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Chlamydophila pneumoniae enhances secretion of VEGF, TGF-β and TIMP-1 from human bronchial epithelial cells under Th2 dominant microenvironment

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Purpose: *Chlamydophila pneumoniae* infection in the airways is thought to be associated with the pathogenesis of asthma, especially in non-atopic severe asthma with irreversible airway obstruction that may be related to airway remodeling. Here, we investigated whether *C. pneumoniae* infection enhances the secretion of critical chemical mediators for airway remodeling, such as VEGF, TGF-β, and TIMP-1, in human bronchial epithelial cells (BECs) in a Th2-dominant microenvironment. **Methods:** Human bronchial epithelial cells (BEAS-2B cells) were infected with *C. pneumoniae* strain TW183 and cultured in both a Th1-dominant microenvironment with INF-γ and a Th2-dominant microenvironment with IL-4 or IL-13 added to the culture medium. The VEGF, TGF-β, and TIMP-1 levels in the culture supernatants were measured using enzyme-linked immunosorbent assays (ELISA). The activation of NF-κB in each experimental condition was determined using an electrophoretic mobility shift assay. **Results:** *Chlamydophila pneumoniae*-infected BECs showed enhanced secretion of VEGF, TGF-β, and TIMP-1 compared with non-infected BECs. The levels of cytokines secreted from BECs were increased more when IL-13 was added to the culture medium. *C. pneumoniae*-infected BECs also showed increased NF-κB activation. **Conclusions:** These results suggest that *C. pneumoniae* may play a role in the pathogenesis of airway remodeling in asthma, revealing a Th2-dominant immune response. Further studies are required to clarify the precise mechanism of *C. pneumoniae* infection in airway remodeling.

Key Words: Asthma; *Chlamydophila pneumoniae*; epithelial cells; vascular endothelial growth factor A; tissue inhibitor of metalloproteinases; transforming growth factor beta

INTRODUCTION

Chlamydophila pneumoniae is a widespread respiratory pathogen that may cause acute and chronic respiratory tract illness.[1,2] Recently, *C. pneumoniae* infection has emerged as one of the important factors in the pathogenesis of asthma because this organism has been reported to be associated with chronic asthma and its severity,[3-5] acute exacerbation,[6,7] and even the development of late-onset asthma in both adults and children.[8,9] In addition, a striking association between *C. pneumoniae* infection and persistent airflow limitation has been suggested in longitudinal and cross-sectional sero-epidemiologic studies.[10,11]

Chlamydophila pneumoniae can infect and then reside and replicate in various structural cells including smooth muscle cells, fibroblasts, endothelial cells, bronchial epithelial cells (BECs), and various immune cells. Infection of various cell types with *C. pneumoniae* induces the increased release of many pro-inflammatory chemical mediators, including tumor necrosis factor alpha (TNF-α), interleukin (IL)-8, IL-6, and basic fibroblast growth factor (bFGF), and upregulates adhesion molecules.[12-14] In addition, a recent study suggested that *C. pneumoniae*-induced inhibition of apoptosis increases the longevity of the host cell, enhancing the survival of *C. pneumoniae* itself.[15] *C. pneumoniae* infection can stimulate the production of IL-10, which can downregulate the expression of major histocompatibility complex class I molecules,[16] and it might partially contribute to the resistance to apoptosis and persistence of infected

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cells. Furthermore, we previously demonstrated that *C. pneumoniae* enhanced the proliferation of human peripheral blood mononuclear cells and reduced their steroid responsiveness via a TNF-α-dependent pathway.[17]

Bronchial epithelial cells (BECs) are a first line of defense in viral and bacterial infection. Growing evidence suggests that epithelial cells actively contribute to the pathogenesis of bronchial asthma through the secretion of chemoattractants and pro-inflammatory mediators, which play an important role in the initiation and perpetuation of airway inflammation. Previous studies demonstrated that *C. pneumoniae* can act as a warning system for local immune cells, and airway inflammation can be induced via the *C. pneumoniae*-induced release of various pro-inflammatory cytokines from BECs.[18,19] Recently, we demonstrated that *C. pneumoniae*-infected BECs enhanced the secretion of CCL20 and VEGF involved in oxidative stress.[20] Taken together, these findings suggest that chronic airway *C. pneumoniae* infection could lead to persistent airway inflammation and may be involved in the airway remodeling observed in the asthmatic airway.

Nevertheless, the exact pathophysiological role of *C. pneumoniae*-infected BECs in the processes of airway remodeling in asthma remains unclear. This study evaluated the effect of *C. pneumoniae* infection on the secretion of important chemical mediators of airway remodeling, such as vascular endothelial growth factor (VEGF), transforming growth factor beta (TGF-β), and tissue inhibitor of metalloproteinase (TIMP-1) in human BECs in a Th2-dominant microenvironment.

MATERIALS AND METHODS

Culture and inoculum preparation of *Chlamydophila pneumoniae*

Chlamydophila pneumoniae was cultured and prepared as described elsewhere.[18,21] In brief, strain TW183 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and propagated in HEp-2 cells (ATCC) grown in Eagle's minimum essential medium (Sigma, St. Louis, MO, USA) supplemented with 1% nonessential amino acids, L-glutamine (2 mM), 10% fetal bovine serum (FBS), non-essential amino acids, gentamicin (10 mg/L), vancomycin (50 mg/L), amphotericin B (2 mg/L), and HEPES buffer solution (GIBCO BRL Life Technologies, Grand Island, NY, USA, 10 mM). Confluent monolayers were infected with *C. pneumoniae* by centrifuging (750× *g*, 35°C, 45 minutes) infectious inoculae onto the host cells. The supernatant was replaced by antibiotic-free growth medium containing cycloheximide (1 mg/L, Sigma). Infected cells were cultured in a 37°C, 5% CO_2-saturated humidified incubator, harvested on day 3, and disrupted by two cycles of freezing and thawing and ultrasonification, and similar harvests were pooled. Cell debris was removed by centrifugation at 800× *g* for 10 minutes at 4°C. Aliquots diluted with an equal volume of sucrose-phosphate-glutamate buffer were stored at -80°C until use.

Preparation of BEAS-2B cells and infection with *Chlamydophila pneumoniae*

The human BEC cell line BEAS-2B was obtained from ATCC (CRL-9606). The cells were subcultured in a 1:1 medium mixture of RPMI 1640 and LHC-9 without serum. We used dishes and plates coated with vitrogen 100 and prewarmed at 37°C for 1 hour. The cells were cultured in a 37°C, 5% CO_2 saturated humidified incubator. Stored *C. pneumoniae* suspensions were thawed, diluted, and inoculated onto BEAS-2B by centrifuging (750× *g*, 35°C, 45 minutes).

Immunofluorescence analysis of *Chlamydophila pneumoniae*–infected BEAS-2B cells

We evaluated the *C. pneumoniae* infection and determined the bacterial titer using an immunofluorescence (IF) assay after 48 and 72 hours, as previously described.[21] In brief, infected cells were fixed with methanol-acetone (1:1) and incubated with antibody to major outer membrane protein (mouse IgG3 anti-*C. pneumoniae* monoclonal antibody (mAb); Dako, Cambridgeshire, UK), followed by fluorescein isothiocyanate (FITC)-conjugated secondary antibody (goat $F(ab')_2$ anti-mouse IgG; Sigma). After counting *C. pneumoniae* inclusions under a fluorescence microscope and correcting for dilution factors, the bacterial titers were expressed as inclusion-forming units (IFU/cell).

Cell proliferation assay

After confirming *C. pneumoniae* infection with the IF assay, we evaluated whether the cellular proliferation after *C. pneumoniae* infection was maintained and determined the proper incubation time and titer of *C. pneumoniae*. BEAS-2B cells were grown to confluence in 96-well plates and were infected with titers of 0, 0.1, 1, and 10 IFU for 96 hours. Then, 10 μL/well of WST-1 reagent was added to each sample. After a 1-hour incubation in a humidified atmosphere (37°C, 5% CO_2), the optical density of the wells was determined by spectrophotometry at a wavelength of 440 nm. The analysis was performed 12, 24, 48, 72, and 96 hours after the initial infection. The results were expressed as the percentage increase in cellular proliferation calculated based on the colony-forming units of each sample.

Analysis of VEGF, TGF-β, and TIMP-1 production

BEAS-2B cells were infected with *C. pneumoniae* (1 IFU/cell determined in a proliferation assay) by centrifuging and cultured as described above. Cell-culture supernatants were collected and analyzed 24 hours after *C. pneumoniae* infection, and the levels of VEGF, TGF-β, and TIMP-1 were determined using commercial enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's guidelines. To evaluate the effect of different microenvironments on the production of cytokines, we added IL-4 (20 ng/mL) or IL-13 (20 ng/mL) for a Th2-dominant

environment and INF-γ (20 ng/mL) for a Th1-dominant environment to the culture medium 30 minutes before *C. pneumoniae* infection.

Evaluation of NF-κB activation

To investigate whether NF-κB activation is associated with the effect of *C. pneumoniae* infection on BECs, an electrophoretic mobility shift assay (EMSA) was performed. First, nuclear protein was isolated and prepared using a described method.[22] Briefly, cells were collected with a scraper and washed twice with phosphate-buffered saline. Then, the cell pellet was suspended in buffer A (10 mM HEPES pH 7.8, 15 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 0.5 mM DTT, 1 mM PMSF, 2 μg/mL leupeptin) supplemented with 1% Igepal. After a 15-minute incubation at 0°C, the lysed cellular suspension was briefly mixed on a vortex and microcentrifuged for 30 seconds at 4°C. After removing the supernatant, the cell pellet was resuspended in buffer B (20 mM HEPES pH 7.8, 50 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 20% glycerol, 0.5 mM DTT, 1 mM PMSF, 2 μg/mL leupeptin) for 30 minutes at 4°C. The cellular suspension was microcentrifuged at 14,000 rpm for 30 seconds. Then, the supernatant was collected and stored at -80°C.

The consensus NF-κB oligonucleotide (sense 5'-AGT TGA GGG GAC TTT CCC AGG C-3', antisense 3'-TCA ACT CCC CTG AAA GGG TCC G-5') was end-labeled with [γ^{32}P]ATP using T4 polynucleotide kinase. EMSA binding reactions were performed by incubating 5 μg of nuclear extract with 1 μg of poly (dI:dC) in a binding reaction mixture (100 mM HEPES, pH 7.8, 60 mM KCl, 1 mM EDTA, 7% glycerol, 1 mM DTT) for 30 minutes at 37°C. For the antibody supershift, NF-κB antibodies p50 and p65 were added to each reaction mixture and allowed to bind for 1 hour at 0°C. The reaction mixture was electrophoresed on 6% polyacrylamide gels before vacuum drying and exposure to a storage phosphor screen for quantification and documentation.

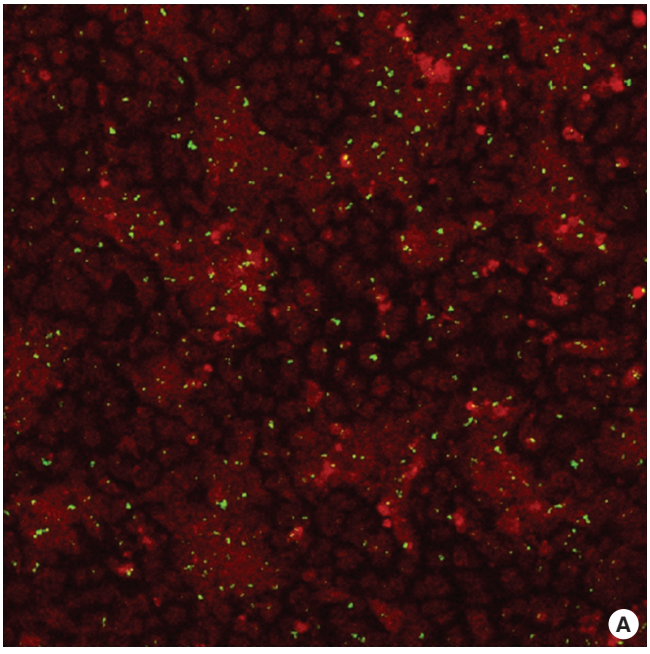
Statistical methods

All data are expressed as the mean±SEM. The comparisons were made using the Mann-Whitney *U*-test. Statistical significance was defined as $P<0.05$.

RESULTS

Identification of *C. pneumoniae* infection in BECs

To confirm the *C. pneumoniae* infection in host cells, we identified inclusion bodies (element body, EB) of *C. pneumoniae* in BEAS-2B cells for 48 hours using an IF assay; most of the cultured cells contained numerous EBs for up to 72 hours (Fig. 1A). Based on the results of the proliferation assay performed to determine the proper incubation time and bacteria titer, the cellular proliferation of cells infected with *C. pneumonia* at a dose of 1 IFU/cell increased continuously, beginning 24 hours after infection, and reached near-maximal levels 48 hours after infection, whereas the cellular proliferation decreased with higher titers of bacteria and more than 72 hours after infection



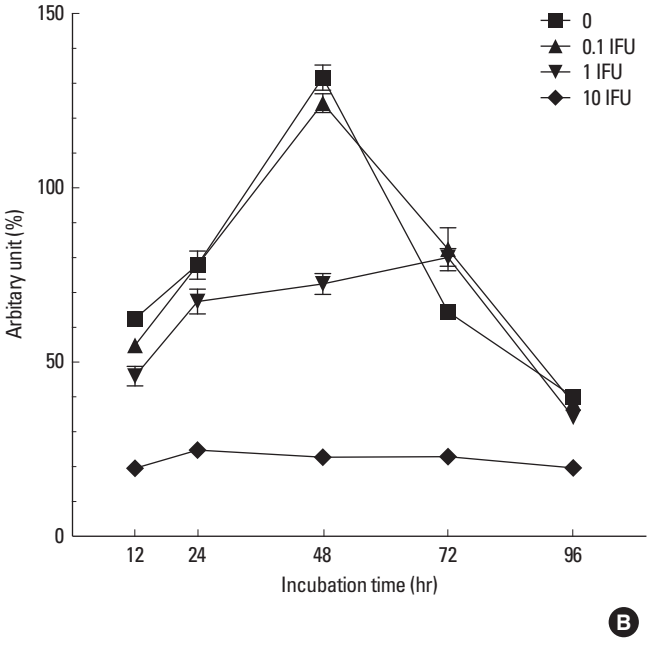


Fig. 1. *C. pneumoniae* strain TW-183 in BEAS-2B cells. (A) BEAS-2B cells grown on cover slips were infected with *C. pneumoniae*. After 24 hr, cells were fixed with methanol, and stained using a fluorescein conjugated, pneumonia IgG-FITC (×200), BEAS-2B cells (red), *C. pneumoniae* (green). Proliferation effect of BEAS-2B cells infected with *C. pneumoniae*. (B) BEAS-2B cells were infected with *C. pneumoniae* at 0 (■), 0.1 (▲), 1 (▼), and 10 (◆) inclusion forming unit (IFU)/cell and cell viability measured at different time points post infection (12, 24, 48, 72, and 96 hr). Results shown are representative of three independent experiments.

(Fig. 1B). Therefore, we decided to use 1 IFU/cell and 24 hours after infection as appropriate experimental conditions for further analyses.

Enhanced secretion of VEGF, TGF-β, and TIMP-1 in *C. pneumoniae*-infected bronchial epithelial cells

Compared with uninfected cells, the levels of VEGF, TGF-β, and TIMP-1 in the supernatants of infected BECs were increased significantly. These results were observed not only in the Th1- and Th2-dominant microenvironments, but also under control conditions (Fig. 2). IL-13 further enhanced the production of VEGF and TGF-β from both *C. pneumonia*-infected and uninfected BECs, whereas treatment with IL-4 and INF-γ did not have a significant effect on the secretion of those cytokines. By contrast, the secretion of TIMP-1 from both *C. pneumonia*-infected and uninfected BECs was higher with the INF-γ treatment.

NF-κB activation in *C. pneumoniae*-infected bronchial epithelial cells

As shown in Figure 3A, the nuclear extracts yielded stronger bands in comparison to the uninfected cells, exhibiting relatively higher levels of NF-κB DNA-binding activity. When the enhanced NF-κB binding was quantified by densitometry, *C. pneumoniae* infection increased the NF-κB binding under Th1 and Th2 microenvironments. Conversely, INF-γ pretreatment reduced the NF-κB binding activity in uninfected cells (Fig. 3B).

DISCUSSION

In this study, we demonstrated that *C. pneumoniae*-infected BECs significantly enhanced the secretion of VEGF, TGF-β, and TIMP-1, which are thought to be the major cytokines involved in airway remodeling. In addition, the levels of these cytokines were increased further under a Th2-dominant microenvironment. These results suggest that *C. pneumoniae* infection in the asthmatic airway may be involved in the pathogenesis of airway remodeling.

Bronchial asthma is a Th2-dominant inflammatory airway disease with variable airway obstruction. In some asthmatics, persistent airflow limitation and a decline in lung function have been observed and could be one of the critical factors contributing to severe asthma. It is also thought that structural changes

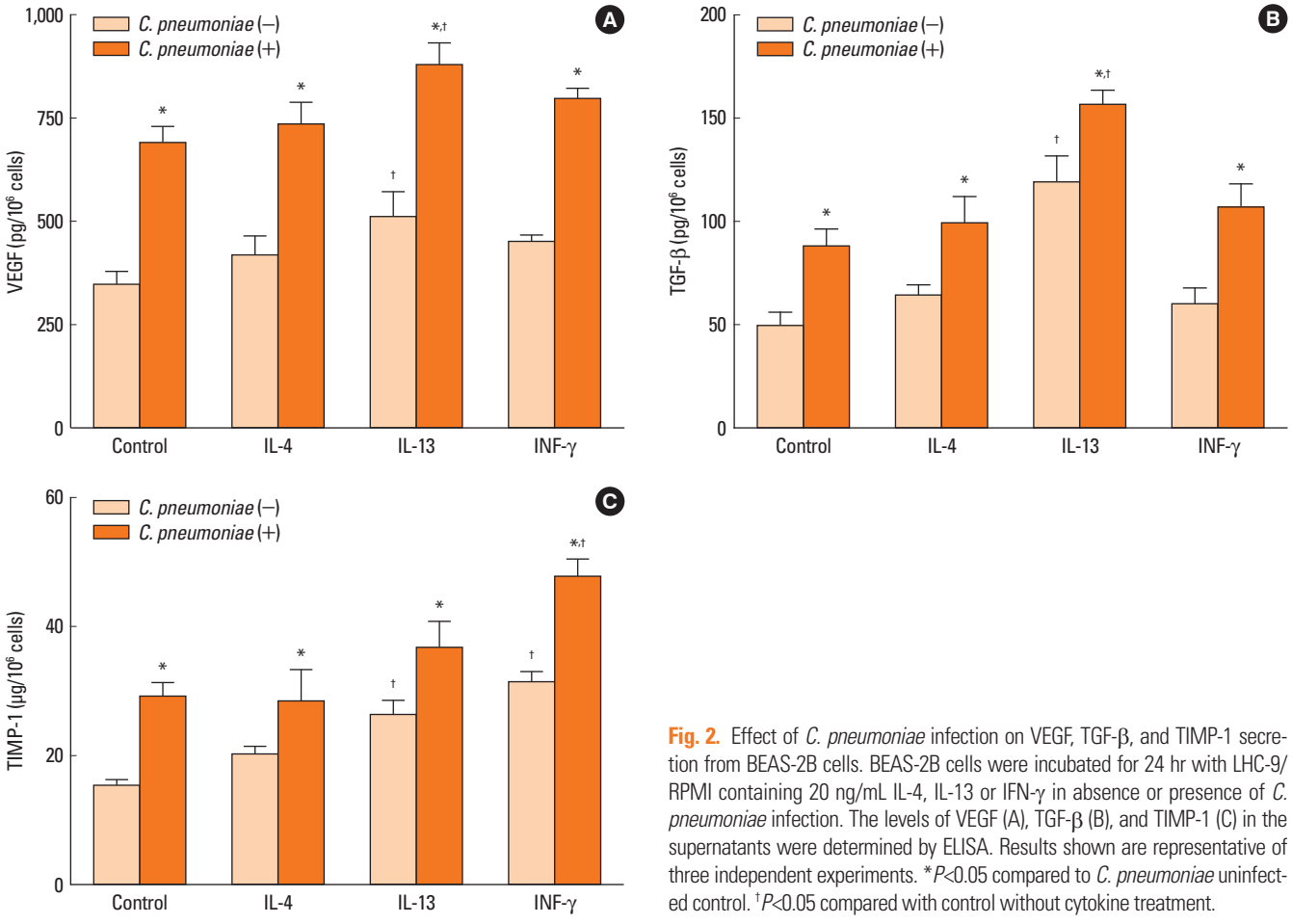


Fig. 2. Effect of *C. pneumoniae* infection on VEGF, TGF-β, and TIMP-1 secretion from BEAS-2B cells. BEAS-2B cells were incubated for 24 hr with LHC-9/RPMI containing 20 ng/mL IL-4, IL-13 or IFN-γ in absence or presence of *C. pneumoniae* infection. The levels of VEGF (A), TGF-β (B), and TIMP-1 (C) in the supernatants were determined by ELISA. Results shown are representative of three independent experiments. **P*<0.05 compared to *C. pneumoniae* uninfected control. †*P*<0.05 compared with control without cytokine treatment.

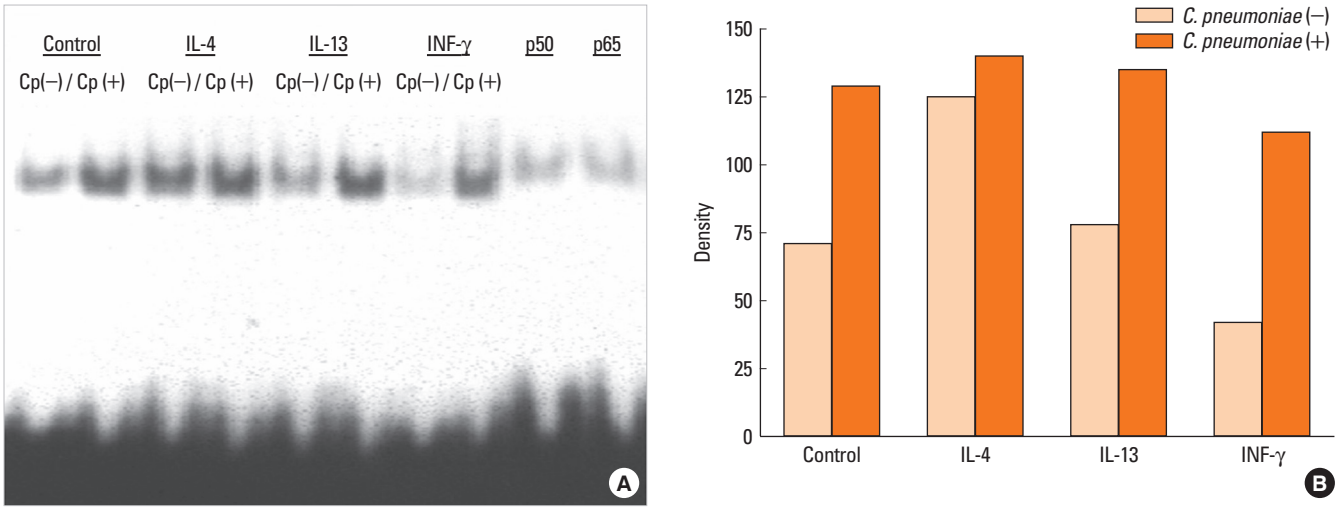


Fig. 3. NF-κB activity by *C. pneumoniae* infection in BEAS-2B cells. Changes of NF-κB binding activity using EMSA (A) gel using g32 P-labeled oligonucleotide probes for NF-κB. Densitometry of NF-κB specific binding activity (B) depicts the densitometric evaluation of NF-κB DNA-binding activity, normalized to the level of standard reaction.

in the airways, widely referred to as airway remodeling, are associated with irreversible fixed airway obstruction. The remodeling in the asthmatic airway is characterized by subepithelial fibrosis, increased airway smooth muscle mass, hyperplasia of mucous glands and goblet cells, angiogenesis, and alterations in the extracellular matrix components.[23] Although chronic inflammation is thought to be a key factor contributing to airway remodeling, the precise mechanisms remain poorly understood.

For decades, studies have suggested a strong association between viral or bacterial infection and asthma exacerbations, as well as chronic persistent asthma. *C. pneumoniae* is an obligate intracellular Gram-negative bacterium that infects humans as a respiratory pathogen. *Chlamydophila* has a biphasic life cycle, existing as either an elementary body (EB) or a reticulate body (RB). The metabolically inactive extracellular infectious EB is internalized by the host cell, where it differentiates into the metabolically active RB. Under certain conditions, RBs do not re-differentiate directly into EBs, but form interim non-replicating persistent bodies, allowing the bacterium to maintain a chronic latent infection.[24] Many ongoing studies are investigating the association between *C. pneumoniae* and the pathogenesis of various chronic inflammatory diseases, such as atherosclerosis, macular degeneration, and Alzheimer's disease.

To our knowledge, this is the first study to investigate the potential role of *C. pneumoniae* infection in enhanced secretion of the cytokines associated with airway remodeling by BECs. Our results demonstrated that *C. pneumoniae*-infected BECs lead to the enhanced secretion of VEGF, TGF-β, and TIMP-1. Although the functions of these cytokines are very diverse, the pivotal features of airway remodeling in asthma, such as enhanced tissue fibrosis, angiogenesis, and hypertrophy of constitutional cells may be attributed to the increased levels of VEGF, TGF-β, and TIMP-1, at least in part. Another study reported that *C. pneumoniae*-infected BECs released more IL-8 and prostaglandin E2 and expressed more ICAM-1 in a time-dependent manner.[18] It was also suggested that the blocking of bacterial adherence reduced the gene expression of IL-8, INF-γ, and TNF-α in BECs.[19] Collectively, these findings indicate that *C. pneumonia*-infected BECs may play an important role in the pathogenesis of asthma through enhanced induction of critical chemical mediators.

In this study, *C. pneumoniae*-infected BECs under a Th2-dominant microenvironment secreted more VEGF, TGF-β, and TIMP-1 than did those under control conditions, especially with the addition of IL-13. Considering the possibility of chronic or recurrent airway infection with *C. pneumoniae* in asthmatic patients, infected epithelial cells could produce various additional pro-inflammatory cytokines under a pre-existing Th2-dominant microenvironment. These results are consistent with a report in which Th2 cytokines enhanced TGF-β and VEGF production.[25] In this context, our results showed that the interaction between the Th2 environment and superimposed *C. pneumoniae* infection is more likely to play an important role in airway remodeling.

In this study, the production of TIMP-1 was enhanced after pretreatment with the Th1-derived cytokine INF-γ, although we also saw a tendency toward enhanced TIMP-1 secretion with IL-13 treatment. Previous studies reported that the levels of INF-γ in the blood and sputum of asthmatics were increased and correlated with asthma severity.[26,27] TIMP-1, which is the major inhibitor of matrix metalloproteinase 9 (MMP-9), has fibrogenic properties and promotes the growth of fibroblasts and myofibroblasts. Some researchers have reported that an excess

of TIMP-1 over MMP-9 in the bronchial secretions of asthmatic patients is associated with chronic airflow obstruction.[28,29] Therefore, INF-γ-induced TIMP-1 secretion may be involved in the pathogenesis of severe asthma with fixed airway obstruction, although the clinical and pathophysiological implications and exact mechanisms are not clear. Unfortunately, we did not measure the MMP-9 levels, so it is hard to draw any conclusions regarding the role of TIMP-1 in the association of INF-γ and *C. pneumonia*. Further studies should investigate the MMP-9 production in *C. pneumonia* infection and the role of INF-γ during the production of this enzyme.

NF-κB was activated in *C. pneumoniae*-infected BECs in our study, whereas stimulation with IL-4, IL-13, or INF-γ had no further effect on the level of NF-κB activation. This increased response of NF-κB activation to the *C. pneumoniae* infection was consistent with previous studies of vascular endothelial cells and epithelial cells.[9,30] Furthermore, the results of a recent study suggested that *C. pneumoniae* triggers a pro-inflammatory signaling cascade involving p38 MARK-dependent NF-κB activation, resulting in subsequent GM-CSF release.[31] Nevertheless, the exact mechanism by which *C. pneumoniae* infection induces VEGF, TGF-β, and TIMP-1 secretion is still unclear.

Interest is growing in developing strategies for preventing airway remodeling. The search for novel therapeutic agents based on a clear understanding of the pathogenesis of airway remodeling is also intensifying. In fact, some evidence suggests that macrolide antibiotics do have beneficial effect in asthma, although it is not clear whether the benefit is due to the anti-inflammatory or antimicrobial effects of these antibiotics. Our data provide additional evidence that macrolide antibiotic treatment could have beneficial effects on reducing pro-inflammatory cytokines and airway remodeling, especially in asthmatic patients with chronic *C. pneumoniae* infection. Further work focusing on the identification of chronic infection is needed.

In conclusion, our results suggest that *C. pneumoniae*-infected BECs may play a role in the pathogenesis of airway remodeling in asthma under a Th2-dominant microenvironment through enhanced secretion of various cytokines.

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