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Bronchial epithelial cells release inflammatory markers linked to airway inflammation and remodeling in response to TLR5 ligand flagellin

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ABSTRACT

Background/Aims: Flagellin, which is abundant in gram-negative bacteria, including *Pseudomonas*, is reported to influence on inflammatory responses in various lung diseases. However, its effect on airway epithelial cells in contribution to asthma pathogenesis is not elucidated yet. We aimed to investigate the effect of TLR5 ligand flagellin on the transcriptomic profile of primary human epithelial cells and to determine the markers of airway inflammation.

Methods: Normal human bronchial epithelial (NHBE) cells were grown and differentiated in air-liquid interface (ALI) culture for 14–16 days. The cells were treated with flagellin *in vitro* at 10 and 100 ng/ml for 3 and 24 h. The conditioned media and cells were harvested to validate inflammatory markers involved in airway inflammation using ELISA, Western blot, and quantitative PCR methods. RNA-sequencing was performed to investigate the transcriptional response to flagellin in ALI-NHBE cells.

Results: Altered transcriptional responses to flagellin in differentiated bronchial epithelial cells were determined, including genes encoding chemokines, matrix metalloproteinases, and antimicrobial biomolecules. Pathway analysis of the transcriptionally responsive genes revealed enrichment of signaling pathways. Flagellin induced the mRNA expressions of proinflammatory cytokines and chemokines, and secretion of GM-CSF, CXCL5, CCL5 and CXCL10. Flagellin enhanced the protein expression of MMP-13 in TGF-β1 and TGF-β2 pretreated cell lysates and Wnt/β-catenin signaling.

Conclusions: These findings suggest that flagellin could be a potent inducer of inflammatory markers that may contribute to airway inflammation and remodeling.

Keywords: Airway, Epithelial cell, Flagellin, Inflammation, TLR5

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INTRODUCTION

The airway epithelium is at the interface between inhaled environment and the host respiratory system, and plays critical functions in host defense mechanisms.[1] The epithelial barrier dysfunction is frequently observed in asthma and increases the susceptibility of airways to environmental irritants.[2] Respiratory infection is one of the common triggers in asthma and is associated with the development of disease onset, severity, and exacerbation.[3]

Microbes of the respiratory tract mediate distinct inflammatory processes and may impact the phenotypes and progression of the disease differentially.[4,5] Innate immune cells respond to infection via members of structurally related receptors termed toll-like receptors (TLRs), which recognize highly conserved molecular structures called pathogen-associated molecular patterns. These transmembrane pattern recognition receptors are typically expressed on various immune cells and airway epithelial cell in response to invading pathogens and initiate innate and adaptive immune responses.[6]

Flagellin is commonly referred to by its contribution to the virulence of pathogenic bacteria providing motility and adhesion to host surfaces.[7] Flagellin activates innate immunity via TLR5 and is produced in Gram-negative β-/γ-proteobacteria and gram-positive Firmicutes bacteria.[8] Microbiome studies have shown that Proteobacteria, particularly *Pseudomonas*, are more common in patients with asthma and chronic obstructive pulmonary disease and are associated with higher use of corticosteroids and disease severity.[5,9–11] Serum flagellin-specific antibodies were reported higher in subjects with asthma,[12] and *TLR5* deficiency was associated with reduced response to flagellin and lower symptom severity.[13,14]

In the previous studies, flagellin showed controversial influence in terms of the development of allergic diseases. In mice, flagellin stimulated ovalbumin-induced T helper type 2 (Th2) responses, and activation of TLR5 could promote asthma by priming the allergic responses to indoor allergens.[12] *In vitro*, *P. aeruginosa*-derived flagellin upregulated proinflammatory interleukin 6 (IL-6) and IL-8 expression in the epithelial cell and induced phosphorylation of mitogen-activated protein kinases,[15] which is critical signaling for immune cell function and differentiation. In contrast, some reports have addressed the protective role of flagellin against allergic responses through TLR5 which was derived from different bacterial species.[16,17] *In vivo*, intranasal administration of flagellin induced mucosal immunoglobulin A (IgA) production,[16] and a therapeutic dose of flagellin suppressed airway eosinophilia and Th2-mediated immune responses in murine models of allergic asthma.[17] Also, decreased expression of TLR5 and downregulation of Th1 and anti-inflammatory cytokines in peripheral blood cells in patients with asthma supports another aspect of the potential contribution of flagellin to airway diseases.[18]

Although previous studies to investigate the effect of bacterial flagellin represent its immunostimulatory properties, models with closer resemblance to human epithelium are needed to better understand the role of flagellin in the pathogenic mechanism of inflammatory airway diseases. Current study aimed to investigate the effect of TLR5 ligand flagellin on the primary human bronchial epithelial (NHBE) cells and the expression of inflammatory markers related to airway inflammation and remodeling. We utilized primary NHBE cells cultured at an air-liquid interface (ALI) which mimic in vivo airway characteristics.[19]

METHODS

Primary bronchial epithelial cell cultures

NHBE cells (Lonza, USA) at passages 2–3 were plated on 12-well transwell plates (Corning, USA) coated with type 1 collagen (Corning, USA) as previously described.[20] When confluent (5–7 days), cells were exposed to air by removing media from the apical surfaces of cells. NHBE cells were fed a 1:1 mixture of bronchial epithelial basal medium (BEBM, Lonza, USA) and Dulbecco Modified Eagle's Medium (DMEM, Corning, USA), and differentiated in ALI culture for 14–16 days.

The basal cell surface of an NHBE cell-ALI culture was treated with purified flagellin from *Pseudomonas aeruginosa* (InvivoGen, USA) at 10 and

100 ng/ml for 3 and 24 h. In the experiments with transforming growth factor beta (TGF-β), 10 ng/ml of TGF-β1 or TGF-β2 (R&D Systems, South Korea) were pretreated 1 h before 100 ng/ml flagellin treatment.

Real-time PCR

Total RNA was extracted with an RNeasy mini kit following the manufacturer's instructions (QIAGEN, Germany). Next, the cDNA was prepared by AccuPower RocketScript RT-PCR PreMix (Bioneer, South Korea) according to the manufacturer's instructions. qPCR was performed with SYBR Green qPCR Master Mix (Applied Biosystems, USA). The following thermocycler parameters were used: amplifications were performed followed by 45 cycles at 95 °C for 40 s, 60 °C for 40 s, and 72 °C for 40 s. Data were normalized by the housekeeper glyceraldehyde 3-phosphate dehydrogenase and was determined by ΔΔ the cycle threshold method. Primers used for qPCR are summarized in Table S1.

RNA sequencing

ALI-NHBE cells were treated with 100 ng/ml flagellin for 3 h and control cells were used for transcriptome analysis. The sequencing procedure of RNA-seq was conducted by Macrogen Inc (Seoul, South Korea). The detailed protocol was provided in Supplementary materials.

Enzyme-linked immunosorbent assay

ELISA was performed using conditioned media from NHBE cells treated with flagellin for 24 h. The concentrations of cytokines (GM-CSF, IL-8, IL-33) and chemokines (CXCL5, CCL5, CXCL10, CXCL11) in culture supernatants were measured with a quantitative sandwich ELISA, according to the manufacturer's instructions. GM-CSF kit was purchased from Koma Biotech, and other kits were purchased from R&D Systems.

Western blot

Western blots were performed using protein (10–30 μg) from treated cell lysates. Samples were separated on 10% SDS-PAGE gels and then transferred to polyvinylidene fluoride membranes. The membrane was blocked with Tris-buffered saline-Tween-20 buffer containing 5% skim milk and incubated with the primary antibodies at 4 °C on a shaker overnight. The primary antibodies used in this experiment were: against MMP-9 (dilution 1:1000, Bioworld, USA), MMP-13 (dilution 1:1000, Bioworld, USA), pGSK3B (dilution 1:1000, Cell Signaling Technology, USA), and β-catenin (dilution 1:1000, Cell Signaling Technology, USA). It was then probed with the secondary antibodies, goat anti-mouse IgG (dilution 1:3000, Abcam, USA) or goat anti-rabbit IgG (dilution 1:3000, Abcam, USA), at room temperature for 2 h. Transferrin (dilution 1:3000, Santa Cruz Biotechnology, USA) was used as a loading control for Western blot analysis. Signals were detected via Image ChemiDoc (Bio-Rad, USA).

Statistical analysis

All experimental data were expressed as the mean ± standard error of the mean (SEM). The Shapiro-Wilk test was applied to assess the normality of the data. The nonparametric Mann-Whitney *U* test was used for statistical comparisons. Data were obtained from at least 3 independent experiments unless otherwise specified. Statistical analysis was performed using SPSS software version 25.0 (IBM, USA). A p-value <0.05 was considered to indicate statistical significance.

RESULTS

RNA sequencing and analysis

Up- and downregulated flagellin-responsive genes were screened using RNA-sequencing. The genes encoding chemokines (*CXCL5*, *CCL5*, *CXCL10*, *CXCL11*), matrix metalloproteinases (*MMP13*, *MMP9*, *MMP7*), and antimicrobial biomolecules (*DEFB4A*, *DEFB4B*, *MUC4*) were frequently observed among the upregulated flagellin-responsive genes (Table 1 and Table S2), while downregulated genes included keratin genes (*KRT16*, *KRT14*, *KRT13*, *KRT17*), and ribosomal protein L21 (*RPL21*), one of the top genes elevated by *Chlamydia pneumoniae* infection in previous study.[21] The top 5 ranking genes were *TINAGL1*, *SERPINA3*, *IGFBP3*, *KRT17*, and *KRT13* (Fig. S1).

Gene ontology (GO) was applied to identify characteristic biological attributes of RNA sequencing data. Separate GO enrichment analysis for up- and downregulated genes was

Gene ID	Transcript ID	Gene	Gene description	Fold change
Upregulated				
3627	NM_001565	*CXCL10*	chemokine (C-X-C motif) ligand 10	13.28
1592	NM_057157 NM_000783	*CYP26A1*	cytochrome P450, family 26, subfamily A, polypeptide 1	11.84
374897	NM_001166035 NM_001166034 NM_198538	*SBSN*	suprabasin	6.07
7018	NM_001063	*TF*	transferrin	6.00
91543	NM_080657	*RSAD2*	radical S-adenosyl methionine domain containing 2	5.30
6352	NM_002985 NM_001278736	*CCL5*	chemokine (C-C motif) ligand 5	5.09
84419	NM_197955 NM_032413	*C15orf48*	chromosome 15 open reading frame 48	5.06
3429	NM_001130080[a]	*IFI27*	interferon, alpha-inducible protein 27	5.02
5653	NM_001012964 NM_001012965 NM_002774	*KLK6*	kallikrein-related peptidase 6	4.61
2537	NM_002038 NM_022872 NM_022873	*IFI6*	interferon, alpha-inducible protein 6	4.28
Downregulated				
6144	NM_000982	*RPL21*	ribosomal protein L21	−5.00
6876	NM_003186, NM_001001522	*TAGLN*	transgelin	−3.98
362	NM_001651	*AQP5*	aquaporin 5	−3.73
3868	NM_005557	*KRT16*	keratin 16	−3.66
101927318	NR_110589, NR_110590, NR_110591	*LOC101927318*	Not applicable	−3.31
103344718	NM_001293171	*HOTS*	H19 Opposite Tumor Suppressor	−2.85
1580	NM_000779, NM_001099772	*CYP4B1*	cytochrome P450, family 4, subfamily B, polypeptide 1	−2.84
8581	NM_003695	*LY6D*	lymphocyte antigen 6 complex, locus D	−2.78
27063	NM_014391	*ANKRD1*	ankyrin repeat domain 1 (cardiac muscle)	−2.76
728066	NR_034169	*FAM133DP*	family with sequence similarity 133, member D, pseudogene	−2.71

Table 1. The top 10 up- and downregulated genes in differentiated NHBE cells.

performed. Upregulated differentially expressed genes (DEGs) were involved in processes including GO terms cytokine-mediated signaling pathway, response to cytokine, cellular response to cytokine stimulus, type 1 interferon signaling pathway, etc (Fig. S2A). While downregulated transcripts were involved in biological processes such as cornification, animal organ development, and intermediate filament cytoskeleton organization (Fig. S2B).

To estimate the number of DEGs contained at different class of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways, pathway enrichment analysis was performed. KEGG pathway analysis showed that upregulated DEGs were mainly enriched in cytokine-cytokine receptor interaction (9 DEGs), IL-17 signaling pathway (8 DEGs), influenza A (7 DEGs), and TNF signaling pathway, whereas downregulated DEGs were enriched in pathways such as *Staphylococcus aureus* infection (5 DEGs), estrogen signaling pathway (4 DEGs), and biosynthesis of amino acids (3 DEGs) (Fig. S3).

Pro-inflammatory cytokines and chemokines are induced by flagellin in NHBE cells

To verify the genes upregulated by flagellin in the RNA-sequencing data, we examined gene expression and protein secretion of chemokines along with inflammatory cytokines involved in the pathogenesis of airway inflammation, particularly asthma. Comparing to control cells, flagellin stimulation enhanced the mRNA expressions of *CSF2*, *CXCL8* and *IL33* (Fig. 1A–C) and *TNF* at 3 h (Fig. S4A). Besides, the expression of *TSLP* was elevated at lower dose followed by a decline at higher dose stimulation (Fig. S4B).

The production of GM-CSF in conditional media was enhanced significantly after stimulation for 24 h (Fig. 1D). However, IL-33 was undetectable in the treated NHBE cells, and IL-8 did not differ significantly compared to controls (Fig. 1E).

Next, we tested 4 chemokine genes that appears to have a role in mediating airway immune response (Fig. 2). After 3 and 24 h of stimulation, the mRNA expression of the *CXCL5* (C-X-C motif chemokine ligand 5), *CCL5* (C–C motif chemokine ligand 5), and *CXCL10* (C-X-C motif chemokine ligand 10) significantly increased by stimulation comparing to control cells (Fig. 2A–C). The protein level of the chemokines of interest was then measured using ELISA assay. The secretions of CXCL5, CCL5, and CXCL10 were significantly elevated in treated NHBE cells (Fig. 2E–G). Th1 response-related chemokine, CXCL11, did not differ significantly at mRNA level (Fig. 2D), but the protein expression declined after stimulation (Fig. 2H).

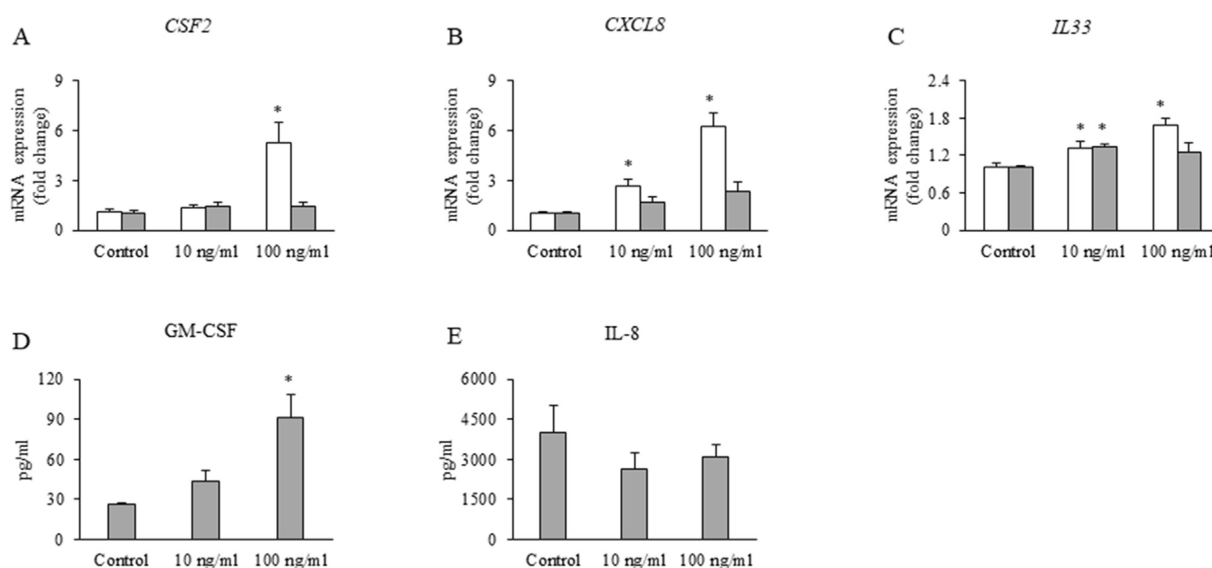

Fig. 1 The expression patterns of gene expression and protein production of cytokines in differentiated NHBE cells stimulated with flagellin. Relative mRNA expression levels of *CSF2* (A), *CXCL8* (B) and *IL33* (C). D-E. Protein levels of GM-CSF and IL-8. Normalization for quantitative PCR was performed using *GAPDH* gene as an internal control. The sampling time points were 3 h (open bar) and 24 h (closed bar) after stimulation. Data are presented as mean ± standard error of the mean (SEM) of 3 independent experiments. *p < 0.05 indicates significant differences from control group.

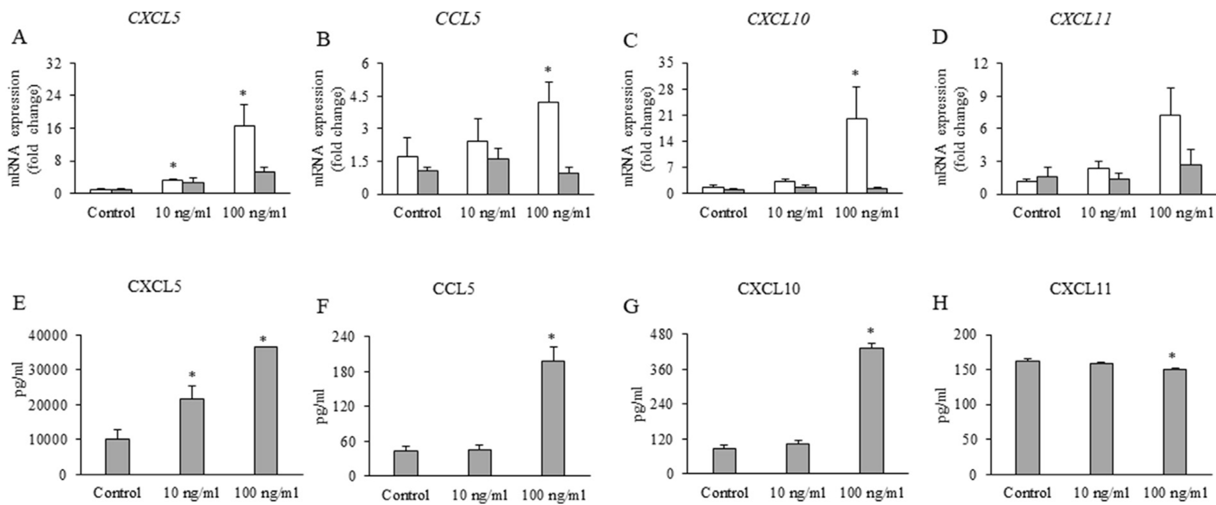

Fig. 2 The expression patterns of gene expression and protein production of chemokines in differentiated NHBE cells stimulated with flagellin. Relative mRNA expression levels of *CXCL5* (A), *CCL5* (B), *CXCL10* (C) and *CXCL11* (D). E-H. Protein levels of chemokines. Normalization for qPCR was performed using *GAPDH* gene as an internal control. The sampling time points were 3 h (open bar) and 24 h (closed bar) after stimulation. Data are presented as mean ± standard error of the mean (SEM) of 3 independent experiments. *p < 0.05 indicates significant differences from control group.

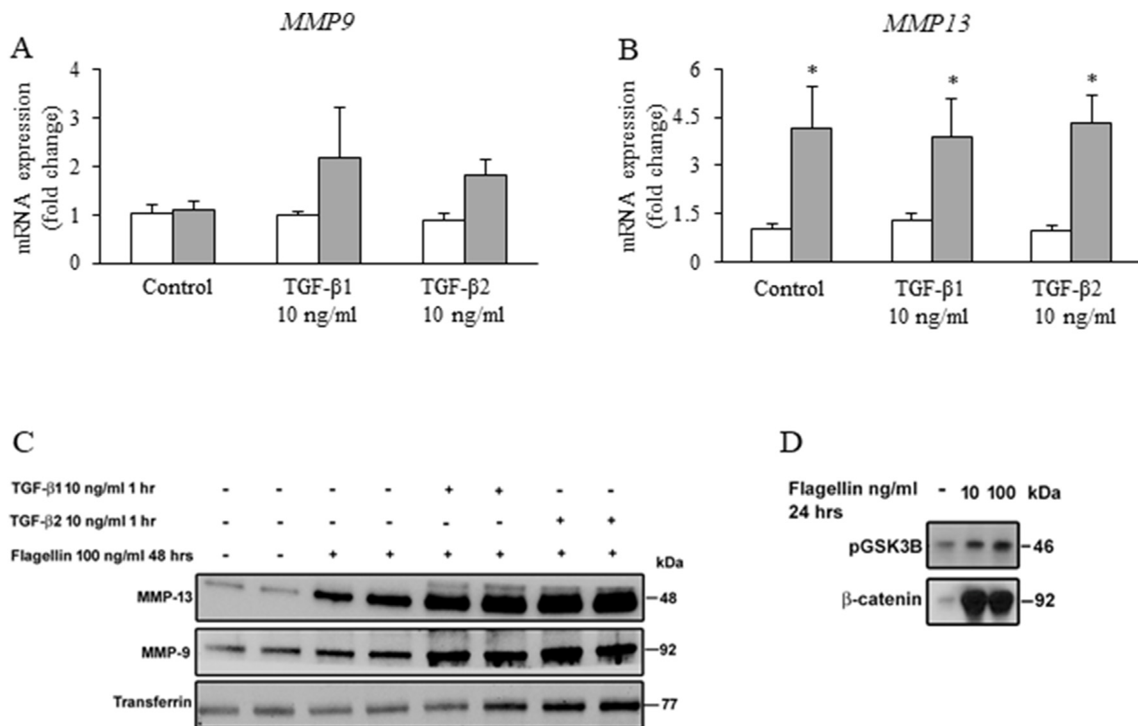

Fig. 3 Expression patterns of 2 representative genes involved in airway remodeling and Wnt/β-catenin signaling pathway. NHBE cells were pretreated with TGF-β1 and TGF-β2 (10 ng/ml for 1 h) and stimulated with flagellin (100 ng/ml for 24 h). Data are presented as mean ± standard error of the mean (SEM) of 2 independent experiments. A-B. The mRNA expression of genes from flagellin unstimulated (open bar) and stimulated (closed bar) samples were evaluated. *p < 0.05 indicates significant differences from unstimulated group. C. Representative Western blot analysis of MMP-9 and MMP-13 with pretreated and stimulated sample. D. Effect of flagellin on Wnt/β-catenin signaling pathway.

MMP13 and Wnt/β-catenin signaling are upregulated in response to flagellin

MMP is of specific interest due to its regulation of airway fibrosis and airway remodeling after release from epithelial cells and fibroblasts.[22] To determine whether MMPs are induced by flagellin to promote airway remodeling, the mRNA and protein expression of transcriptionally upregulated MMP-9 and MMP-13 were assayed with or without stimulation with TGF-β1 and TGF-β2. Flagellin enhanced the mRNA expression of *MMP13*, but not *MMP9* in NHBE cells. However, pretreated with TGF-β1 and TGF-β2 did not significantly promote the mRNA expression of *MMP9* and *MMP13* (**Fig. 3**A and B). MMP-13 protein expression was increased after flagellin stimulation, which were augmented by pretreatment of TGF-β1 and TGF-β2 (**Fig. 3**C). To reveal the impact of flagellin on Wingless/Integrase-1 (Wnt)/β-catenin signaling, western blots of Wnt/β-catenin signaling molecules in NHBE cells were assessed after stimulation. Flagellin induced the production of phosphorylated glycogen synthase kinase 3 beta (pGSK3B) and β-catenin in NHBE cells (**Fig. 3**D).

DISCUSSION

P. aeruginosa-derived flagellin is an important stimulator for activating the expression of inflammatory markers in human bronchial epithelial cells. In this study, we applied an ALI culture of primary bronchial epithelial cells that recapitulate the morphological and physiological features of the airway epithelia. Air-exposure of the cultured NHBE cells is essential for the ciliogenesis, and ALI-cultured NHBE cells have similar transcriptional profiles and protein compositions to the human bronchial epithelium.[19,23,24] We also conducted RNA sequencing analysis to identify transcription profiles of bronchial epithelial cells induced by TLR5 ligand flagellin and experimental study to verify the differentially expressed genes of transcriptomics.

In transcriptome analysis, flagellin stimulation in differentiated bronchial epithelial cells upregulated the expression of chemokines, host defense molecules and metalloproteinases involved in immune response/inflammation, and airway remodeling, but in addition, other regulatory genes were also found to be modulated. This finding together with the previous study of transcriptomic and secretomic analysis[25] suggests that the most predominantly upregulated genes in response to flagellin were chemokine genes which play a pivotal role in orchestrating both the innate and adaptive immunity. Our functional annotation analysis of the transcriptome revealed that the upregulated differentially expressed genes were enriched in the gene sets of the cytokine-cytokine receptor interaction, IL-17 signaling, TNF signaling, TLR signaling, and chemokine signaling pathway. IL-17 induces the expression of chemokines or β-defensin in human airway epithelial cells, and might cooperate with other cytokines such as TNF to promote the expression of inflammatory genes that contribute to the chronic airway inflammation.[26] IL-17 and TLR signaling coordinate to synergistically induce proinflammatory gene expression in the human airway epithelium, and may exacerbate infection-induced chronic lung diseases.[27]

In the experimental study, we observed differences in gene expression and protein secretion of epithelial cytokines along with chemokines in response to flagellin in bronchial epithelial cells. Enhanced gene expressions of *CSF2*, *CXCL8*, *IL33*, and *TNF* and release of GM-CSF were observed in the stimulated cells. In the airways, epithelial cells are a source of IL-8 which recruits neutrophils into the infected airways and prevents a bacterial invasion.[28] On the other hand, excessive neutrophil influx into airways contributes to the development of chronic airway inflammatory diseases, including asthma and chronic obstructive pulmonary disease. GM-CSF is a strong antigen-presenting cell activator in response to a variety of environmental stimuli,[29] and GM-CSF secreted from flagellin-activated nasal epithelial cells contributed to TLR5-mediated dendritic cell activation and IgA enhancement.[30] Furthermore, GM-CSF induces intracellular IL-33 expression in the lung, and GM-CSF/IL-33 pathway may increase the susceptibility to develop allergic asthma precipitated by environmental exposures.[31] We also observed increased expression of *TNF* mRNA and transcript of TNFα-induced protein *TNFAIP2* which provides further evidence of the role of these markers in flagellin-induced airway inflammation. In addition, the mRNA expression of *TSLP* was enhanced at a lower dose at early exposure, followed by a decline

at the higher dose. In a previous study, low-dose lipopolysaccharide (LPS) induced Th2 cytokines and recruitment of eosinophilic and neutrophilic inflammation, while high-dose LPS exhibited Th1 immune response and neutrophil accumulation in murine asthma.[32,33] These findings suggest that TLR ligands have a distinct immunoregulatory effect based on exposure level, resulting in different clinical outcomes.

Pretreatment of TGF-β1 and TGF-β2 in flagellin-stimulated bronchial epithelial cells promoted the expression of MMP-13, which was one of the upregulated metalloproteinases in transcriptome analysis. TGF-β is released from damaged epithelial cells and plays a central role in tissue fibrosis and remodeling in the asthmatic lung.[34] TGF-β activation interfere with other pro-repair signaling pathway Wnt/β-catenin, that synergistically affects lung epithelial repair.[35] In the current study, Wnt pathway antagonist *DKK3* (dickkopf Wnt signaling pathway inhibitor 3) was downregulated in transcriptome analysis, and β-catenin protein expression was dose dependently increased in response to flagellin. Taken together, these results show that Wnt/β-catenin signaling might be involved in the flagellin-induced inflammation in airway epithelial cells.

A better understanding of the role of airway epithelium playing in early immune responses is critical to the future development of microbiome-targeted therapeutic interventions. The present study has some potential limitations. Firstly, genes and cytokines of interest were selected from differentially expressed genes based on their involvement in asthma pathogenesis. However, these markers play an important role in orchestrating the inflammatory processes and structural changes of the airway in both asthma and COPD. Secondly, current study used NHBE cells derived from normal donors to evaluate how the immune response induced by bacterial flagellin could promote airway inflammation. Further studies are needed to clarify their interaction with other inflammatory and structural cells obtained from donors with healthy and diseases state. In conclusion, TLR5 ligand flagellin is an active inducer of a variety of gene expression, including proinflammatory cytokines, chemokines, host-defense proteins and metalloproteinases that may result in prolongation of the inflammatory phase and airway remodeling of bronchial epithelial cells in airway diseases.

Abbreviations

ALI, air-liquid interface; CCL5, C–C motif chemokine ligand 5; CSF2, colony-stimulating factor 2; CXCL, C-X-C motif chemokine ligand; DEGs, differentially expressed genes; ELISA, enzyme-linked immunosorbent assay; GM-CSF, granulocyte-macrophage colony-stimulating factor; GO, gene ontology; Ig, immunoglobulin; IL, interleukin; KEGG, Kyoto Encyclopedia of Genes and Genomes; MMPs, matrix metalloproteinases; mRNA, messenger RNA; NHBE cell, normal human bronchial epithelial cell; pGSK3b, phospho-glycogen synthase kinase-3 beta; qPCR, quantitative PCR; RNA, ribonucleic acid; TGF-β, transforming growth factor-β; Th cells, T helper cells; TLR5, toll-like receptor 5; TNF, tumor necrosis factor; TSLP, thymic stromal lymphopoietin; Wnt, wingless-related integration site

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Availability of data and materials

The raw sequencing data has been deposited in NBCI sequence read archive (SRA) under the accession number PRJNA792916. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author contributions

Conceptualization: PL, YSC, SHK; Methodology and analysis: MHJ, JHK, BKK, JEY; Interpretation of data: JPC, JEY, JHS; Writing the manuscript: PL, YSC, SHK.

Ethics approval

Not applicable.

Consent for publication

The authors have read and approved the final version of the manuscript, its content, and its submission to WAO Journal. We confirm that the manuscript is original and has not been considered or published elsewhere.

Declaration of competing interest

The authors report no competing interest.

Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.waojou.2023.100786.

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