



## Flagellin/Toll-like receptor 5 response was specifically attenuated by keratan sulfate disaccharide via decreased EGFR phosphorylation in normal human bronchial epithelial cells

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### ABSTRACT

Bacterial or viral infection of the airway plays a critical role in the pathogenesis and exacerbation of chronic obstructive pulmonary disease (COPD) which is expected to be the 3rd leading cause of death by 2020. The induction of inflammatory responses in immune cells as well as airway epithelial cells is observed in the disease process. There is thus a pressing need for the development of new therapeutics. Keratan sulfate (KS) is the major glycosaminoglycans (GAGs) of airway secretions, and is synthesized by epithelial cells on the airway surface. Here we report that a KS disaccharide, [SO<sub>3</sub> – 6]Galβ1–4[SO<sub>3</sub> – 6]GlcNAc, designated as L4, suppressed the production of Interleukin-8 (IL-8) stimulated by flagellin, a Toll-like receptor (TLR) 5 agonist, in normal human bronchial epithelial (NHBE) cells. Such suppressions were not observed by other L4 analogues, *N*-acetyllactosamine or chondroitin-6-sulfate disaccharide. Moreover, treatment of NHBE cells with L4 inhibited the flagellin-stimulated phosphorylation of epidermal growth factor receptor (EGFR), the down stream signaling pathway of TLRs in NHBE cells. These results suggest that L4 specifically blocks the interaction of flagellin with TLR5 and subsequently suppresses IL-8 production in NHBE cells. Taken together, L4 represents a potential molecule for prevention and treatment of airway inflammatory responses to bacteria infections, which play a critical role in exacerbation of COPD.

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### 1. Introduction

#COPD, which is predicted to be the third leading cause of death by 2020 [1], is associated with specific inflammatory responses around small airways [2]. COPD exacerbation occurs in some patients mostly due to bacterial or viral infections of the lung and air pollution [3,4]. In fact, highly significant levels of bacteria are observed in the airway of exacerbated COPD as compared to those in healthy adults [5]. The most commonly isolated bacteria are *Haemophilus influenzae*, *Streptococcus pneumoniae* and *Moraxella catarrhalis*, while there is growing evidence that *Pseudomonas aeruginosa* is particularly involved in advanced COPD [6,7]. Bacterial infection is associated with the pathogenesis and exacerbation of

COPD partly via the induction of host inflammatory responses [4]. Thus, it can be a potent therapeutic target for COPD to suppress airway inflammatory responses triggered by bacterial infection.

Airway inflammatory responses are mediated by immune cells, such as macrophages, neutrophils and lymphocytes, as well as by airway epithelial cells [8]. Airway epithelial cells actively promote airway inflammatory responses by releasing various inflammatory mediators, including a neutrophil chemo attractant, IL-8 [9,10]. Indeed, increased levels of neutrophils, their degradation products, such as myeloperoxidase, elastase, and IL-8 are detected in sputum or bronchoalveolar lavage fluid from COPD patients with bacterial infection, as compared to patients without infections [11–14]. The production of IL-8 in airway epithelial cells is initiated by the recognition of bacterial pathogens through TLRs, as is the case with immune cells [15,16]. However, in airway epithelial cells, TLRs-stimulated IL-8 production is mediated by the activation of EGFR [17,18].

Oligosaccharides are well known for their function in the progress of diseases. Recently, we reported that reduction of α1,6

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fucosylation resulted in an elevated sensitivity to cigarette-smoke induced emphysema of mice [19] that is related to the frequency of exacerbation and the decline of lung function among COPD patients [20]. Moreover, the extracellular GAGs, important matrix components, are considered to be essential for normal lung function and the response to injury by regulating such signal transduction events [21–23]. GAGs are linear unbranched polysaccharides, composed of repeating disaccharide unit, and classified into four groups: hyaluronan, chondroitin sulfate/dermatan sulfate, keratan sulfate and heparan sulfate/heparin. GAGs are identified to be endogenously expressed in the airways [23]. Among these GAGs, KS is present at the apical border of NHBE cells and on the apical surface of ciliated cells in human trachea tissue sections [24]. KS inhibits the expression and activation of MMP-2 in corneal and skin explant cultures [25]. In addition, KS disaccharide L4 suppresses IL-12 production in macrophages stimulated with LPS [26].

In order to better understand the role of KS in COPD exacerbation and airway inflammation, we investigated their effects on TLRs-stimulated inflammatory responses of airway epithelial cells using L4, the key-disaccharide-unit of KS. Namely, we examined the effects of L4 on the production of IL-8 and the phosphorylation of EGFR in NHBE cells stimulated with a variety of TLR agonists.

## 2. Materials and methods

### 2.1. Cells and culture conditions

NHBE cells were purchased from Takara Bio (Shiga, Japan), and cultured in bronchial epithelial basal medium (Takara Bio) supplemented with defined growth factors and antibiotics. The cultures were maintained at 37 °C in a humidified incubator containing 5% CO<sub>2</sub> in the air.

### 2.2. TLR agonists

NHBE cells were plated at approximately  $3.0 \times 10^4$  cells/cm<sup>2</sup> in culture dishes, grown to reach complete confluence, and then cultured without any growth factors for 24 h. Growth factors-starved cells were stimulated with TLR1/2 agonist Pam3CSK4 (1 µg/ml), TLR2 agonist heat-killed *Listeria monocytogenes* (HKLM) ( $10^8$  - cells/ml), TLR3 agonist Poly(I:C) (10 µg/ml), TLR4 agonist LPS (10 µg/ml), TLR5 agonist flagellin (50 ng/ml), TLR6/2 agonist FSL1 (1 µg/ml), TLR7 agonist Imiquimod (10 µg/ml), TLR8 agonist ssRNA40 (10 µg/ml) or TLR9 agonist ODN2006 (5 µM) contained in Human TLR1-9 Agonist kit (InvivoGen, San Diego, CA) for the indicated times.

### 2.3. L4 and other disaccharides

LacNAc, lactose, chondroitin-6-sulfate and L4 were supplied by Seikagaku Corp. (Tokyo, Japan). L4 was isolated from the keratanase II digest of shark fin KS, by sequential steps of anion-exchange and gel-permeation chromatography. The structure has been identified by mass spectrometry [27]. When the effects of disaccharides on TLR agonists-stimulated inflammatory responses were examined, growth factors-starved cells were first treated with disaccharides (1 mg/ml) for 1 h, and then TLR agonists were added to the cultures. Structures of each disaccharide used in this study are shown in Supplementary Fig. 1.

### 2.4. Measurement of IL-8 production in NHBE cells

After 24 h from the onset of stimulation with TLR agonists with or without L4, culture media were collected, and IL-8 concentrations

in the media were measured using OptEIA Human IL-8 ELISA Set (BD Biosciences, NJ, USA).

### 2.5. Reverse-transcriptase-polymerase chain reaction (PCR)

Total cellular RNA was extracted and purified from NHBE cells by the acid guanidinium phenol chloroform method using RNAiso Plus (Takara Bio). One µg aliquot of total RNA was converted to single-stranded cDNA using Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science, Indianapolis, IN). The cDNA (1 µl) was subjected to PCR using ExTaq DNA polymerase (Takara Bio), and amplified in the following condition: denaturing at 94 °C for 30 s; annealing at 55 °C for 30 s; and extension at 72 °C for 30 s. The PCR products were electrophoresed in 1.5% agarose gel containing 1 µg/ml ethidium bromide. The primers used in this study are listed in Supplementary Table 1.

### 2.6. Western blotting

At the end of flagellin and/or disaccharide treatment, NHBE cells were collected and the cell lysates were analyzed by 10% SDS-polyacrylamide gel electrophoresis. Gels were blotted onto PVDF membranes, and the membranes were incubated with the primary antibodies against EGFR (Cell Signaling Technology, Danvers, MA) and against phospho-EGFR (Cell Signaling Technology) overnight at 4 °C. Following washing and exposing to HRP-conjugated second antibodies (Jackson Immuno Research Laboratories, PA, USA) for 30 min at room temperature, membrane was detected with enhanced chemiluminescent reagent (GE Healthcare, Buckinghamshire, UK).

### 2.7. Statistical analysis

Experimental data are presented as means ± standard error of the mean (SEM). Equivalence of group means was tested by one-way analysis of variance (ANOVA). *Post hoc* comparisons to significant differences among three groups and over were performed by the Bonferroni test. Differences were considered significant when  $P < 0.05$ .

## 3. Results

### 3.1. L4 suppressed IL-8 production in NHBE cells stimulated with flagellin

We first confirmed the reactivity of NHBE cells to a variety of TLR agonists. NHBE cells produced IL-8 in response to Pam3CSK4 (TLR1/2 agonist), Poly(I:C) (TLR3 agonist), flagellin (TLR5 agonist), FSL1 (TLR6/2 agonist) and ODN2006 (TLR9 agonist). Poly I:C and flagellin induced the highest production of IL-8 (Supplementary Fig. 2). And after 24-h-stimulation of flagellin, the changes on both the mRNA and protein expression level of IL-8 could be detected easily (Supplementary Fig. 3).

Next we examined if L4 suppressed the IL-8 production in NHBE cells stimulated with those agonists. L4 did not affect the IL-8 production in NHBE cells stimulated with Pam3CSK4, Poly(I:C), FSL1 and ODN2006 in both protein and mRNA levels (Fig. 1A and B). However, L4 significantly suppressed flagellin-stimulated IL-8 production in NHBE cells (Fig. 1C). In addition, similar results were observed in IL-8 mRNA level (Fig. 1D). These results suggest that L4 specifically suppresses flagellin/TLR5-stimulated IL-8 production in NHBE cells at the gene transcription level.

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