERD phenotypes.

Conflicts of Interest

The authors have no competing interests.

Acknowledgments

This research was supported by a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI) and funded by the Ministry of Health & Welfare, Republic of Korea (Grant no. HI16C0992).

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Hindawi
Mediators of Inflammation
Volume 2017, Article ID 6249685, 10 pages
https://doi.org/10.1155/2017/6249685

Research Article

MBD2 Regulates Th17 Cell Differentiation and Experimental Severe Asthma by Affecting IRF4 Expression

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Received 17 April 2017; Accepted 31 May 2017; Published 20 July 2017

Academic Editor: Younghyo Kim

Th17 cells and IL-17 participate in airway neutrophil infiltration characteristics in the pathogenesis of severe asthma. Methyl-CpG binding domain protein 2 (MBD2) expression increased in $CD4^+$ T cells in peripheral blood samples of asthma patients. However, little is known about that epigenetic regulation of MBD2 in both immunological pathogenesis of experimental severe asthma and $CD4^+$ T cell differentiation. Here, we established a neutrophil-predominant severe asthma model, which was characterized by airway hyperresponsiveness (AHR), BALF neutrophil granulocyte (NEU) increase, higher NEU and IL-17 protein levels, and more Th17 cell differentiation. In the model, MBD2 and IRF4 protein expression increased in the lung and spleen cells. Under overexpression or silencing of the MBD2 and IRF4 gene, the differentiation of Th17 cells and IL-17 secretion showed positive changes. IRF4 protein expression showed a positive change with overexpression or silencing of the MBD2 gene, whereas there was no significant difference in the expression of MBD2 under overexpression or silencing of the IRF4 gene. These data provide novel insights into epigenetic regulation of severe asthma.

1. Introduction

Asthma is a complex chronic inflammatory disease characterized by AHR, reversible airflow obstruction, and airway inflammation [1]. IL-17 (also known as IL-17A) is a representative cytokine produced by Th17 cells, which can be induced by the release of IL-8, CXC, and other neutrophil chemokines to raise and activate neutrophils [2–4], and animal and clinical analysis of samples have proved that Th17 cells and IL-17 play important roles in the pathogenesis of asthma. These asthma cases are characterized by predominantly neutrophilic and mixed granulocytic types and are associated with severe asthma and poor response to corticosteroids [5–9]. It is therefore very important to establish a

NEU predominant inflammatory phenotype asthma model, and we tried using 100 μg of HDM and OVA combined with 15 μg LPS to establish a neutrophil-predominant severe asthma model and to further study the relationship between the changes of Th17 cells and the epigenetic alterations.

MBD2 (methyl-CpG binding domain protein 2) can specifically bind to the promoter region of a target gene and change in the posttranscriptional modification of histone through the recruitment of other molecules, thus changing the chromatin structure and regulating the expression of target genes. In our preliminary study, we found that the expression of MBD2 increased obviously after accepting stimulus differentiation in splenic $CD4^+$ T cell in mice. Through the analysis of peripheral blood samples of asthma patients, we also found that MBD2 expression increased in $CD4^+$ T cells in peripheral blood compared with healthy people, meaning that MBD2 has a close relationship with both the immunological pathogenesis of asthma and $CD4^+$ T cell differentiation. Therefore, MBD2 could play an important role in neutrophil-predominant severe asthma, and to find which cytokine it impacts is our next research goal.

IRF4 (interferon regulatory factor 4) is strictly expressed in immune cells. In IRF4 research on the role of Th17 cell differentiation, Brüstle found that mice knockout IRF4 failed to develop experimental autoimmune encephalomyelitis, T cell and RORgamma T were expressed lower, and Th17 cell differentiation was impaired [10]. So, IRF4 is therefore an important factor in neutrophil-predominant severe asthma.

At present, there is little research on the role of MBD2 in severe asthma, and the relationship between MBD2 and IRF4 in severe asthma is not known. Here, through the above data review, we hypothesize that MBD2 would regulate Th17 cell differentiation and experimental severe asthma by affecting IRF4.

2. Materials and Methods

2.1. Ethics Statement. All studies were performed in compliance with the Second Xiangya Hospital and Central South University Animal Care and Use Committee guidelines.

2.2. Mice. Female C57BL/6 mice (provided by animal center of the Second Xiangya hospital of Central South University, China), aged 6-7 weeks, weighing 18–20 grams, were used in the experiments. All mice were bred and housed in a SPF facility with a 12/12 h light/dark cycle.

2.3. Experimental Reagents and Equipment. The HDM were supplied by GREER; the LPS, aluminum hydroxide gel, and OVA were supplied by Sigma; small animal cough- and asthma-inducing instrument (YLS-8A) was purchased from Beijing Zhongshidichuang Science and Technology Development Co. Ltd.; mice spirometer (MAX 1320) was purchased from Buxco®, USA; low-speed centrifuge (5810 R) was purchased from Eppendorf®, Germany; optical microscope (DMI3000B) was from Olympus, Japan (Leica®); and pathological image analyzer (Leica Application Suite V4) was purchased from Leica, Germany.

2.4. Mice Asthma Model. Female C57BL/6 mice were grouped according to a random number table, with six mice in each group. The group of severe asthma model mice were given an intraperitoneal sensitization injection with 100 μg HDM + 100 μg OVA+ 15 μg LPS + 2 mg aluminum hydroxide on days 0, 1, and 2 [11] and then challenged with OVA solution atomized for 30 minutes before HDM intranasal excitation on days 14, 15, 18, and 19. For the saline group, mice received saline only. The group of conventional asthma model mice were given a sensitization intraperitoneal injection with 25 μg OVA + 1 mg aluminum hydroxide on days 0 and 7 and then challenged with OVA solution atomized excitation for 30 minutes on days 14, 15, 16, 17, 18, 19, and 20 [12]. Mice were sacrificed on day 21.

2.5. Ethology Observed. Mice ethology was observed everyday as follows: fur luster, nose and ear scratching, irritability, sneezing, rapid breathing, and incontinence.

2.6. Airway AHR. Methacholine- (Mch-) induced airway resistance was measured on day 21 (24 h after the final challenge) by direct plethysmography (Buxco Electronics, RC System, USA). The procedures were the same as previously described [13]. Mice were anesthetized, tracheotomized, and then intubed. At first, the baseline of lung resistance (RL0) was recorded for one minute. Then, mice were given 10 μl saline and 10 μl Mch with increasing doses of 0.39 mg/ml (dose 1), 0.78 mg/ml (dose 2), 1.56 mg/ml (dose 3), and 3.12 mg/ml (dose 4) atomized to stimulate the airway, and the changes of lung resistance (RLX) were then recorded. The ratios of RLX/RL0 were used as the final results for analysis.

2.7. BALF Cell Count. BALF collection procedures were the same as previously described [13]. After eliminating red blood cells, by centrifugation and precipitation, total BALF cell counts were determined by a haemocytometer. Then, cell slices were made using a Biping settlement system. BALF NEU and EOS cell counts were determined in 200 total BALF cells after cell slices underwent H&E staining.

2.8. Histopathological Analysis of Inflammatory and Structural Changes. The right lungs were incubated in 4% paraformaldehyde for 24 h and level dehydration: 70% ethanol (5 min), 75% ethanol (5 min), 80% ethanol (5 min), 90% ethanol (5 min), 95% ethanol (5 min), 100% ethanol I (10 min), 100% ethanol II (10 min), xylene liquid I (10 min), and xylene liquid II (10 min) and then embedded in paraffin.

2.8.1. Lung Tissue Inflammation Score. Lung tissues were stained with H&E, and 3~4 H&E stained sections were chosen per group under a single-blind procedure. Two pathologists assessed the lung tissue inflammation score under a microscope as previously described [14]: a score of 0 represented no inflammatory cell infiltration; a score of 1 represented little inflammatory cell infiltration; a score of 2 represented 1 layer of inflammatory cells around the airway; a score of 3 represented 2~4 layers of inflammatory cells around the airway; and a score of 4 represented 4 or more layers of inflammatory cells around the airway.

2.8.2. Immunohistochemistry for NEU, EOS, IL-17A, IL-4, MBD2, and IRF4. Lung tissues were stained for immunohistochemistry (neutrophil-specific antibody (anti-Gr1, Biolegend), eosinophil antibody (anti-ECP, Biorbyt), IL-17A antibody (Proteintech), IL-4 antibody (ABBIOTEC), MBD2 antibody (Abcam), and IRF4 antibody (Proteintech)), and 2~4 H&E stained sections per group were chosen under single-blind conditions to detect and localize NEU, EOS, IL-17A, IL-4, MBD2, and IRF4 protein expression. Using a microscope and computer image processing system (Image-Pro Plus 6.0), two pathologists assessed the NEU, EOS, IL-17A, IL-4, MBD2, and IRF4 scores.

2.9. Western Blotting. Total proteins were prepared using RIPA lysis buffer supplemented with protease inhibitors. Western blotting was carried out by probing the membranes with indicated primary antibodies followed by incubating with an HRP-conjugated secondary antibody. The NEU, EOS, IL-17A, IL-4, MBD2, and IRF4 antibodies were the same as above.

2.10. Bronchial Lung Tissue Suspension Cells. Mice bronchial lungs were collected, washed once with 5x antibiotic, and washed 2 times with PBS. Then, the lungs were cut as little as possible, and subsequently, digestive juice consisting of collagenase1 (0.5 mg/ml Sigma, unit/ml) + 10 μg/ml DNase in RPMI medium was added and kept for 1 hour at 37°C in a water bath shaking incubator. After the digestion of the lung tissue and straining with a 70 μm filter Cell Strainer (BD Falcon), cells were centrifugated and resuspended in 10% FBS culture medium.

2.11. T Cell Purification, Activation, and Staining. Spleen $CD4^+$ T cells of model mice were selected by using microbead sorting (130-049-201, Miltenyi Biotec, Germany) and were then seeded in 12-well flat bottom plates. In the next 5 hours, the cells were restimulated with 50 ng/ml phorbol-12-myristate-13-acetate (PMA) (Multi Sciences Company, China), 1 μg/ml ionomycin (Multi Sciences Company, China), and 3 μg/ml monensin (Multi Sciences Company, China). The lung cells were then stained for surface marker FITC-antiCD4 cytokine antibody (BioLend, USA) followed by fixation and permeabilization with fixation and permeabilization buffer (Multi Sciences Company, China) 15 min. After washing with permeabilization buffer, the lung and spleen cells were stained with intracellular markers APC-anti-IL-17 and PE-anti-IL-4 cytokine antibodies (BioLend, USA) in the permeabilization buffer for 20 min. Flow cytometry was conducted and the data were analyzed using the FACSCalibur and FlowJo version X software.

2.12. Th17 Cell Differentiation. For directed differentiation of Th17 cells, 1×10^6 $CD4^+$ T cells from C57BL/6 mice were activated with 2 μg/ml anti-CD3, 2 μg/ml anti-CD28 for 3 days in the presence of 5 ng/ml TGF-beta, 20 ng/ml IL-6 and 20 ng/ml IL-23. For neutralization of IL-4 and IFN-gamma, 10 μg/ml anti-IL-4 and 10 μg/ml anti-INF-gamma were added into the cultures [15, 16]. All cytokines were purchased from BioLend Co. (BioLend, USA).

2.13. Lentiviral Transduction of Th17 Cells. Splenic $CD4^+$ Th17 lymphocytes were transfected with chemosynthesis MBD2 siRNA sequence (S) 5′-GTTTGGCTTAACACA TCTCAA-3′ and IRF4 siRNA sequence (S) 5′-GCCA GACAACTGTATTACTTT-3′. The Th17 cells were cultured in 1640 medium without serum. The cells were resuspended according to 1.5×10^6/tube and MOI = 20, and the appropriate amount of virus was used for transfection. The cells were seeded in 12-well flat-bottom plates for 72 h. In the last 5 h, the cells were restimulated with PMA, ionomycin, and monensin as above.

3. Results

3.1. The Severe Asthma Mice Model Was Established. We first observed sleepiness in severe asthma mice on the 4th day of stimulation, compared to the 5th day for conventional asthma mice. When Mch was inhaled, the pulmonary resistance (RL) of the severe group showed a sharp rise (Figure 1(a)). Through BALF of the three groups, we found that total cell counts and neutrophil granulocyte counts of the severe group were the highest, but the eosinophil granulocyte counts of the conventional group were the most (Figure 1(b)). Histological analyses of lungs from the severe group exhibited markedly enhanced peribronchial inflammation with infiltrated neutrophils, but eosinophils were not obviously different compared with the conventional group (Figure 1(c)). Results of Western blot analyses were the same as those of histological analyses (Figure 1(d)).

3.2. The Severe Asthma Mice Was Mediated by Th17 Cells. IL-17 is the main representative cell factor of Th17 cells, as is IL-4 for Th2 cells. Histological analyses of lungs from the severe group exhibited markedly enhanced cells with stained IL-17, but cells with stained IL-4 were not obviously different compared with the conventional group (Figure 2(a)). Results of Western blot analyses were the same as histological analyses (Figure 2(b)). Th17 cells were the most in bronchial lung tissue suspension cells and splenocytes from the severe group compared with the conventional group (Figure 2(c)).

3.3. Expression of MBD2 in Severe Asthma Mice. Histological analyses of lungs from the severe group exhibited markedly enhanced cells with stained MBD2 compared with the conventional group (Figure 3(a)). MBD2 expression in lungs and splenic $CD4^+$T cells from the severe group were significantly increased compared with the conventional group (Figures 3(b) and 3(c)).

3.4. IL-17 Expression and Th17 Cell Differentiation under MBD2 Gene Silencing or Overexpression. We conducted Western blot analyses and demonstrated either MBD2 gene silencing (M(−)) or overexpression (M(+)) in splenic $CD4^+$T cells successfully. With MBD2 gene silencing, IL-17 expression was significantly lower than that of the empty transfection group (M(0)); and when the MBD2 gene was overexpressed, IL-17 expression was markedly increased compared to that of the empty transfection group (Figure 4(a)). Th17 cells were obviously decreased or increased under MBD2 gene silencing or overexpression (Figure 4(b)).

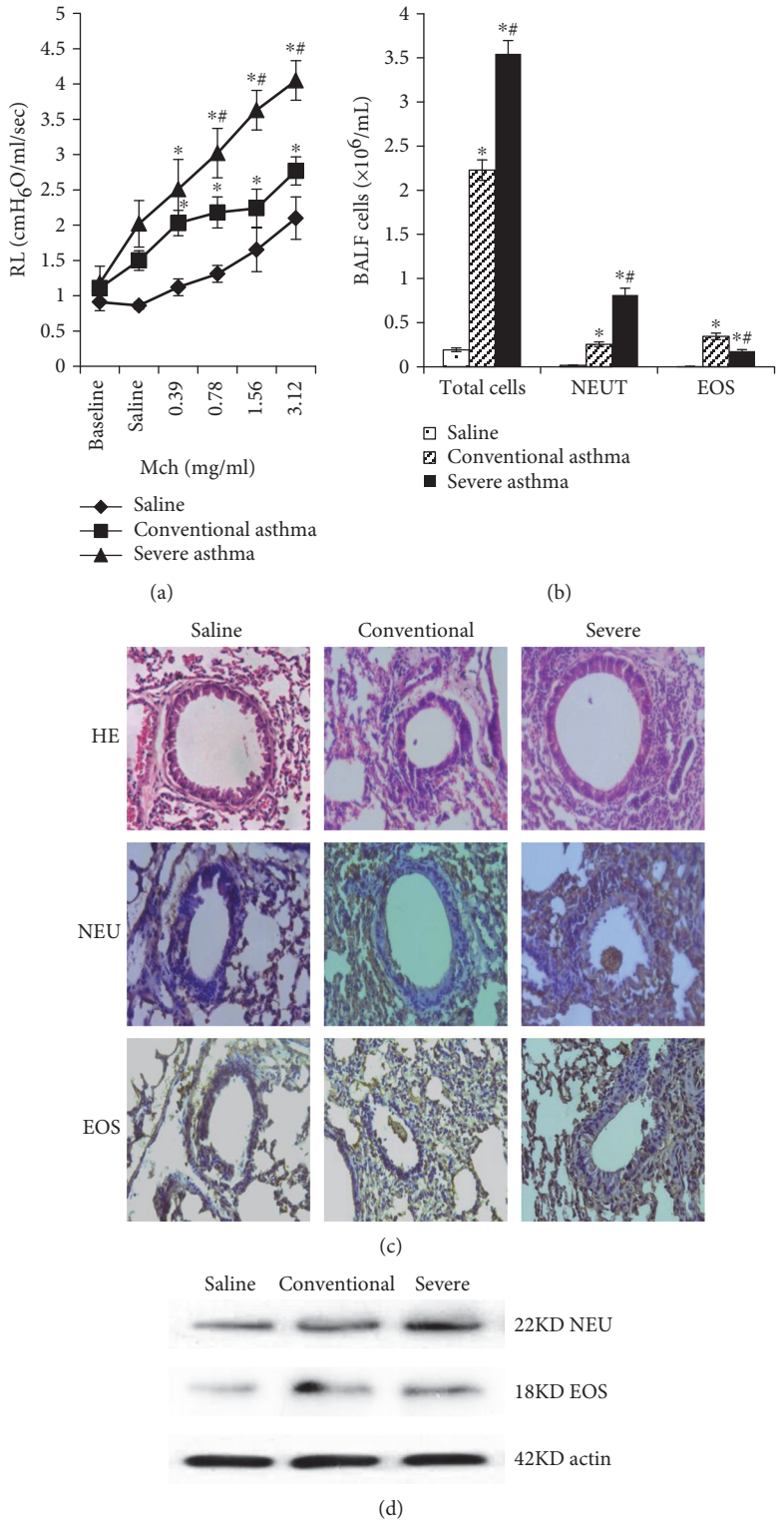

Figure 1: The severe asthma mice model was established. (a) Pulmonary resistance in the saline, conventional, and severe groups. When Mch was inhaled, the RL of the conventional and severe groups showed a rise, and the RL values of the severe group were higher than those of the conventional group. (b) The total, NEU, and EOS cells of BALF from the three groups. The total and NEU cells of BALF from the severe group were higher than those from the conventional group, but EOS cells showed an opposite result. (c) Lung tissues were stained with H&E, immunohistochemistry (neutrophil-specific antibody (anti-Gr1), eosinophil antibody (anti-ECP)) of the three groups. Histological analyses of lungs from the severe group exhibited markedly enhanced peribronchial inflammation with infiltrated neutrophils, but eosinophils were not obviously different compared with the conventional group. (d) Western blot analysis detected NEU and EOS protein expression in the three groups. The NEU protein expression of the lungs from the severe group were higher than those from the conventional group, whereas EOS protein expression values were not obviously different between them. $^{*}p < 0.05$ as compared with the control group. $^{\#}p < 0.05$ as compared with the conventional group.

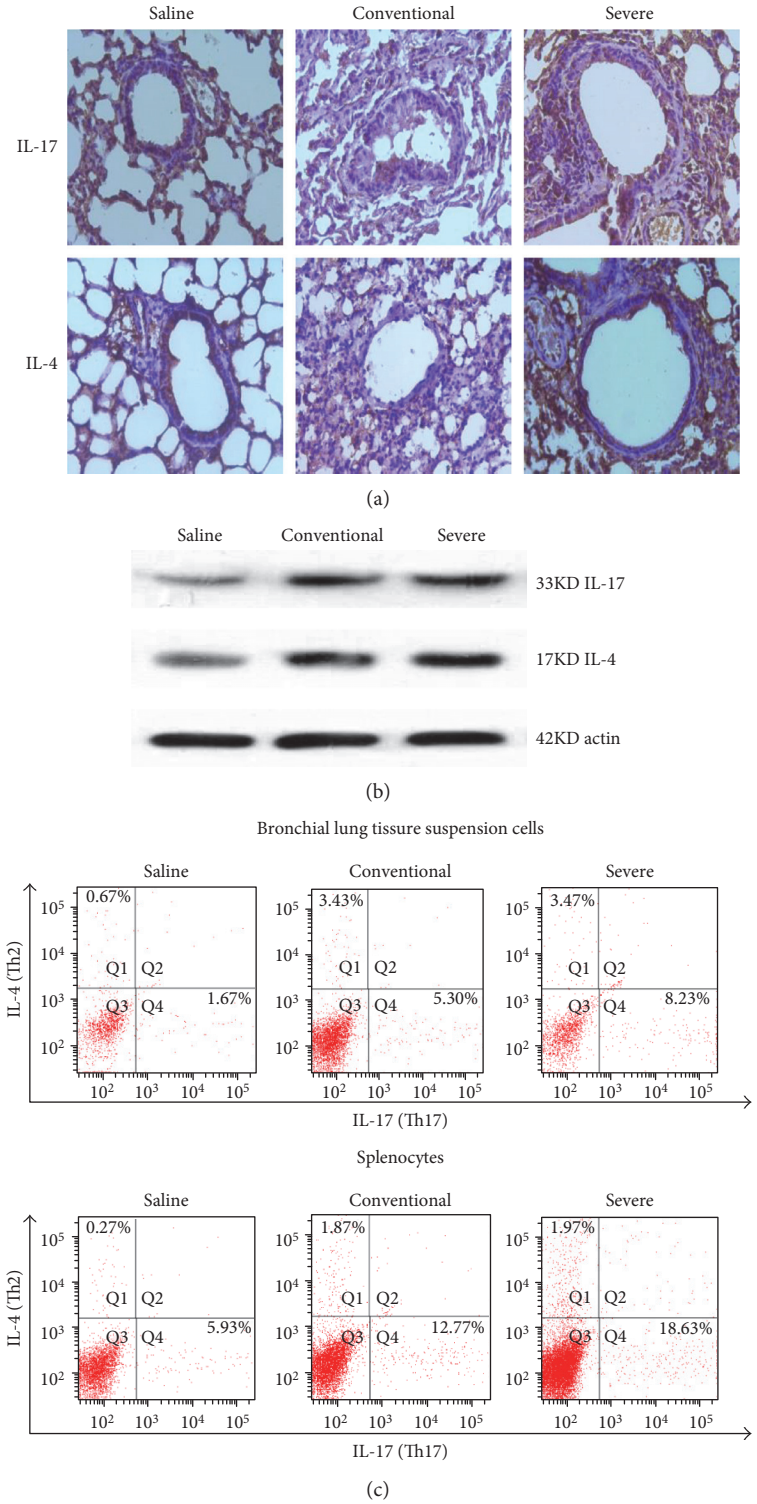

FIGURE 2: The severe asthma mice were mediated by Th17 cells. (a) Lung tissues were stained for immunohistochemistry (anti-IL-17, anti-IL-4) of the three groups. Histological analyses of lungs from the severe group exhibited markedly enhanced cells with stained IL-17 positively, but cells with stained IL-4 positively were not obviously different compared with those from the conventional group. (b) Western blot analysis detected IL-17 and IL-4 protein expression in the three groups. The IL-17 protein expression of the lungs from the severe group was higher than that from the conventional group, whereas IL-4 protein expression values were not obviously different between them. (c) Th17 and Th2 cells were tested in bronchial lung tissue suspension cells and splenocytes, and the cells were then subjected to intracellular staining of APC-anti-IL-17 and PE-anti-IL-4 by flow cytometry analyses. Th17 cells were the most in bronchial lung tissue suspension cells and splenocytes from the severe group compared with the conventional group, whereas Th2 cells were not obviously different between them.

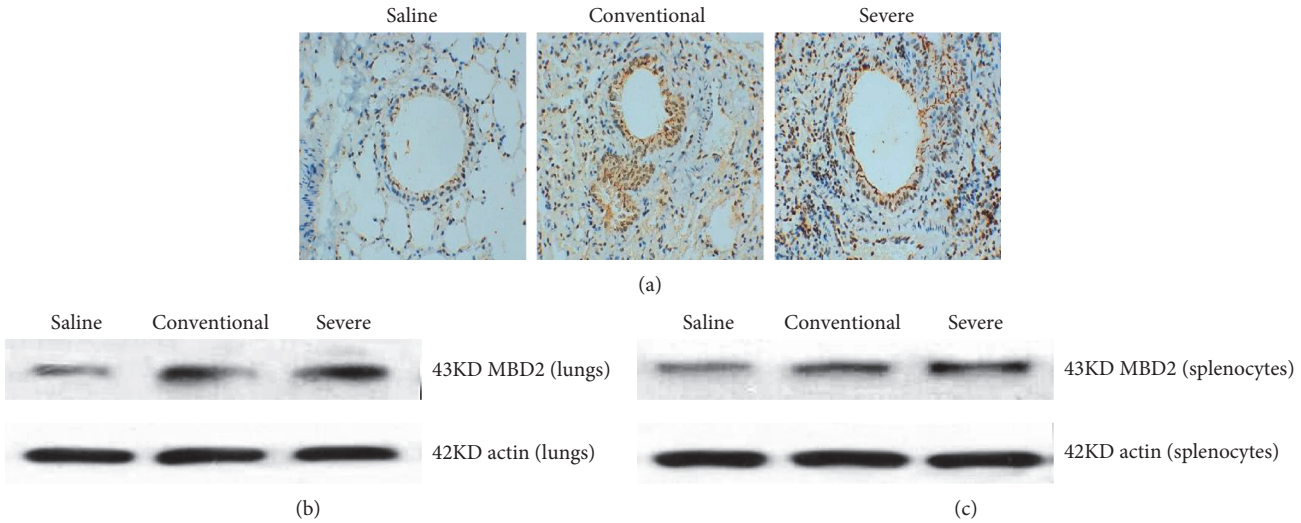

Figure 3: Expression of MBD2 in three groups. (a) Lung tissues were stained for immunohistochemistry (anti-MBD2) of the three groups. Histological analyses of lungs from the severe group exhibited more cells with stained MBD2 positively than those from the conventional group. (b) Western blot analyses detected MBD2 protein expression in the lungs of the three groups. The MBD2 protein expression of the lungs from the severe group was higher than that from the conventional group. (c) Western blot analyses detected MBD2 protein expression in the splenocytes of the three groups. The MBD2 protein expression of the lungs from the severe group was higher than that from the conventional group.

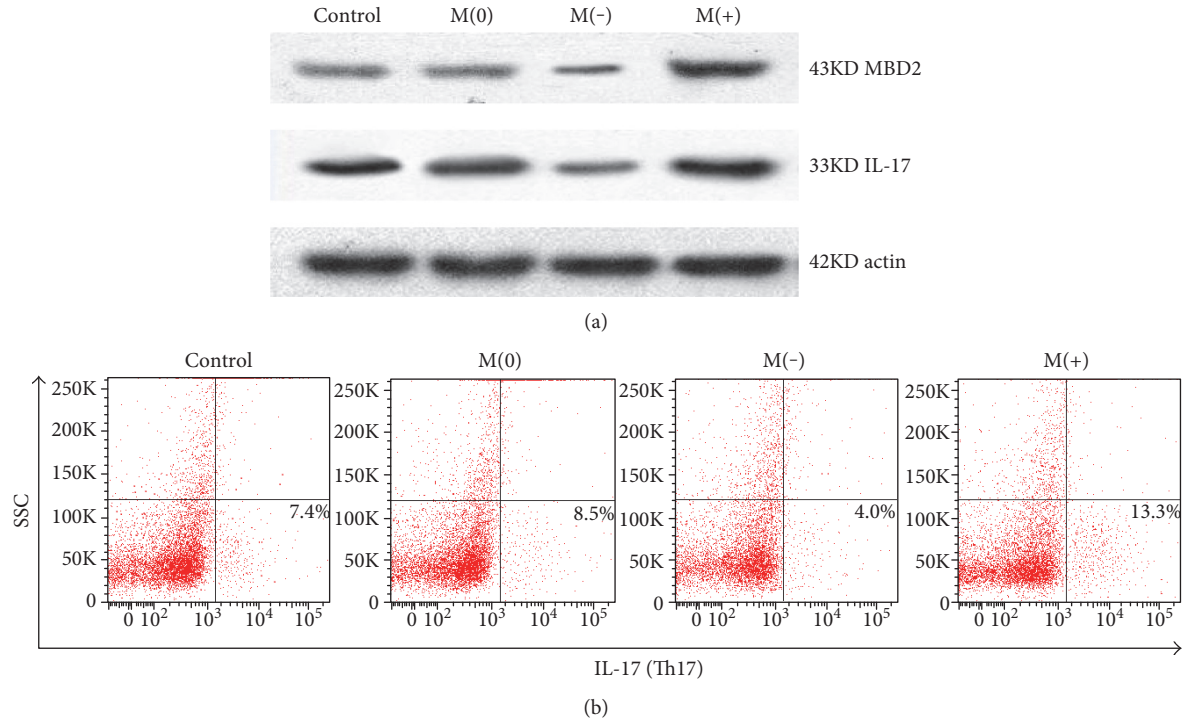

Figure 4: IL-17 expression and Th17 cell differentiation under MBD2 gene silencing or overexpression. (a) Under MBD2 gene silencing (M(−)), IL-17 and MBD2 protein expression was significantly lower than that of the empty transfection group (M(0)) by Western blot analyses; with MBD2 gene overexpression (M(+)), IL-17 and MBD2 protein expression was markedly increased than that of M(0). (b) Under MBD2 gene silencing (M(−)), Th17 cell differentiation was significantly lower than that of M(0) according to flow cytometry analyses; with MBD2 gene overexpression (M(+)), Th17 cell differentiation was markedly increased compared to that of M(0).

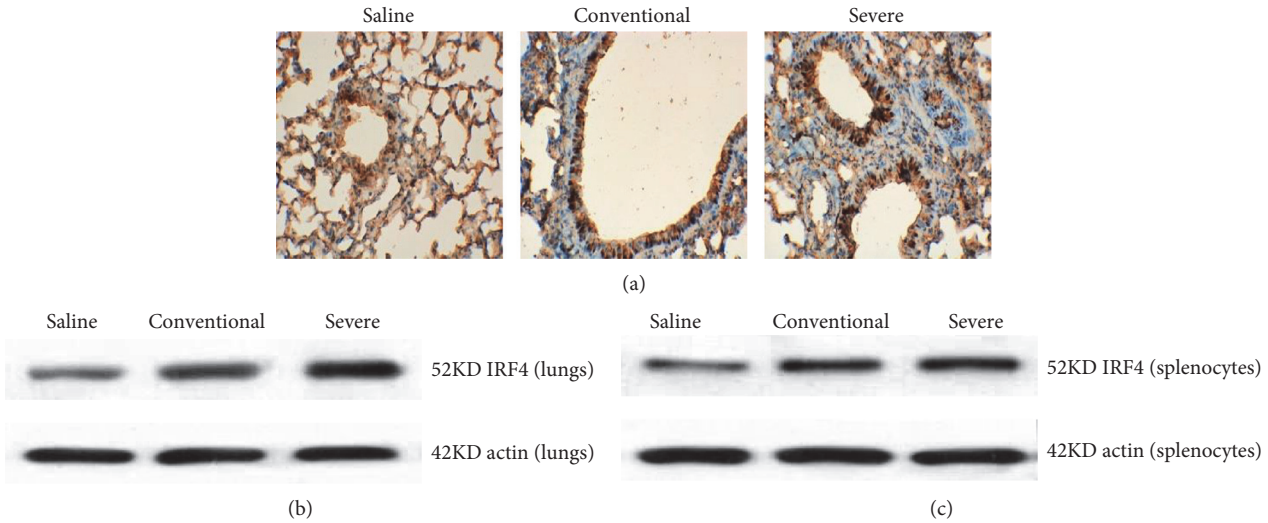

Figure 5: Expression of IRF4 in three groups. (a) Lung tissues were stained for immunohistochemistry (anti-IRF4) of the three groups. Histological analyses of lungs from the severe and conventional groups exhibited markedly enhanced cells with stained IRF4 compared with those of the saline group. (b) Western blot analyses detected IRF4 protein expression in the lungs of the three groups. The IRF4 protein expression from the severe and conventional groups were higher than that from the saline group. (c) Western blot analyses detected IRF4 protein expression in the splenocytes of the three groups. The IRF4 protein expression from the severe and conventional groups were higher than that from the saline group.

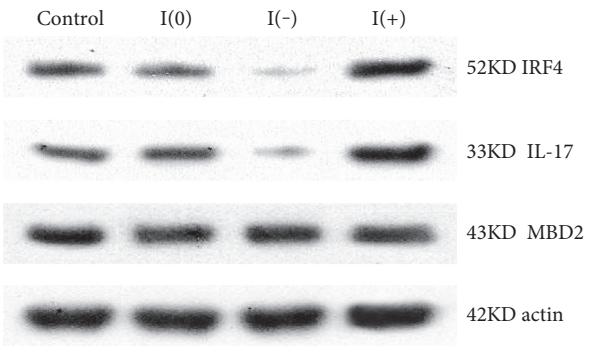

Figure 6: IL-17 and MBD2 expression under IRF4 gene silencing (I(−)) or overexpression (I(+)). Under I(−), IL-17 and IRF4 protein expression were significantly lower than that of the empty transfection group (I(0)) according to Western blot analyses; under I(+), IL-17 and IRF4 protein expression were markedly increased compared to that of I(0). Under I(−) or I(+), MBD2 protein expression showed no significant difference.

3.5. Expression of IRF4 in Severe Asthma Mice. Histological analyses of the lungs from the severe and conventional groups exhibited markedly enhanced cells with stained IRF4 compared with those of the saline group (Figure 5(a)). IRF4 expression in the lungs and splenic $CD4^+$T cells from the two groups were significantly increased compared with that of the saline group, but there was no significant difference between the two groups (Figures 5(b) and 5(c)).

3.6. IL-17 and MBD2 Expression under IRF4 Gene Silencing or Overexpression. We demonstrated that IRF4 gene silencing (I(−)) or overexpression (I(+)) in splenic $CD4^+$T cells was successful. Under IRF4 gene silencing, IL-17 expression was significantly lower than that of the empty transfection group (I(0)), and under IRF4 gene overexpression (I(+)), IL-17 expression was markedly increased compared to that of the empty transfection group. Under IRF4 gene silencing or overexpression, no significant difference in MBD2 expression was observed (Figure 6).

3.7. IL-17 Expression and Th17 Cell Differentiation under Joint MBD2 and IRF4 Gene Silencing or Overexpression. We found that IL-17 expression was significantly the lowest when both MBD2 and IRF4 underwent joint gene silencing, while IL-17 expression was the highest when both MBD2 and IRF4 underwent joint gene overexpression (Figure 7(a)). Th17 cells were obviously the least or the most while both MBD2 and IRF4 underwent joint gene silencing or overexpression (Figure 7(b)).

4. Discussion

In order to better study the mechanisms of severe asthma, we urgently need to build a neutrophil-predominant severe asthma model. While OVA is a classic asthma model allergen, exposure to LPS is also known to lead to an increased risk of asthma-like symptoms [17] and the onset of asthma exacerbations [18, 19]. Meanwhile, the development of allergic asthma is strongly associated with the exposure to HDM [20, 21]. When OVA-induced asthmatic mice are re-exposed to HDM, the pathomechanism is different from OVA exposure alone [22]. Although there have been lots of similar experiments, there are so little successful severe asthma models. In our previous study, we tried different doses of HDM and OVA combined with 15 μg LPS to establish a neutrophil-predominant severe asthma model and found that 100 μg HDM + 100 μg OVA + 15 μg LPS successfully

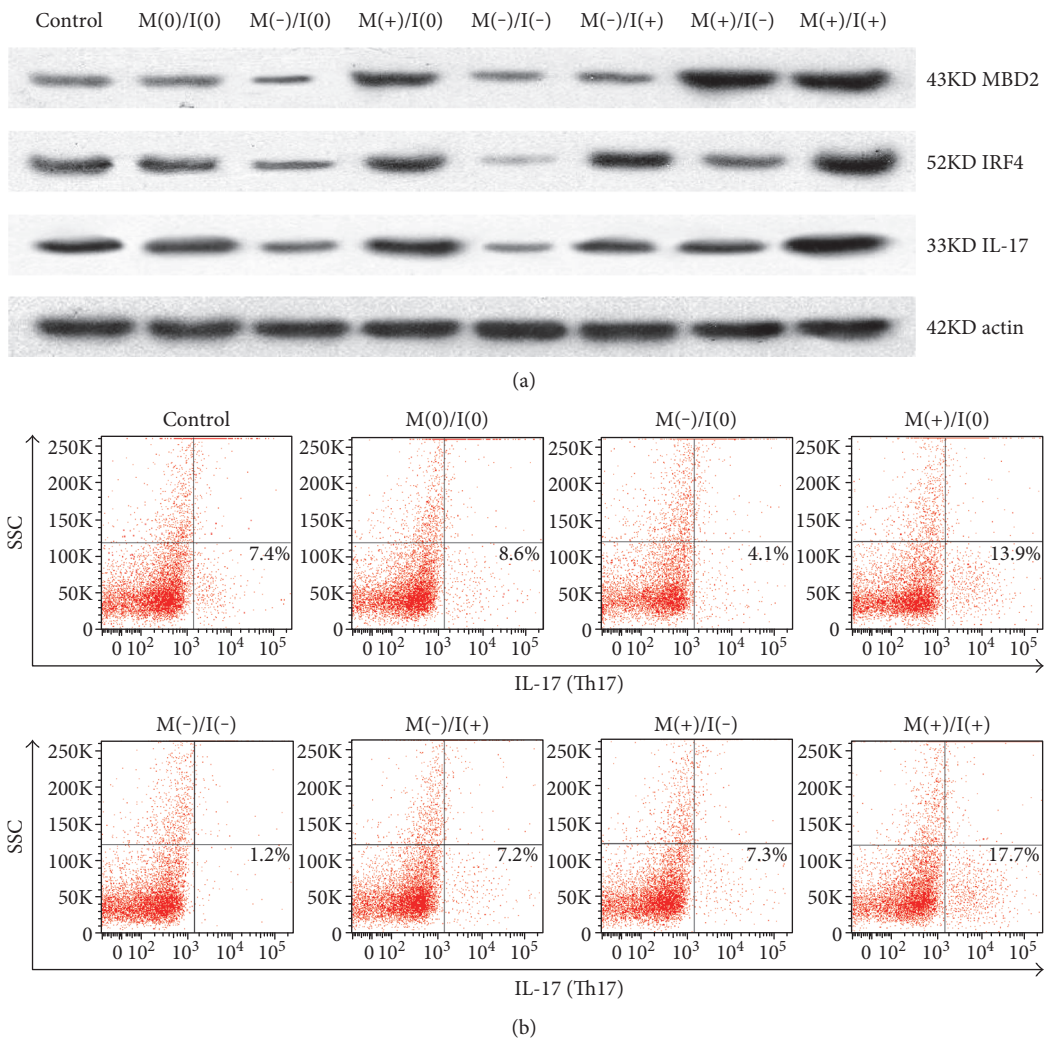

Figure 7: IL-17 expression and Th17 cell differentiation under joint MBD2 and IRF4 gene silencing or overexpression. (a) Under joint M(−)/I(−), IL-17 protein expression was significantly the lowest according to Western blot analyses; under joint M(−)/I(0), IL-17 protein expression was higher than M(−)/I(−). While under joint M(+)/I(+), IL-17 protein expression was significantly the highest; under joint M(+)/I(0), IL-17 protein expression was lower than M(+)/I(+). IL-17 protein expression showed no significant difference between the control group, M(0)/I(0), M(−)/I(+), and M(+)/I(−). (b) Under joint M(−)/I(−), Th17 cell differentiation was significantly the lowest according to flow cytometry analyses; under joint M(−)/I(0), Th17 cell differentiation was higher than M(−)/I(−). Under joint M(+)/I(+), Th17 cell differentiation was significantly the highest; under jointM(+)/I(0), Th17 cell differentiation was lower than M(+)/I(+). No significant difference in Th17 cell differentiation was observed between the control group, M(0)/I(0), M(−)/I(+), and M(+)/I(−).

established a NEU predominant inflammatory phenotype severe asthma model. Here, compared with the conventional asthma mice sensitized by OVA only (named the OVA group), among BALF total cell counts, BALF NEU was significantly increased in the severe asthma mice sensitized by HDM/OVA/LPS, but BALF EOS showed an opposite tendency. Similarly, analysis of lung tissue sections revealed that the HDM/OVA/LPS sensitized group caused a significant increase of both histological inflammation scoring and NEU expression compared to the OVA group, whereas there was no difference in the expression of EOS between the two groups. Compared to moderate asthma, IL-4 expression was decreased [7] and IL-17A expression was increased [23, 24] in severe asthma. In the present study, lung tissue IL-17A expression was significantly increased in the HDM/OVA/LPS sensitized group when compared with the OVA group, whereas there was no difference in the expression of IL-4 between the two groups. The spleen is the classical observation target for immune cells, and Th17 cells were the most in the splenocytes from severe asthma mice compared with conventional asthma mice according to flow cytometry. The lung is one of the effector organs of immunity, and Th17 cells were the most in the lung tissue suspension cells from severe asthma mice compared with conventional asthma mice according to flow cytometry. This showed that neutrophilic predominant severe asthma mice first exhibited mainly Th17 cell differentiation followed by an increase of IL-17 secretion, and the recruitment of NEU, thus promoting the development of asthma, in a way distinctly different from the classic EOS allergic asthma

involving IL-4 recruitment. Therefore, the neutrophil-predominant severe asthma model was established successfully.

MBD2, as an epigenetic regulation element [25], can regulate T cells to differentiate into Th17 cells by methylating T-bet/Hlx [26], so it could be involved in the pathogenesis of severe asthma. Considering that Th17 cell is mainly involved in the inflammatory response, after being induced by oligodendrocytes, EAE mice would develop severe paralysis, but MBD2 null mutant mice were completely protected from EAE induction [26]. In our study, MBD2 showed significantly increased expression in the splenocytes of the severe asthma model mice, representing the classical observation target as immune cells. The lung is one of the effector organs of immunity, and MBD2 showed a higher expression in the lung tissue of severe asthma model mice compared with the conventional asthma model mice. To ascertain whether MBD2 is involved in the pathogenesis of severe asthma, we conducted in vitro splenocyte experiments and found that IL-17 protein expression increased significantly along with overexpression of the MBD2 gene and decreased with the silencing of the MBD2 gene. At the same time, the number of Th17 cells showed a consistent change with the overexpression or silencing of the MBD2 gene. Through these tests, MBD2 is shown to participate in severe asthma by affecting the differentiation of Th17 cells and IL-17 secretion.

Next, we needed to analyze whether MBD2 can affect IRF4 and participate in asthma. IRF4, as an essential controller of Th17 differentiation, participates in many immune diseases [27]. IRF4-deficient mice are totally resistant to the development of a Th17-mediated disease [10]. Overexpressed IRF4 in naive $CD4^+$ T cells derived from relapsing remitting multiple sclerosis patients significantly increased their ability to secrete IL-17A, IL-17F, IL-21, and IL-22 [28]. We found that both IRF4 and IL-17 had a significantly increased expression in the lung and the spleen cells of severe asthma model mice. From the in vitro cell experiment, IL-17 protein expression increased significantly with overexpression of the IRF4 gene and decreased with the silencing of the IRF4 gene. These findings show that IRF4 participates in severe asthma by affecting the differentiation of IL-17 secretion and is consistent with previous studies. Then, from the in vitro splenocyte experiment, we found that IRF4 protein expression increased significantly along with overexpression of the MBD2 gene and decreased with the silencing of the MBD2 gene, whereas there was no significant difference in the expression of MBD2 under overexpression or silencing of the IRF4 gene. This shows that MBD2 can affect IRF4 expression in severe asthma.

Finally, in conditions of joint overexpression of both MBD2 and IRF4 genes, the levels of expression of IRF4, differentiation of Th17 cells, and IL-17 secretion were the highest, while joint silencing of MBD2 and overexpression of IRF4 genes, the levels of expression of IRF4, differentiation of Th17 cells, and IL-17 secretion were declined. Under conditions of joint silencing of both genes, the levels of expression of IRF4 and differentiation of Th17 cells and IL-17 secretion were the lowest, while joint overexpression of MBD2 and silencing of IRF4 genes, the levels of expression of IRF4, and differentiation of Th17 cells and IL-17 secretion were increased. This indicates that MBD2 can participate in the differentiation of Th17 cells and IL-17 secretion through IRF4 expression and so participate in the pathogenesis of the neutrophil-predominant severe asthma model.

Abbreviations

MBD2: Methyl-CpG binding domain protein 2
BALF: Bronchoalveolar lavage fluid
AHR: Airway hyperresponsiveness
IRF4: Interferon regulatory factor 4
HDM: House dust mite
OVA: Ovalbumin
LPS: Lipopolysaccharide
NEU: Neutrophil granulocyte
EOS: Eosinophilic granulocyte
RL: Pulmonary resistance.

Conflicts of Interest

The authors report no conflicts of interest.

Authors' Contributions

Yueling Wang and Aijun Jia contributed equally to this work.

Acknowledgments

The authors thank Professor Cong-Yi Wang, Center for Biomedical Research, Tongji Hospital, Huazhong University of Science and Technology, China, for the data analysis and guiding the manuscript writing. This study was supported by the National Natural Science Foundation of China (81370128) and the Natural Science Foundation of Hunan Province (14JJ2028).

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Hindawi
Mediators of Inflammation
Volume 2017, Article ID 8123281, 9 pages
https://doi.org/10.1155/2017/8123281

Research Article

Chemical Chaperone of Endoplasmic Reticulum Stress Inhibits Epithelial-Mesenchymal Transition Induced by TGF-β1 in Airway Epithelium via the c-Src Pathway

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Received 25 April 2017; Accepted 27 June 2017; Published 19 July 2017

Academic Editor: Younghyo Kim

Epithelial-mesenchymal transition (EMT) is a biological process that allows epithelial cells to assume a mesenchymal cell phenotype. EMT is considered as a therapeutic target for several persistent inflammatory airway diseases related to tissue remodeling. Herein, we investigated the role of endoplasmic reticulum (ER) stress and c-Src in TGF-β1-induced EMT. A549 cells, primary nasal epithelial cells (PNECs), and inferior nasal turbinate organ cultures were exposed to 4-phenylbutylic acid (4PBA) or PP2 and then stimulated with TGF-β1. We found that E-cadherin, vimentin, fibronectin, and α-SMA expression was increased in nasal polyps compared to inferior turbinates. TGF-β1 increased the expression of EMT markers such as E-cadherin, fibronectin, vimentin, and α-SMA and ER stress markers (XBP-1s and GRP78), an effect that was blocked by PBA or PP2 treatment. 4-PBA and PP2 also blocked the effect of TGF-β1 on migration of A549 cells and suppressed TGF-β1-induced expression of EMT markers in PNECs and organ cultures of inferior turbinate. In conclusion, we demonstrated that 4PBA inhibits TGF-β1-induced EMT via the c-Src pathway in A549 cells, PNECs, and inferior turbinate organ cultures. These results suggest an important role for ER stress and a diverse role for TGF-β1 in upper airway chronic inflammatory disease such as CRS.

1. Introduction

Epithelial-mesenchymal transition (EMT) is a biological process that transforms a polarized epithelial cell into a mesenchymal cell phenotype. EMT is characterized by enhanced mobility and invasiveness and increased the production of extracellular matrix (ECM) components. EMT makes epithelial cells lose characteristics such as cell-to-cell adhesion and apical-basal polarity [1]. EMT contributes to the formation of tissues and organs during development and to wound healing by altering cell-ECM interactions, cytoskeletal organization, and cellular metabolism in a well-coordinated manner [2, 3]. However, dysregulated EMT can occur during chronic inflammation, leading to the disruption of epithelial integrity, disorganization of epithelial tissue, and production of malfunctioning mesenchymal cells and causing pathological remodeling and cancer progression [4–6]. Recently, there has been an emerging opinion that the remodeling and EMT observed in chronic rhinosinusitis (CRS) are the cause of disease recalcitrance [7, 8].

The endoplasmic reticulum (ER) is an organelle where secretory and membrane proteins are assembled into their secondary and tertiary structures. Several pathological conditions such as hypoxia, nutrient deprivation, and infection lead to accumulation of unfolded proteins in the ER (a process known as ER stress). Under ER stress, cells activate signaling cascades known as the unfolded protein response (UPR). The UPR comprises three major signaling pathways mediated by protein kinase-like ER kinase (PERK), activating transcription factor 6 (ATF6), and inositol-requiring enzyme 1 (IRE1) [9, 10]. Through these signaling pathways, UPR leads to attenuation of ER stress and restores ER function

via general translational suppression, induction of ER chaperones, and upregulation of ER-associated degradation. XBP-1 and GRP78 are related to IRE1 activation and transcriptional activation of the UPR and are therefore recognized as reliable indicators of ER stress [11].

Tumor growth factor- (TGF-) β1 is a multifunctional peptide that regulates proliferation, differentiation, adhesion, migration, and other cellular functions and is involved in the pathogenesis of a variety of airway diseases related to remodeling, including CRS [12]. In a previous study, we confirmed that TGF-β1 causes EMT in the airway epithelium and nasal tissues [13]. Recent studies showed that ER stress also induces EMT in a variety of different cell types, such as alveolar epithelial cells and thyroid epithelial cells [14–16]. c-Src, a member of the Src family of tyrosine kinases, has been proposed as a possible target of UPR to induce EMT [17]. Based on these facts, we hypothesized that ER stress could be involved in TGF-β1-induced EMT and that c-Src could be involved in this process in cells and tissues of airway including the nose. Thus, in the present study, we investigated the role of ER stress and c-Src in TGF-β1-induced EMT.

2. Materials and Methods

2.1. Materials. Human recombinant TGF-β1 was obtained from R&D Systems (Minneapolis, MN) and dissolved in 0.1% bovine serum albumin (Millipore Inc., Billerica, MA). 4-PBA and PP2 (Sigma, St. Louis, MO, USA) were dissolved in DMSO (Sigma) and then diluted to the desired concentrations with complete medium. DMSO (<0.1%) was added to the medium.

2.2. Harvesting Nasal Polyps and Inferior Turbinates. Six patients with chronic rhinosinusitis with nasal polyps were recruited from the Department of Otorhinolaryngology, Korea University College of Medicine, Korea. Twelve inferior turbinate tissues were harvested during endoscopic sinus surgery for benign tumors. Half of them were used for control, and others were used for organ culture and cell culture. The patients that have any history of allergies, asthma, or aspirin sensitivity or received medications such as steroids, NSAIDs, antihistamines, or antibiotics within 4 weeks prior to tissue harvest were excluded. The Institutional Review Board of Korea University Guro Hospital approved the present study (KUGGR-12041-001), and informed consent was obtained from each patient.

2.3. Cell Culture. A549 cells, a human cell line derived from the respiratory epithelium, were purchased from the American Type Culture Collection (Manassas, VA). A549 cells were cultured in RPMI-1640 medium supplemented with 10% (*v*/*v*) heat-inactivated fetal bovine serum (Invitrogen, Carlsbad, CA), 1000 μg/mL streptomycin (Invitrogen), and 1000 unit/mL penicillin.

For culture of primary nasal epithelial cells (PNECs), nasal tissues were washed with phosphate-buffered saline (PBS), digested in dispase (Stem Cell Technologies, Vancouver, Canada) for 4 hours, and then filtered through a mesh. PNECs were cultured in Bronchial Epithelial Cell Growth Medium (Lonza, Basel, Switzerland).

2.4. Inferior Turbinate Organ Culture. Inferior turbinates were dissected into three 3 mm pieces with scissors in sterile manner. Dissected tissues were washed three times with PBS and then placed onto a hydrated gelatin sponge (10 mm × 10 mm × 1 mm; Spongostan, Johnson & Johnson, San Angelo, TX) in 6-well plates. Each well was filled with 1.5 mL Dulbecco's Modified Eagle Medium (Invitrogen) including 2% fetal bovine serum (Invitrogen). Inferior turbinate tissues were stimulated with TGF-β1 (5 ng/mL) with or without 4PBA or PP2. Plates were incubated at 37°C in 5% CO_2.

2.5. MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl Tetrazolium Bromide) Assay. A549 cells or PNECs were placed onto 96-well plates at a density of 4×10^5 cells/mL with various concentrations of 4PBA (0–20 mM) for 72 hours. Cells were incubated with MTT (Sigma) for 4 hours, and the reaction was stopped by the addition of acidified isopropanol. The results were measured by a fluorescence microplate reader (F2000; Hitachi Ltd., Tokyo, Japan) at 570 nm.

2.6. Immunofluorescence. Cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100 in 1% bovine serum albumin for 10 minutes, blocked with 5% bovine serum albumin for 1 hour at room temperature, and incubated overnight at 4°C with monoclonal (vimentin and α-SMA) or polyclonal (E-cadherin and fibronectin) primary antibodies (Santa Cruz, CA). Cells were incubated with Alexa 488 anti-mouse IgG antibody or Alexa 555 anti-rabbit IgG secondary antibodies (Invitrogen, Carlsbad, CA) and counterstained with 4′,6-diamidino-2-phenylindole (Invitrogen, Carlsbad, CA). Images of immunostained cells were captured using a confocal microscope (LSM700; Zeiss, Oberkochen, Germany).

2.7. Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total RNA was extracted using TRIzol reagent (Invitrogen), according to the recommendations of the manufacture. RT was carried out with 2 μg RNA for each sample. MMLV reverse transcriptase (Invitrogen) was used according to the manufacturer's protocol. PCR was performed using the following primers: *GRP78* (sense sequence 5′- GTT CTT GCC GTT CAA GGT GG -3′ and antisense sequence 5′- TGG TAC AGT AAC AAC TGC ATG GG -3′, 180 bp), *XBP-1s* (sense sequence 5′- CCT GGT TGC TGA AGA GGA GG -3′ and antisense sequence 5′- CCA TGG GGA GAT GTT CTG GAG -3′, 138 bp), and *GAPDH* (sense sequence 5′- GTG GAT ATT GTT GCC ATC AAT GAC C -3′ and antisense sequence 5′- GCC CCA GCC TTC TTC ATG GTG GT -3′, 271 bp). Gels were visualized using a Molecular Imager ChemiDoc XRS+ (Bio-Rad, Hercules, CA).

2.8. Western Blot Assay. PRO-PREPTM protein extraction solution (iNtRON Biotechnology, Seongnam, Korea) was used to lyse cells or tissues. Proteins were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis

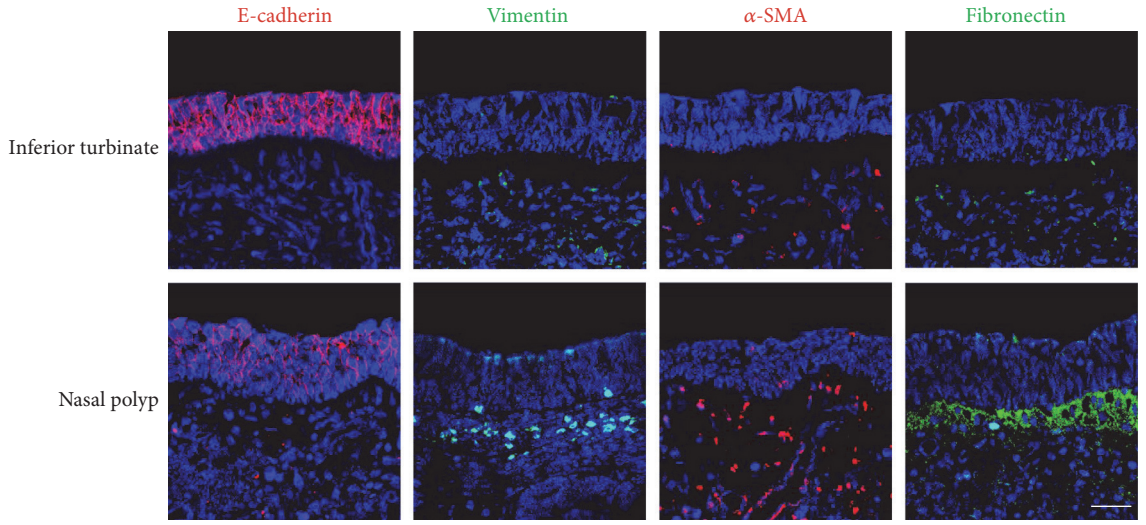

Figure 1: Expression of epithelial-mesenchymal markers E-cadherin, vimentin, α-smooth muscle actin, and fibronectin in nasal polyp and control (normal inferior turbinate) tissues determined by immunofluorescence. Scale bar = 50 μm.

and transferred onto polyvinyl difluoride membranes (Millipore Inc., Billerica, MA). Membranes were blocked with 5% skim milk and incubated with the following antibodies: E-cadherin, vimentin, α-SMA, fibronectin, GRP78, XBP-1s, phosphorylated c-Src, and β-actin (Santa Cruz). The blots were detected after incubation with horseradish peroxidase-conjugated secondary antibodies by an enhanced chemiluminescence detection system (Pierce, Rockford, IL).

2.9. Cell Migration Scratch Assay. On 6-well tissue culture dishes, A549 cells were plated and cultured to confluence. Through the cells, a straight scratch was made with a pipette tip. Scratched cells were washed immediately with PBS, and culture medium was added. Cells were treated only with TGF-β1 (5 ng/mL) or in combination with 4PBA or PP2. After 48 hours, images were captured with a microscope (Olympus BX51; Olympus, Tokyo, Japan).

2.10. Transwell Migration Assay. Cells were placed onto the top chamber of a transwell chambers (Corning Life Sciences, MA). Chamber was filled with RPMI-1640 medium supplemented with 10% (*v*/*v*) heat-inactivated fetal bovine serum, 1000 μg/mL streptomycin, and 1000 unit/mL penicillin (Invitrogen). TGF-β1 (5 ng/mL) alone or in combination with 4PBA or PP2 was added in the bottom chamber for 48 hours. Cells on the upper surface of the membrane were removed using a cotton swab, and cells on the lower surface were stained with Diff-Quik stain (Sysmex, Kobe, Japan). Images of stained cells from five selected fields of view were obtained using a microscope at 400x magnification.

2.11. Statistical Analysis. At least three independent experiments provided the results in this study. Unpaired two-way analysis of variance (ANOVA) test or one-way ANOVA followed by Tukey's test (GraphPad Prism, version 5, GraphPad Software, San Diego, CA) was used to confirm the Statistical significance of differences between control and experimental data. A 95% confidence level established significance. *P* values less than 0.05 were accepted as statistical significant.

3. Results

3.1. Reduced Expression of Epithelial Markers and Increased Expression of Mesenchymal EMT Markers in Nasal Polyp Tissues. To investigate whether EMT occurs in CRS tissues in vivo, we examined the fluorescent immunocytochemical expression of the EMT markers including E-cadherin, vimentin, α-SMA, and fibronectin—in six nasal polyps and six normal inferior turbinates. E-cadherin expression was reduced in epithelial cells from nasal polyps compared to inferior turbinate epithelial tissues, as characterized by weak membrane staining. Staining for the mesenchymal markers vimentin, α-SMA, and fibronectin in nasal polyps was strongly positive in submucosal tissues and weaker on the apical epithelial side. However, in the inferior turbinate tissues, both epithelium and submucosa were negative for these mesenchymal markers (Figure 1).

3.2. 4-PBA Reduces TGF-β1-Induced Expression of ER Stress Markers in A549 Cells. To determine whether TGF-β1 causes ER stress in A549 cells, expression levels of the ER stress markers, GRP78 and XBP-1s, were measured using RT-PCR and Western blot. TGF-β1 induced the expression of GRP78 and XBP-1s mRNA after 24 hours, and protein expression was increased after 48 hours. Next, we investigated the effects of 4-PBA, a chemical chaperone, on TGF-β1-induced ER stress in A549 cells. Prior to treatment with 4-PBA, an MTT assay was performed on A549 cells and nasal epithelial cells to examine the effects of 4-PBA on cell survival. Cells were examined after treatment with 4-PBA concentrations ranging from 0 to 20 mM, and cell survival was not affected below 10 mM (Figure 2). After pretreatment with 4-PBA (1.24–5 mM) for 1 hour, cells were treated with TGF-β1 for 24 or 48 hours and the expression of GRP78 and XBP mRNA and protein were measured. Pretreatment

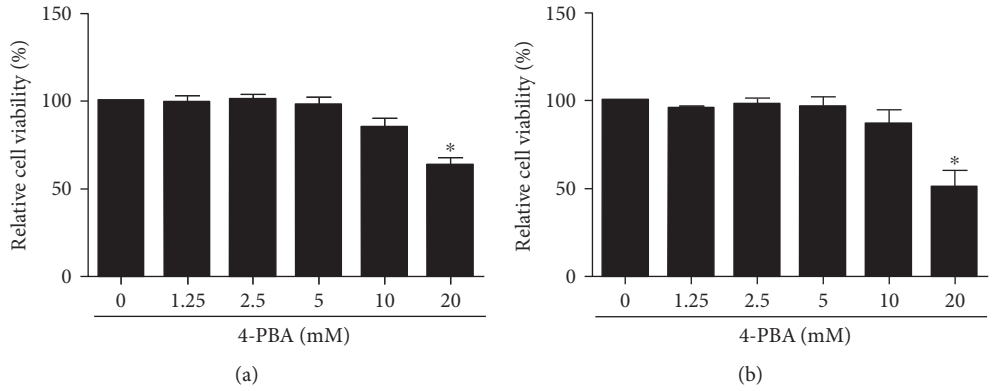

Figure 2: Cytotoxicity of 4-PBA determined by MTT assay in A549 cells (a) and in primary nasal epithelial cells (b). 4-PBA, 4-phenylbutyric acid. MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide. $^{*}P < 0.05$ versus control.

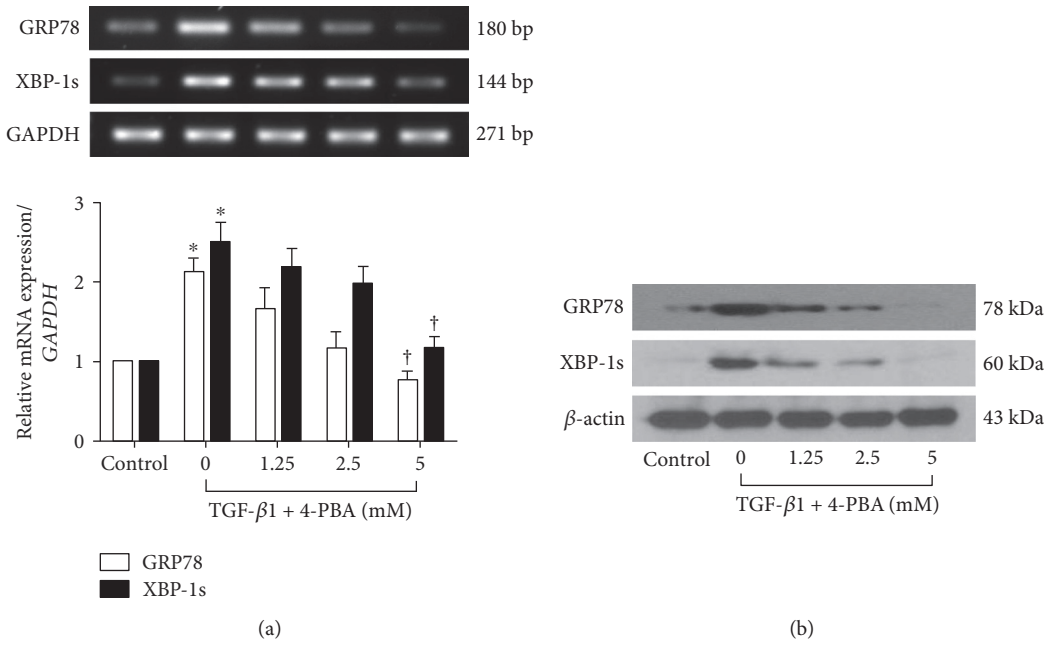

Figure 3: Effect of 4-PBA on GRP78 and XBP-1s mRNA and protein expression in TGF-β1-stimulated A549 cells determined by RT-PCR (a) and Western blotting (b) (representative of independent experiments). Values expressed as mean ± SEM of independent experiments. $^{*}P < 0.05$ versus control. $^{\dagger}P < 0.05$ versus TGF-β1 alone. 4-PBA, 4-phenylbutyric acid. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

with 4-PBA reduced the TGF-β1-induced expression of GRP78 and XBP-1s in a dose-dependent manner (Figure 3).

3.3. 4-PBA Inhibits TGF-β1-Induced EMT in A549 Cells. In a previous study, we showed that TGF-β1 induces EMT in the primary airway epithelial cells [13]. Cells were treated with 5 ng/mL TGF-β1 for 72 hours, and morphological changes of the cells were observed by phase-contrast microscopy. After 72 hours of TGF-β1 treatment, the normal cobblestone-like appearance of the epithelial cells was converted into a morphology of mesenchymal cells with an abnormal elongated appearance. Pretreatment of these TGF-β1-stimulated A549 cells with 4-PBA for 1 hour restored the morphology of normal epithelial cells (Figure 4(a)). We examined E-cadherin, vimentin, α-SMA, and fibronectin protein expression, as markers of EMT, using Western blot and fluorescent immunocytochemical staining (Figures 4(b) and 4(c)). TGF-β1 treatment for 72 hours decreased E-cadherin expression and increased vimentin, fibronectin, and α-SMA expression. Thus, 4-PBA reversed the effects of TGF-β1 on EMT in A549 cells.

3.4. TGF-β1-Induced ER Stress and EMT Are Mediated through c-Src Kinase Activation in A549 Cells. To evaluate the contribution of c-Src kinase to TGF-β1-induced ER stress and EMT, we used the c-Src kinase inhibitor, PP2 (2 μM). We first confirmed that phosphorylation of c-Src kinase is stimulated by TGF-β1 (5 μM) in A549 cells and then determined whether it was blocked by PP2 treatment. Phosphorylation of c-Src kinase was increased in TGF-β1-treated

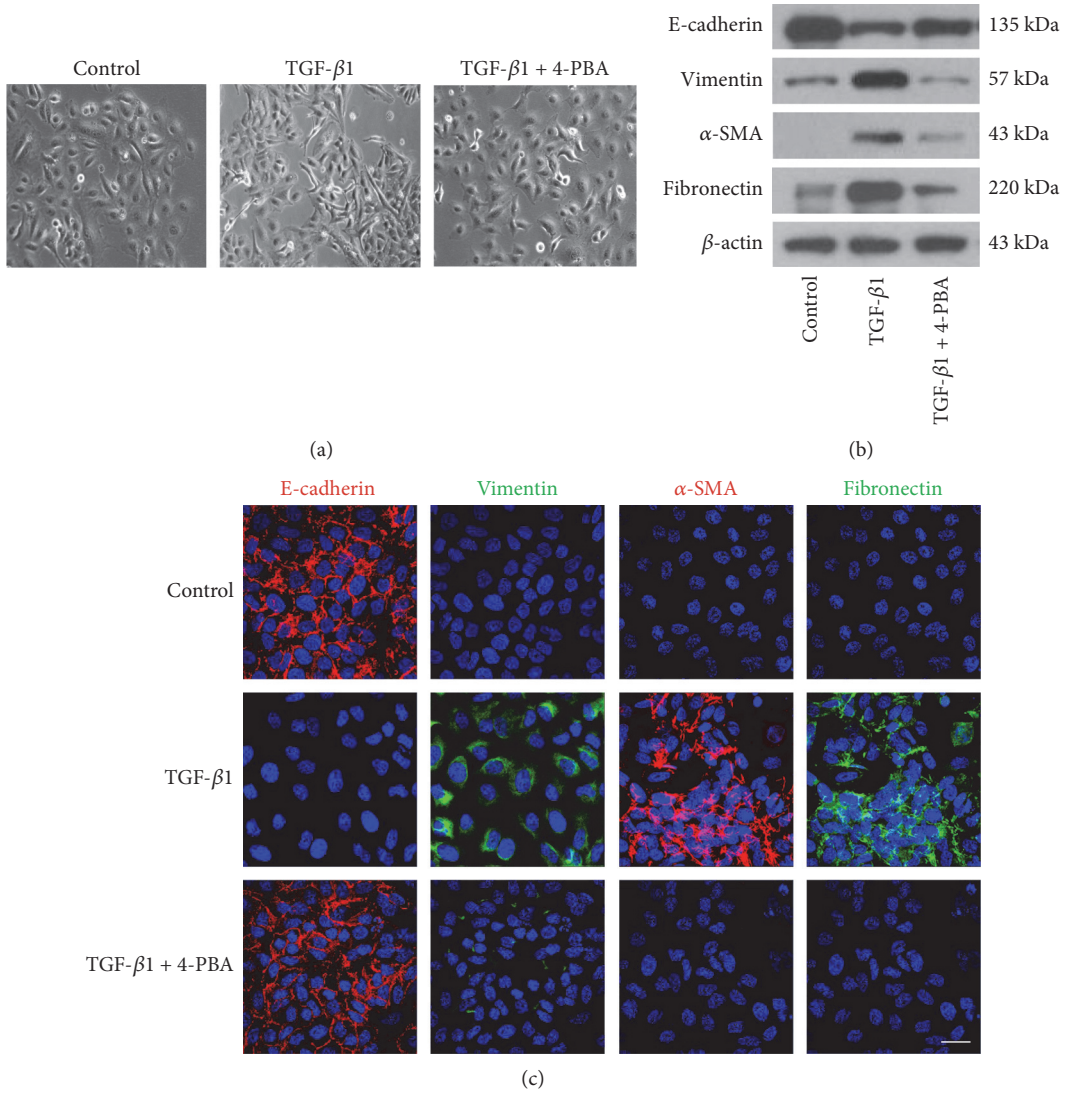

Figure 4: (a) Effect of 4-PBA on morphology of TGF-β1-stimulated A549 cells as observed under a phase-contrast microscope. Effects of 4-PBA on E-cadherin, vimentin, α-smooth muscle actin protein, and fibronectin expression in TGF-β1-stimulated A549 cells determined by Western blotting (b) and immunofluorescence (c). Representative of independent experiments. Scale bar = 50 μm. 4-PBA, 4-phenylbutyric acid.

A549 cells after 48 hours, as shown by Western blotting, and this was reduced by PP2 (2 μM) but not by 4-PBA (5 mM) (Figure 5(a)). PP2 treatment inhibited the TGF-β1-induced changes in ER stress marker proteins (GRP78 and XBP-1) after 48 hours and in EMT marker proteins (E-cadherin, vimentin, α-SMA, and fibronectin) after 72 hours (Figures 5(b) and 5(c)). Localization of the EMT markers was observed using fluorescent immunocytochemical staining, and these results mirrored those from the Western blotting (Figure 5(d)). These data indicate that c-Src is involved in TGF-β1-induced ER stress and EMT in A549 cells.

3.5. 4-PBA and PP2 Inhibit Migration of TGF-β1-Induced A549 Cells. As one of the important functional characteristics of mesenchymal cells is increased migratory ability, cell migration assay was done to measure changes in the migratory capacity of A549 cells. We made a straight scratch in the middle of adherent cells on the plate using a pipette tip. And then, we stimulated the cells with TGF-β1, with or without 4-PBA or PP2, and determined the distance from the initial boundary to the moved cells. After 48 h, cells had moved meaningfully further from the boundary of the initial scratch in TGF-β1-treated cells, compared to controls. This cell migration was inhibited following pretreatment with either 4-PBA or PP2 (Figures 6(a) and 6(b)). We reevaluated the inhibitory effect of 4-PBA and PP2 on the increased migratory ability of TGF-β1-induced A549 cells by performing a transwell invasion assay. Cells were treated with TGF-β1 with or without 4-PBA or PP2 for 48 h, and the number of cells that had moved through the filter and adhered to the underside of the well was counted. These results confirm that pretreatment with 4-PBA and PP2 blocks the enhanced cell invasion seen in TGF-β1-treated cells (Figure 6(b)). In summary, the increased migratory ability induced by TGF-

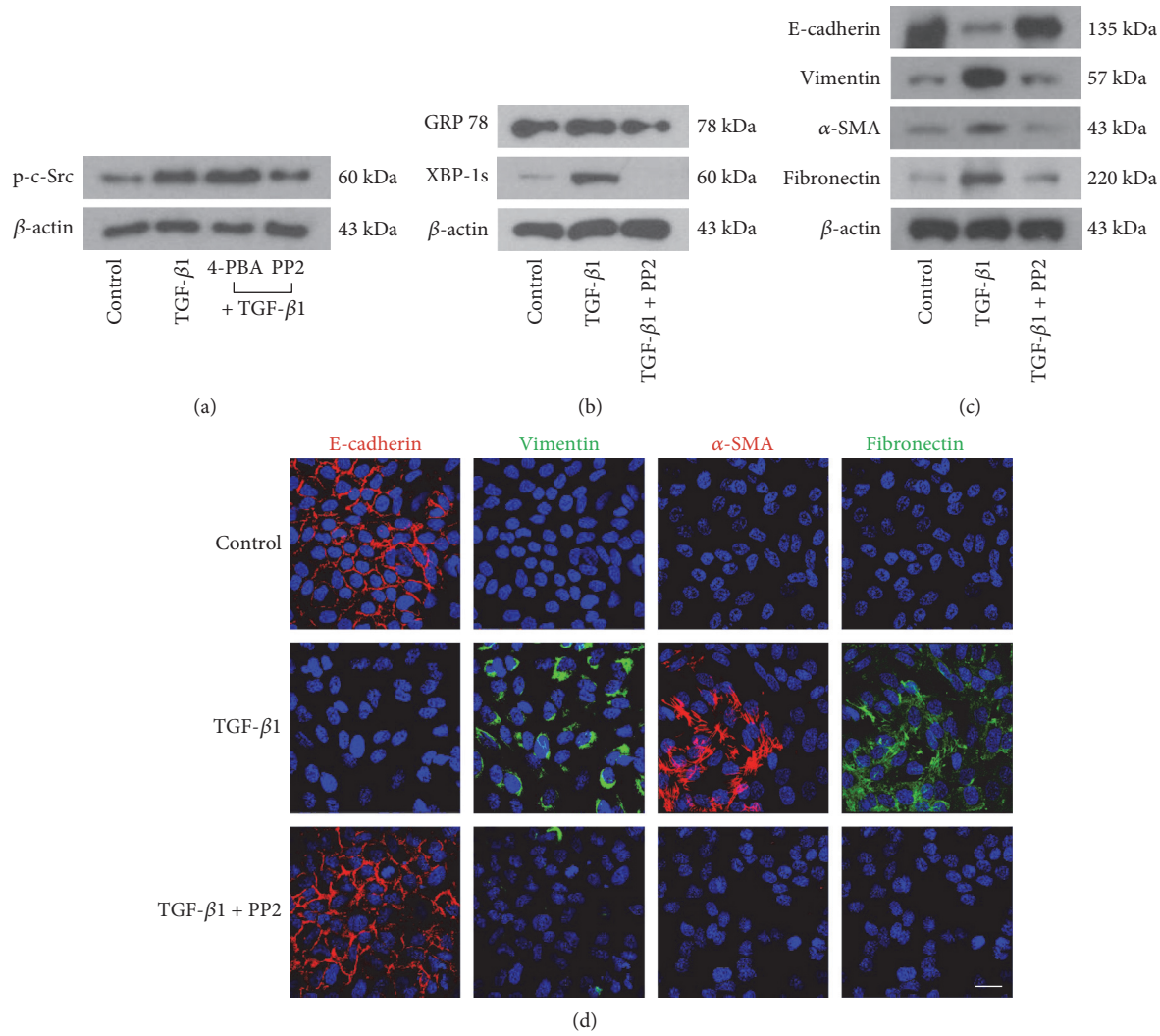

Figure 5: (a) Effect of 4PBA and PP2 on c-Src phosphorylation in TGF-β1-stimulated A549 cells determined by Western blotting (representative of independent experiments). (b) Effects of PP2 on GRP78 and XBP-1s protein expression in TGF-β1-stimulated A549 cells determined by Western blotting (representative of independent experiments). Effects of PP2 on E-cadherin, vimentin, α-smooth muscle actin protein, and fibronectin expression in TGF-β1-stimulated A549 cells determined by Western blotting (c) and immunofluorescence (d). Representative of independent experiments. 4-PBA, 4-phenylbutyric acid.

β1 is blocked by a chemical chaperone of ER stress and inhibition of c-Src phosphorylation.

3.6. 4-PBA and PP2 Inhibit TGF-β1-Induced EMT in PNACs and Nasal Inferior Turbinate Organ Cultures. To determine whether the blockage of TGF-β1-induced EMT by PBA and PP2 in A549 cells is also seen in nasal tissue, we repeated our experiments in PNECs and inferior turbinate organ cultures. To access whether TGF-β1 causes EMT in PNECs, cells were treated with 5 ng/mL TGF-β1 for 72 hours and then we observed expression of fibronectin, α-SMA, vimentin, and E-cadherin protein using a fluorescence microscope. The cells showed increased vimentin, α-SMA, and fibronectin expression and decreased E-cadherin expression. 4-PBA or PP2 pretreatment for an hour blocked the effects of TGF-β1 on EMT in PNECs (Figure 7(a)). In nasal inferior turbinate organ cultures, tissues were exposed to TGF-β1 for 72 hours, with or without 4-PBA or PP2, and expression levels of α-SMA, fibronectin, vimentin, and E-cadherin protein were assayed using Western blot. Expression levels of α-SMA, vimentin, fibronectin were increased, and E-cadherin expression was decreased in TGF-β1-treated inferior turbinate organ cultures, compared to controls. However, pretreatment with PBA or PP2 inhibited the effect of TGF-β1 on expression of EMT markers. These results show that 4-PBA or PP2 pretreatment ameliorate EMT induced by TGF-β1 in cells and tissues of the nose (Figure 7(b)).

4. Discussion

In the present study, we first confirmed that features of EMT are increased in nasal polyp tissues and then showed that a chemical chaperone of ER stress, 4PBA, inhibits TGF-β1-induced EMT in A549 cells, PNECs, and inferior turbinate organ cultures. TGF-β1 increased mRNA and protein expression levels of ER stress markers (XBP-1s

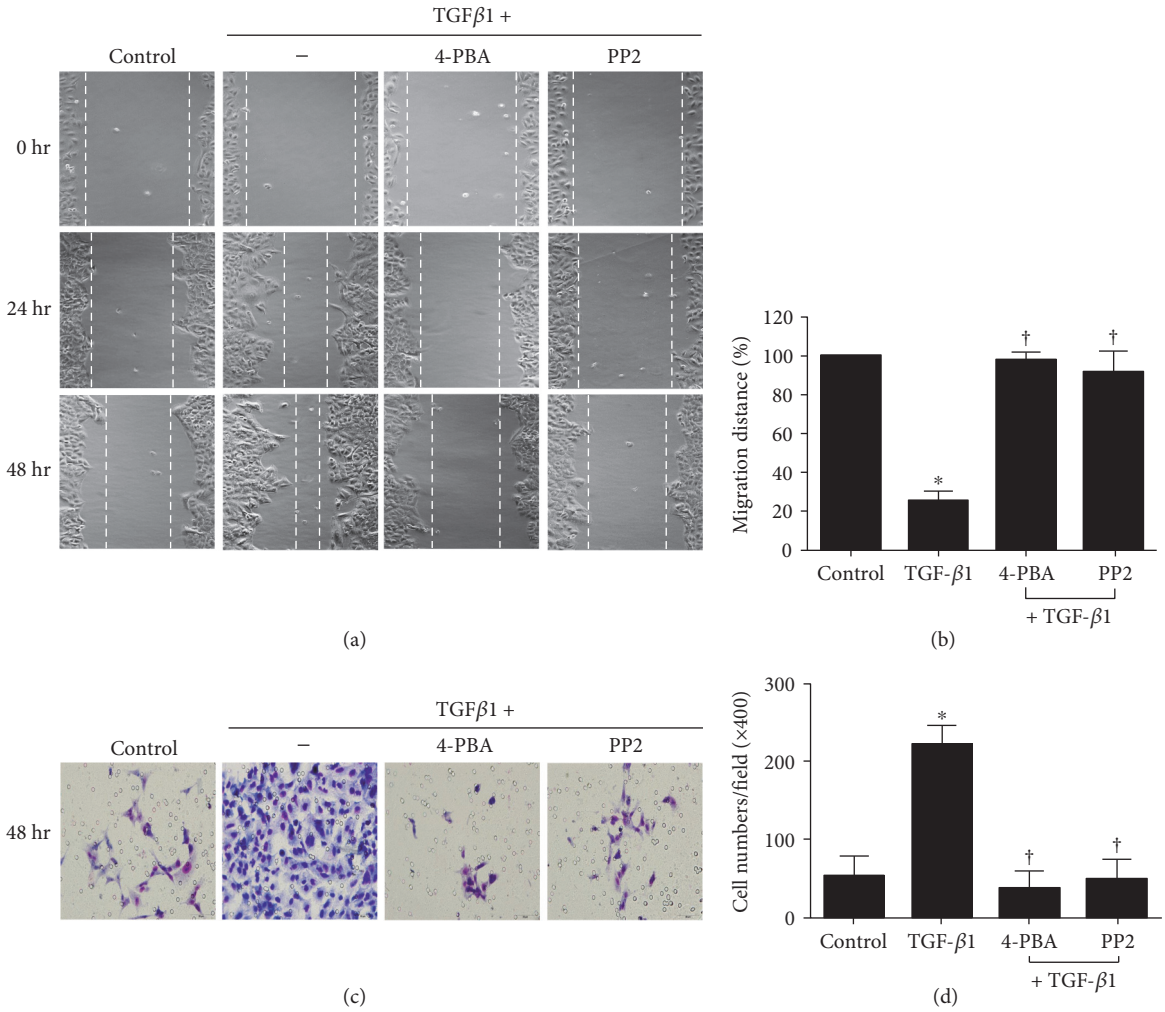

Figure 6: Effect of 4-PBA and PP2 on migratory ability of TGF-β1-stimulated A549 cells measured using cell migration (a, b) and transwell invasion (c, d) assays. Values expressed as mean ± SEM of independent experiments. $^{*}P < 0.05$ versus control. $^{\dagger}P < 0.05$ versus TGF-β1 alone. Scale bar = 50 μm.

and GRP78) and also altered expression levels of EMT markers, namely, E-cadherin, vimentin, fibronectin, and α-SMA. Pretreatment with 4PBA reversed the effect of TGF-β1 on EMT in A549 cells, while pretreatment with PP2 reversed the effect on both ER stress and EMT. However, 4PBA treatment did not show inhibitory effects on c-Src phosphorylation in TGF-β1-induced A549 cells. 4PBA and PP2 also reversed the stimulatory effect of TGF-β1 on the migratory and invasive ability of the cells, which was a characteristic of mesenchymal cells in both a cell migration and transwell invasion assay. In experiments using PNECs and inferior turbinate tissues, 4PBA and PP2 suppressed the changes in EMT marker expression levels that were induced by TGF-β1.

Inflammation leads to a varied degree of tissue injury, depending on the disease and its severity. This means that remodeling occurs in all inflammatory disease, as remodeling is an essential process in the healing and repair of injured tissue. Remodeling that occurs in response to a minor inflammatory condition usually leads to a normal reconstructive process. On the contrary, dysregulated remodeling, such as that caused by severe or chronic long-lasting inflammation, can cause pathological reconstruction and formation of pathological tissue [18]. Pathological remodeling of the lower airways has received considerable attention as it is one of the major features of asthma and chronic obstructive pulmonary disease. As a result, much progress has been made towards understanding these diseases [19]. The role of remodeling in the upper airway chronic inflammatory disease, such as CRS, has received less attention. This could be due in part to the fact that remodeling of the upper airway does not result in the same fatal airflow limitations that occur in patients with asthma and chronic obstructive pulmonary disease. Recently, however, there has been increased concern regarding the role of remodeling in the upper airway diseases, including CRS, for two main reasons. The first is that the upper airway remodeling can lead to irreversible structural changes, which could explain the recalcitrance of disease [20, 21]. The other is that remodeling begins early in the development of disease and therefore plays an important role in the disease progression. Although remodeling in CRS has been considered a secondary process that results from persistent inflammation over a long period of time, a

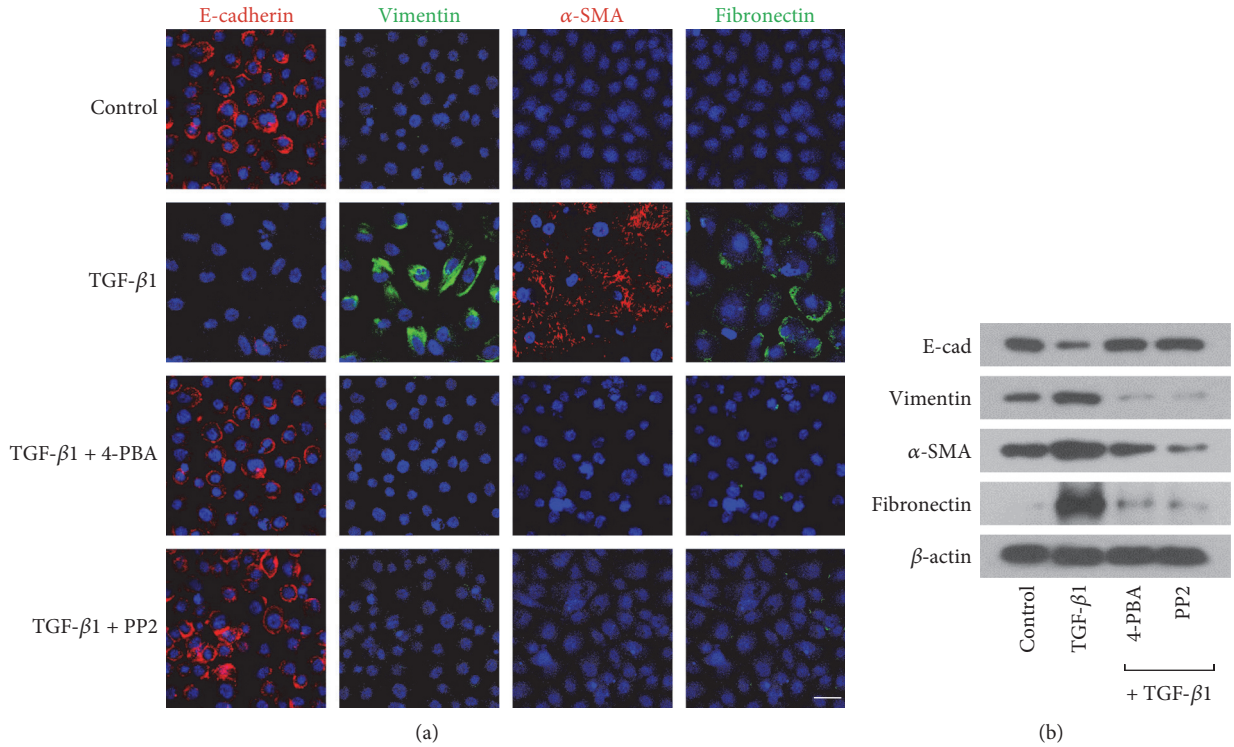

Figure 7: (a) Effect of 4-PBA and PP2 on E-cadherin, vimentin, α-smooth muscle actin protein, and fibronectin expression in TGF-β1-stimulated primary nasal epithelial cells determined by immunofluorescence. (b) Effects of 4-PBA and PP2 on E-cadherin, vimentin, α-smooth muscle actin protein, and fibronectin expression in TGF-β1-stimulated inferior turbinate tissues determined by Western blotting. Representative of independent experiments. Scale bar = 50 μm. 4-PBA, 4-phenylbutyric acid.

recent study showed that remodeling actually occurs in parallel with inflammation [22]. As a result, remodeling is now thought to be one of the main reasons for disease recalcitrance, and EMT is receiving great attention as a convergence point between inflammation and pathological remodeling in many progressive fibrotic diseases. Accumulating evidence has confirmed that ongoing EMT leads to loss of epithelial barrier function, which would also contribute to disease progression [12].

Endoplasmic reticulum (ER) stress refers to the state where protein folding in the ER is disrupted by alterations in homeostasis within the ER lumen. UPR is the main mechanism by which cells adapt to ER stress and maintain homeostasis. In recent years, ER stress has been implicated in fibrotic remodeling during chronic inflammatory disease [23, 24]. Furthermore, studies suggest that ER stress induces EMT in a number of biological systems [25]. Based on these facts, we sought to evaluate the effect of TGF-β1, a known strong inducer of EMT, on ER stress. In present study, we showed that TGF-β1 simultaneously affected the expression levels of ER stress markers and EMT markers in A549 cells and that these effects were reversed by the chemical chaperon of ER stress, 4PBA. To our knowledge, this is the first study to demonstrate a role for TGF-β1 as an inducer of both ER stress and EMT.

The Src protein nonreceptor tyrosine kinase family is a group of cytoplasmic tyrosine kinases capable of communicating with a large number of different receptors and thus regulates many cellular events [26]. c-Src is a member of the Src tyrosine kinase family, and it plays a critical role as a switch in mediating signal transduction via interactions with multiple proteins and protein complexes. Abnormal activation of c-Src is involved in apoptosis, proliferation, cell adhesion, cell migration, and invasion, and all of which are related to tumor progression [27]. Dysfunction of E-cadherin caused by c-Src could also contribute to our understanding of EMT. In fact, increased Src activity promotes EMT, while c-Src inhibition suppresses this process [28]. In the present study, we showed that c-Src inhibition by PP2 reversed the effect of TGF-β1 on EMT in airway epithelial cells. Moreover, PP2 also inhibited the expression of ER stress markers, XBP-1 and GRP78. We can therefore assume that c-Src is involved in TGF-β1, UPR, and the EMT axis at a higher level than UPR.

5. Conclusions

We demonstrated that 4PBA inhibits TGF-β1-induced EMT via the c-Src pathway in A549 cells, PNECs, and inferior turbinate organ cultures. These results suggest an important role for ER stress and a diverse role for TGF-β1 in the upper airway chronic inflammatory diseases such as CRS. In the near future, it will also be necessary to demonstrate additional level of evidences done with diseased tissue instead of TGF-β1-induced tissue. We hope these approach elucidate the role of ER stress modulation in the treatment of recalcitrant CRS related to tissue remodeling.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Authors' Contributions

Heung-Man Lee and Ju-Hyung Kang contributed equally to this work.

Acknowledgments

This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT and Future Planning (R1513641).

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Hindawi
Mediators of Inflammation
Volume 2017, Article ID 3549375, 7 pages
https://doi.org/10.1155/2017/3549375

Research Article

A Role of the ABCC4 Gene Polymorphism in Airway Inflammation of Asthmatics

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Received 14 March 2017; Accepted 10 April 2017; Published 4 June 2017

Academic Editor: Younghyo Kim

The ATP-binding cassette subfamily C member 4 gene encodes a transmembrane protein involved in the export of proinflammatory molecules, including leukotriene, prostaglandin, and sphingosine-1-phosphate across the plasma membrane. Those metabolites play important roles in asthma. We investigated the potential associations between *ABCC4* gene polymorphisms and asthma phenotype. In total, 270 asthma patients and 120 normal healthy controls were enrolled for a genetic association study. Two polymorphisms (−1508A>G and −642C>G) in the *ABCC4* promoter were genotyped. The functional variability of the promoter polymorphisms was analyzed by luciferase reporter assay. Inflammatory cytokine levels were measured by enzyme-linked immunosorbent assay. Serum and urinary eicosanoid metabolites, sphingosine-1-phosphate, were evaluated by quadrupole time-of-flight mass spectrometry. Asthma patients carrying the G allele at −1508A>G had significantly higher serum levels of periostin, myeloperoxidase, and urinary levels of 15-hydroxyeicosatetraenoic acid and sphingosine-1-phosphate ($P = 0.016$, $P = 0.027$, $P = 0.032$, and $P = 0.010$, resp.) compared with noncarrier asthma patients. Luciferase activity was significantly enhanced in human epithelial A549 cells harboring a construct containing the −1508G allele ($P < 0.01$ for each) compared with a construct containing the −1508A allele. A functional polymorphism in the *ABCC4* promoter, −1508A>G, may increase extracellular 15-hydroxyeicosatetraenoic acid, sphingosine-1-phosphate, and periostin levels, contributing to airway inflammation in asthmatics.

1. Introduction

Multidrug resistance protein 4 (ABCC4) is a member of the family of ATP-binding cassette transporters required for the active transport of many bioactive substrates across the cell membrane [1]. ABCC4 pumps various substrates, including eicosanoids, cyclic nucleotides, bile salts, steroids, and other drugs, out of the cell to control multiple cellular signaling processes, including inflammation, cancer, cardiovascular homeostasis, platelet function, endothelial barrier function, vascular smooth muscle cell proliferation, and vasodilation [1–6].

Eicosanoids such as prostaglandin E2 (PGE_2) and cysteinyl leukotriene (LT) E4, along with cyclic adenosine monophosphate (cAMP), are exported via ABCC4, and these molecules play important roles in airway inflammation [7, 8]. Previous studies have demonstrated that intracellular elevation of cAMP exerts an anti-inflammatory effect and PGE_2 induces cAMP [9, 10]. The exposure of eosinophils to LTE4 also induces cAMP production [11]. Additionally, cAMP is involved in the induction and regulation of T helper (Th2) immunity, particularly in allergic asthma via dendritic cells [12]. Pharmacological inhibition of ABCC4 reduces the migration of human dendritic cells, indicating an important role for ABCC4 in human immunology [13]. Therefore, ABCC4 may play an important role in inflammatory diseases, particularly in asthma, by regulating the intracellular concentration of cAMP [12]. In addition, ABCC4 facilitates the transport of sphingosine-1-phosphate (S1P), the main active metabolite of sphingolipids, from the platelets [14].

ABCC4 is ubiquitously expressed, with particularly high expression in hematopoietic stem cells and blood cells [15]. Limited data on the functions of its variants are available despite the fact that *ABCC4* is a highly polymorphic gene. *ABCC4* variants are associated with various diseases; however, no report has implicated an association between *ABCC4* and immunological diseases. Copsel et al. demonstrated that an *ABCC4* polymorphism regulates the cellular levels of cAMP and controls human leukemia cell proliferation and differentiation, indicating its role in cellular processes [16]. Therefore, *ABCC4* variants may also play an important role in the pathogenesis of asthma.

There is little evidence regarding associations of *ABCC4* polymorphisms with asthma in Korean patients. Therefore, we investigated the potential associations between asthma and *ABCC4* polymorphisms in a Korean population.

2. Materials and Methods

2.1. Study Subjects. We enrolled 270 asthma patients and 120 normal healthy controls (NCs) from the Department of Allergy and Clinical Immunology, Ajou University Hospital, Suwon, Korea. Written informed consent was obtained from each subject, and the study was approved by the Institutional Review Board of Ajou University Hospital (AJIRB-GEN-SMP-13-108).

Methacholine bronchial challenge tests were performed as described previously [17]. NCs were selected from the general population using a screening questionnaire. Participants with a history of respiratory symptoms or aspirin hypersensitivity were excluded. All NC subjects exhibited a forced expiratory volume 1 (FEV_1) > 80% of the predicted value, a provocation concentration (PC_{20}) of methacholine > 25 mg/mL and normal findings on chest radiographs. Atopy was defined as one or more positive reactions on a skin prick test of 12 common aeroallergens (Bencard Co., Brendford, UK); histamine and saline served as controls. Serum total IgE levels were measured using the UniCAP system (Thermo Scientific, Uppsala, Sweden) according to the manufacturer's instructions. The threshold cut-off value for a specific IgE level was 0.35 kU/L, as measured by UniCAP. The presence of rhinosinusitis and nasal polyps was determined using paranasal sinus X-ray and rhinoscopy.

2.2. DNA Extraction, Single Nucleotide Polymorphism (SNP) Identification, and Genotyping. Each of the twenty Korean asthma patients and NCs was used for SNP identification. Total genomic DNA was isolated from peripheral blood samples using the Puregene DNA Purification Kit (Gentra, Minneapolis, MN, USA) according to the manufacturer's protocol. Our objective was to screen for promoter and 5′-untranslated region (UTR) SNPs. Based on previous findings and sequencing results, we chose two SNPs: one in the promoter and one in the 5′UTR of *ABCC4* (−1508A>G and −642C>G, resp.). The two SNPs were genotyped using the TaqMan Allelic Discrimination assay with TaqMan probes (rs868853, −1508A>G assay ID c__7461591_10; rs869951, −642C>G, c__7461587_10; Applied Biosystems, Foster City, CA, USA).

2.3. Quantification of Serum and Urinary Metabolites. For the serum and urinary metabolites, we enrolled 60 and 31 asthmatic patients for measurement, respectively. The serum and urinary levels of S1P and five eicosanoid metabolites, LTE4, prostaglandin F2α (PGF2α), thromboxane B2 (TXB2), 15-hydroxyeicosatetraenoic acid (15-HETE), and eoxin C4, were determined using the Agilent 6530 quadrupole time-of-flight (Q-TOF) mass spectrometer. The device settings have been described in detail in a previous study [18].

2.4. Measurement of Serum Inflammatory Cytokines. Several inflammatory biomarkers, including myeloperoxidase (MPO), interleukin- (IL-) 8, IL-18, eotaxin-1, and eotaxin-2, were measured by enzyme-linked immunosorbent assay (ELISA) (Quantikine, R&D Systems, Minneapolis, MN, USA). Serum periostin levels were measured using a proprietary sandwich ELISA kit (Shino-test, Kanagawa, Japan) [19]. Each sample was run in duplicate. Serum samples were stored at −80°C prior to use.

2.5. Activity of the ABCC4 Promoter Constructs. Human mast cells (HMC-1) were cultured in Iscove's Modified Dulbecco's Medium (Gibco, Grand Island, NY, USA). A549, human alveolar type II epithelial-like, and U937, human leukemic monocyte lymphoma cell lines, were cultured in Roswell Park Memorial Institute-1640 Medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/mL penicillin G sodium, and 100 μg/mL streptomycin sulfate (Gibco) at 37°C in a 5% CO_2 incubator.

A 1681 bp fragment of the human *ABCC4* gene was amplified from the genomic DNA of −1508GG and −1508AA homozygous subjects by PCR using the following primers: (forward) 5′- TCTATCGATAGGTACGGCCATGCTTAGACATAGG CTTA-3′ and (reverse) 5′- GATCGCAGATCTCGAAGAA CACGCGTGAGCAGAGGTT-3′. PCR products were gel purified using an Agarose Gel Purification Kit (GeneAll Biotechnology, Seoul, Korea) and ligated into the pGL3-basic vector (Promega, Madison, WI, USA) after digestion with KpnI and XhoI (Takara, Shuzo, Japan) using the In-Fusion® HD Cloning Kit (Clontech Laboratories Inc., Mountain View, CA, USA). All constructs were confirmed by a restriction enzyme analysis and DNA sequencing. Plasmid DNAs were prepared from these constructs using the Endo-Free Plasmid Maxi Kit (Qiagen, Hilden, Germany), and the concentration and purity were assessed by UV spectrophotometry and agarose gel electrophoresis. Before transfection, the constructs were verified by direct sequencing.

The constructs were transfected into A549, HMC-1, and U937 cells using Lipofectamine (Invitrogen) according to the manufacturer's protocol. Briefly, 1×10^5 cells were seeded into 12-well plates and, after reaching 70–80% confluency, were transfected with 1 μg of the reporter construct, 5 ηg Renilla plasmid DNA and 5 μL Lipofectamine. Forty-eight hours after transfection, the cells were lysed and assayed for firefly luciferase activity according to the manufacturer's instructions (Promega). Transfection and luciferase assays were repeated three times according to the method described above.

2.6. Statistical Analyses. Statistical analyses were performed using SPSS version 22.0 (SPSS Inc., Chicago, IL, USA). Differences in clinical characteristics among the groups were examined using the independent *t*-test for continuous variables and the χ^2 test for categorical variables. Genotype frequency was examined between the subject groups using a χ^2 test, and differences in clinical characteristics, cytokines and metabolites, according to genotype were examined using a logistic regression analysis with codominant, dominant, and recessive models after accounting for age and sex as covariables. Statistical significance was established at $P < 0.05$.

3. Results

3.1. Clinical Characteristics of the Study Subjects. The clinical characteristics of the study population are summarized in Table 1. The mean age of the asthma patients was 43.8 (±13.85) years and that of the NCs was 27.04 (±7.17) years ($P < 0.001$). The percentage of males was significantly higher in the asthma group (58.21%) than in the NC group (38.35%) ($P < 0.001$). In the asthma group, atopy was observed in 48.50% of patients, rhinosinusitis in 83.75%, and nasal polyps in 39.44%.

3.2. No Association of the ABCC4 Promoter Polymorphisms with Asthma. Two promoter polymorphisms of *ABCC4* gene (−1508A>G and −642C>G) were examined in this study. Linkage disequilibrium analysis was performed between the two *ABCC4* SNPs. Three common haplotypes, ht1 [AC], ht2 [AG], and ht3 [GG], were constructed using the EM algorithm (Table 2), which revealed the genotype and haplotype frequencies of SNPs in the study subjects. There were no significant differences with respect to genotype or haplotype between the study groups.

3.3. Associations between the ABCC4 −1508A>G Polymorphism and Urinary Levels of Metabolites. We next examined potential associations between *ABCC4* gene polymorphisms and serum and urinary eicosanoid metabolites and S1P (Table 3).

Among five eicosanoid metabolites (LTE4, PGF2α, TXB2, 15-HETE, and eoxin C4), the urinary 15-HETE level was significantly associated with the *ABCC4* −1508A>G polymorphism; asthma patients carrying the −1508G allele showed a significantly higher level than that of noncarriers (332 ± 99.31 versus 271.91 ± 89.87 pmol/mg creatinine [pmol/mg Cr], $P = 0.032$; Figure 1(a)). Serum levels of LTE4, PGF2α, TXB_2, and eoxin C4 did not differ significantly between −1508G carriers and noncarriers among asthma patients (Table 3).

Regarding to S1P, asthma patients carrying the −1508G allele showed a significantly higher level of the urinary level of S1P than that of noncarriers (41.5 ± 9.35 versus 32.56 ± 8.25 pmol/mg Cr, $P = 0.010$; Figure 1(b)).

3.4. Associations between the ABCC4 −1508A>G Polymorphism and Clinical Characteristics, Serum Periostin and MPO. Several inflammatory cytokines that are important biomarkers for asthma were also measured (Table 3). Among the cytokines examined, asthma patients carrying the −1508G allele showed significantly higher serum MPO levels (150.91 ± 94.13 versus 108.26 ± 79.5 mg/L, $P = 0.027$) and serum periostin level than those of noncarrier asthma patients (91.83 ± 50.85 versus 71.07 ± 33.62 ng/mL, $P = 0.016$), (Figures 1(c) and 1(d)).

3.5. Effects of the ABCC4 Polymorphisms on Transcriptional Activity. The luciferase reporter assay was performed using constructs containing two different *ABCC4* alleles, −1508A

Table 1: Clinical characteristics of study subjects.

	Asthmatics ($n = 270$)	NC ($n = 120$)	*P* value
Age (years)†	43.80 ± 13.85	27.04 ± 7.17/98	**<0.001**
Male (%)‡	58.21	38.35	**<0.001**
Atopy (%)†	48.50	NA	NA
Total IgE (kU/L)†	0.431 ± 0.9	NA	NA
Rhinosinusitis (%)‡	83.75	NA	NA
Nasal polyp (%)‡	39.44	NA	NA
FEV_1 (%)†	85.69 ± 20.30	NA	NA
PC_{20} methacholine (mg/mL)	9.17 ± 15.99	NA	NA
Asthma duration (years)†	7.69 ± 15.97	NA	NA

NC: normal control; *n*: number of subjects; NA: not applicable; FEV_1: forced expiratory volume in 1 s; PC_{20} methacholine: provocative concentration of methacholine producing a 20% fall in FEV_1. †This value was presented as mean ± SD, whereas ‡ value was shown as percentage. Values in bold indicate significant *P* value. Each *P* value <0.05 was considered to be significant. *P* value was obtained by *t*-test for continuous variables and the χ^2 test for categorical variables.

Table 2: Distribution of genotypes and haplotypes of *ABCC4* gene polymorphisms.

SNP	Genotype	Asthmatics ($n = 270$)	NC ($n = 120$)	*P* value
−1508A>G (rs868853)	AA	224 (83.3)	102 (86.4)	0.288
	AG	47 (17.5)	16 (13.6)	0.417
	GG	2 (0.74)	2 (1.69)	0.648
−642C>G (rs869951)	CC	94 (34.9)	52 (44.1)	0.61
	CG	139 (51.7)	47 (39.8)	0.101
	GG	36 (13.4)	19 (16.1)	0.068
ht1 [AC]	+/+	94 (34.9)	52 (44.0)	0.171
	+/−	139 (51.7)	47 (39.8)	0.705
	−/−	36 (13.4)	19 (16.1)	0.068
ht2 [AG]	+/+	17 (6.3)	14 (11.9)	0.077
	+/−	156 (57.9)	50 (42.3)	0.094
	−/−	96 (35.7)	54 (45.7)	0.366
ht3 [GG]	+/+	2 (0.74)	2 (1.7)	0.404
	+/−	17 (6.3)	3 (2.5)	0.75
	−/−	250 (92.9)	113 (95.7)	0.454

NC: normal healthy control; *n*: number of subjects; ht: haplotype. *P* value was obtained by logistic regression analysis with age and sex as covariates.

Table 3: Association of *ABCC4* −1508A>G polymorphism with clinical features, metabolite, and cytokine profiles in asthmatic patients.

Clinical features	AA ($n = 305$)	AG/GG ($n = 48$)	P value
Age (years)†	39.01 ± 14.34	40.92 ± 15.09	0.735
Sex, male (%)‡	32.14	28.57	0.335
Atopy (%)‡	57.46	51.18	0.928
Total IgE (IU/mL)†	451.48 ± 965.78	278.32 ± 319.68	0.240
Rhinosinusitis (%)‡	81.21	18.78	0.143
Nasal polyp (%)‡	81.11	18.88	0.889
FEV_1 (%)†	86.45 ± 19.66	87.54 ± 14.57	0.638
PC_{20} methacholine (mg/mL)	10.19 ± 17.07	5.82 ± 8.04	0.119
Total IgE	451.48 ± 965.78	278.32 ± 319.69	0.24
Asthma duration (years)†	6.30 ± 6.03	12.81 ± 32.36	0.237
Inflammatory cytokines	AA ($n = 121$)	AG/GG ($n = 26$)	P value
MPO (μg/L)†	108.26 ± 79.5	150.91 ± 94.13	**0.027**
IL-8 (pg/mL)†	16.82 ± 14.45	14.47 ± 7.5	0.616
IL-18 (pg/mL)†	247.77 ± 166.06	281.92 ± 197.10	0.560
Eotaxin-1 (ng/mL)†	85.67 ± 63.67	85.22 ± 65.11	0.841
Eotaxin-2 (ng/mL)†	1146.92 ± 802.14	1104.95 ± 641.09	0.650
Periostin (ng/mL)†	71.07 ± 33.62	91.83 ± 50.85	**0.016**
Serum metabolites (ng/mL)	AA ($n = 43$)	AG/GG ($n = 17$)	P value
15-HETE	265.98 ± 185.69	334.34 ± 132.47	0.195
LTE4	18.22 ± 17	18.8 ± 14.71	0.731
PGF2α	23.55 ± 9.3	21.96 ± 8.69	0.653
TXB2	0.41 ± 0.42	0.39 ± 0.17	0.952
Eoxin C4	4.82 ± 9.5	8.14 ± 11.95	0.224
S1P	111.75 ± 42.16	120.91 ± 42.88	0.419
Urinary metabolites (pmol/mg Cr)	AA ($n = 23$)	AG/GG ($n = 8$)	P value
15- HETE	271.91 ± 89.87	332.5 ± 99.31	**0.032**
LTE4	7648.29 ± 14378.31	2601.73 ± 3043.59	0.616
PGF2α	8618.51 ± 22942.8	1678.71 ± 978.76	0.65
TXB2	5686.61 ± 4305.7	4798.25 ± 3957.21	0.765
Eoxin C4	144.78 ± 94.07	124.75 ± 46.65	0.602
S1P	32.56 ± 8.25	41.5 ± 9.35	**0.010**

†Values were presented as mean ± SD, whereas ‡ values were shown as percentage. Cr: creatinine; LTE4: leukotriene E4; PGF2α: prostaglandin F2α; TXB2: thromboxane B2; 15-HETE: 15-hydroxyeicosatetraenoic acid; MPO: myeloperoxidase; S1P: sphingosine-1-phosphate. P value was obtained by logistic regression analysis with age and sex as covariates.

and −1508G, to determine the transcriptional effects of the *ABCC4* −1508A>G polymorphism. The constructs comprised of the *ABCC4* sequence and a luciferase reporter gene were transfected into A549, U937, and HMC-1 cells. The reporter activities of the −1508G allele and −1508A allele constructs were compared. Luciferase activity was significantly enhanced in the construct with the −1508G allele compared with the −1508A allele in all cell lines ($P < 0.01$ for each, Figure 2).

4. Discussion

Asthma is a complex, chronic respiratory disease with a wide clinical spectrum, with contributions from several environmental and genetic factors [20, 21]. Recent studies have shown that various gene polymorphisms influence the onset and progression of asthma [22, 23]. In the present study, we selected two SNPs within the *ABCC4* gene to examine their potential roles in asthma pathogenesis based on their relationships with eicosanoid, sphingolipid metabolites, and proinflammatory cytokines. To date, the roles of these genes in asthma have not been determined.

Most studies on ABCC4 have focused on its role in cancer chemotherapy, particularly its ability to confer clinical drug resistance [24]. Diverse studies have shown that ABCC4 induces the extrusion of cyclic nucleotides in various cell types; however, it has emerged as the main transporter of cAMP [25]. van de Ven et al. reported that ABCC4 plays an important role in dendritic cell migration in humans and that inhibition of ABCC4 activity decreases dendritic cell migration in the skin [13]. However, no association study to date has examined the relationships between this gene and immunological diseases such as asthma, although this gene is associated with immunological processes.

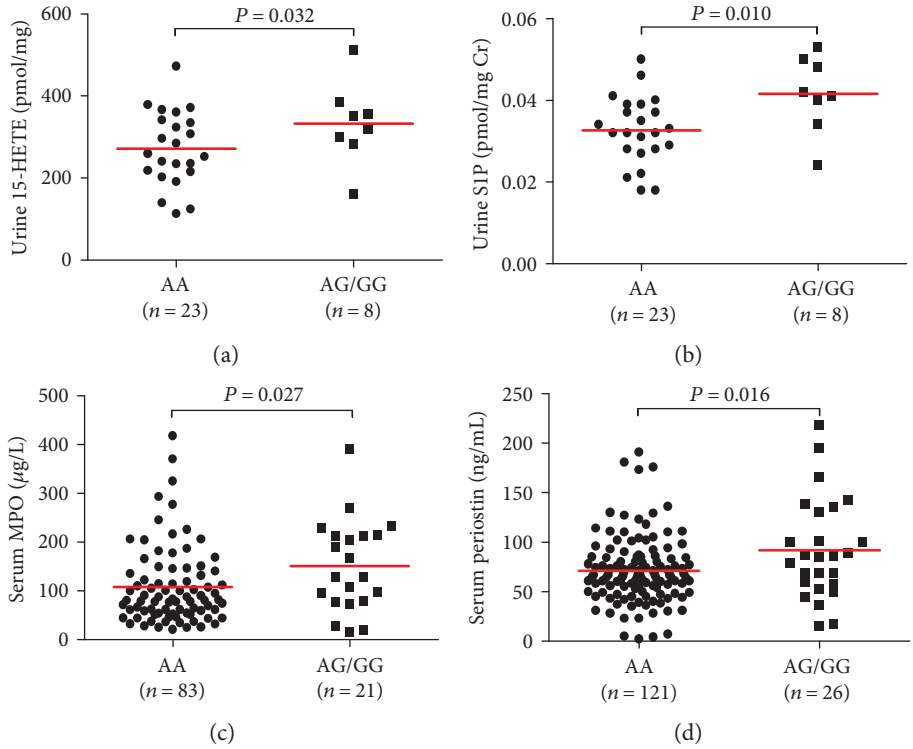

Figure 1: Asssociation of the baseline levels of (a) urinary 15-HETE, (b) urinary S1P, (c) serum MPO, and (d) serum periostin with the *ABCC4* −1508A>G polymorphisms in asthma patients. *ABCC4*: ATP-binding cassette subfamily C member 4; 15-HETE: 15-hydroxyeicosatetraenoic acid; MPO: myeloperoxidase; S1P: sphingosine-1-phosphate. *P* values were obtained by logistic regression with age and sex as covariates. The data represented as mean values ± SD.

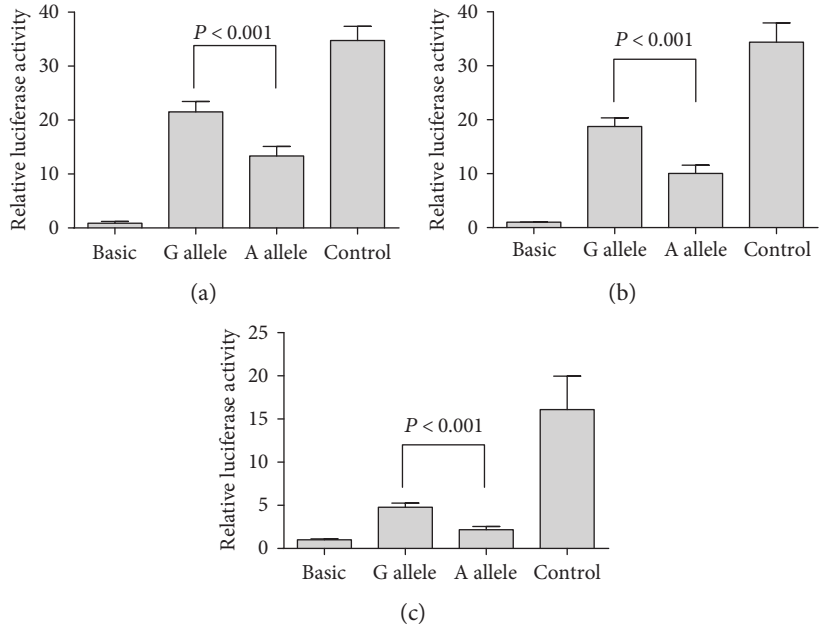

Figure 2: Effect of the *ABCC4* −1508A>G polymorphism on the promoter activity. Transfection of plasmid constructs carrying *ABCC4* −1508A or G allele into (a) A549, (b) U937, and (c) HMC-1 cells. All *P* values were obtained by Mann–Whitney *U* test. Data represent the mean values of three independent experiments ± SD. Each experiment was conducted in triplicate.

We first identified a significant association between the *ABCC4* −1508A>G polymorphism and the urinary levels of metabolites, including 15-HETE and S1P. 15-HETE is the major metabolite of arachidonic acid in the 15-lipoxygenase pathway [26]. 15-HETE was recently proposed as a biomarker for asthma severity, as its levels were 5-fold higher in eosinophils from severe asthmatics than from mild asthmatics [27]. 15-HETE undergoes reaction to produce 14,15-epoxides, designated eoxins A4, C4, D4, and E4 in eosinophils, mast cells, and nasal polyps from allergic subjects [28]. Similar to cysteinyl leukotrienes, eoxins are potent pro-inflammatory agents [28]. We found a tendency toward increased eoxin C4 levels in asthmatics with the −1508G allele, although no significance was observed, suggesting inflammation of the airways in eosinophilic asthma. Beside, S1P, a major metabolite of sphingolipid pathway, has been identified as a biomarker for asthma in our previous study [29]. S1P is suggested to contribute to airway hyperreactivity and release of IL-4 and IL-13, thereby involving in asthma pathogenesis [30, 31]. Inhibitors of ABCC4 block the release of S1P from platelet granules [14]. Therefore, the −1508G allele of *ABCC4* polymorphism may be associated with the transport of metabolites from immune cells.

Secondly, we found enhanced serum periostin levels in asthma patients carrying the −1508G allele [32]. Periostin is a multifunctional protein expressed in many types of inflammatory cells, such as epithelial cells, mast cells, and so forth [33]. Recent studies have suggested that periostin modulates Th2-mediated asthma pathogenesis by assisting in the recruitment of inflammatory cells, particularly eosinophils, to the lungs [34]. Moreover, periostin is known to facilitate the activation of dendritic cells, thereby rendering airway hyperresponsiveness and airway inflammation in mice [35, 36]. Beside, we discovered an increased serum level of MPO in the *ABCC4* −1508G allele carriers comparing to that of noncarriers. Activated neutrophils secrete MPO which then induces oxidative stress, thereby resulting in oxidative damage of respiratory cells, lung inflammation, cytotoxicity, airway obstruction, and decrease of lung function [37, 38]. A polymorphism of the *MPO* gene was proposed to be associated with asthma susceptibility [38]. Taken together, the *ABCC4* −1508G allele may also interfere the release of periostin and MPO from inflammatory cells.

Although we did not find any genetic association in asthma patients in the present study, we found that a functional polymorphism of the *ABCC4* gene (−1508A>G) may affect its promoter activity, thereby affecting release of 15-HETE, S1P, periostin, and MPO from innate immune cells in asthma.

5. Conclusions

To our knowledge, this is the first study to provide evidence of associations between *ABCC4* and 15-HETE, S1P, periostin, and MPO in asthma patients. The present findings further suggest that ABCC4 represents a new potential target of asthma therapy. However, further studies are required to understand the functional mechanism of *ABCC4* polymorphisms on airway inflammation in asthmatics.

Conflicts of Interest

The authors have no conflict of interest to declare.

Authors' Contributions

Sailesh Palikhe and Eun-Mi Yang performed the experiments. Udval Uuganbayar and Hoang Kim Tu Trinh performed the statistical analysis and participated in writing the manuscript. Ga-Young Ban recruited patients and normal healthy controls. Hae-Sim Park and Seung-Hyun Kim interpreted the data, revised the manuscript, and supervised all the steps of this study.

Acknowledgments

This research was supported by the National Research Foundation of Korea (NRF) grant, funded by the Korean government (MSIP) (Grant no. 2015R1C1A2A01053492) and the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI) grant, and funded by the Ministry of Health and Welfare, Republic of Korea (Grant no. HI16C0992).

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