Biochemical and Biophysical Research Communications 435 (2013) 460–465

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

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

Ken Shirato [a,b,1], Congxiao Gao [a,c,1], Fumi Ota [a,1], Takashi Angata [a], Hidehiko Shogomori [a], Kazuaki Ohtsubo [a,c], Keiichi Yoshida [a], Bernd Lepenies [d], Naoyuki Taniguchi [a,c,*]

[a] Systems Glycobiology Research Group, RIKEN-Max Planck Joint Research Center, RIKEN Global Research Cluster, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan
[b] Faculty of Human Sciences, Waseda University, 2-579-15 Mikajima, Tokorozawa, Saitama 359-1192, Japan
[c] RIKEN Alliance Laboratory, The Institute of Scientific and Industrial Research, Osaka University, 8-1 Mihogaoka, Ibaraki, Osaka 567-0047, Japan
[d] Max Planck Institute of Colloids and Interfaces, Arnimallee 22, 14195 Berlin, Germany

ARTICLE INFO

Article history:
Received 2 May 2013
Available online 14 May 2013

Keywords:
Keratan sulfate disaccharide
Flagellin
Interleukin-8
Epidermal growth factor receptor
Airway epithelial cells

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_3^- – 6]Galβ1–4[SO_3^- – 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.

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

* Corresponding author at: Systems Glycobiology Research Group, RIKEN-Max Planck Joint Research Center, RIKEN Global Research Cluster, 2-1 Hirosawa, Wako, Saitama 351-0198, Japan. Fax: +81 48 467 9617.
E-mail address: tani52@wd5.so-net.ne.jp (N. Taniguchi).
[1] These authors equally contributed to this work.

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.05.009

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_2 in the air.

2.2. TLR agonists

NHBE cells were plated at approximately 3.0×10^4 cells/cm^2 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.

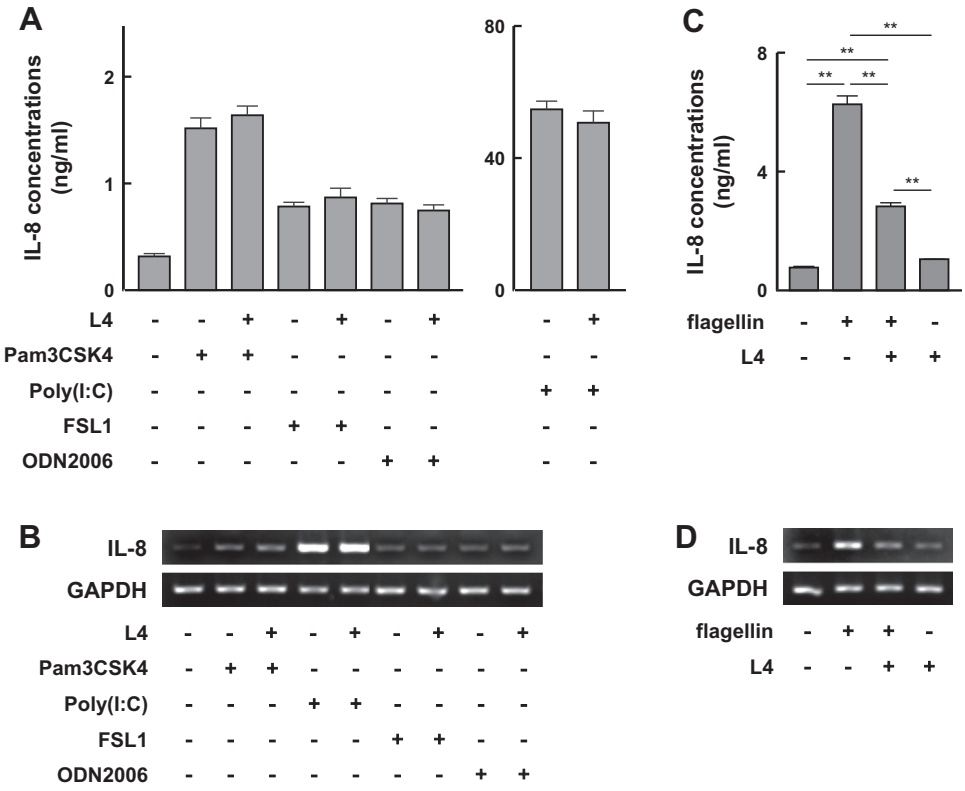

Fig. 1. Effects of L4 on TLR agonists-stimulated IL-8 production in NHBE cells. (A) NHBE cells were treated with or without 1 mg/ml L4, and 1 h later, Pam3CSK4, Poly(I:C), FSL1 or ODN2006 was added. After 24 h, the concentrations of IL-8 in the media were determined by ELISA. Values: means ± SEM (n = 3 for each data). (B) From the cells, total RNA was prepared and converted to cDNA, subsequently the levels of IL-8 mRNA were analyzed by PCR. (C) NHBE cells were treated with or without 1 mg/ml L4, and flagellin was added 1 h later. After 24 h, the concentrations of IL-8 in the media were measured by ELISA. Values: means ± SEM (n = 3 for each data). $^{**}P < 0.01$. (D) From the cells, total RNA was prepared and converted to cDNA, subsequently the levels of IL-8 mRNA were analyzed by PCR.

3.2. The sulfation and conformation of L4 are essential for its suppressive effect on flagellin-induced IL-8 production

To elucidate if the suppressive effect of L4 on flagellin-stimulated IL-8 production is specific, NHBE cells were treated with LacNAc, a disaccharide composed of galactose and GlcNAc, or lactose, a disaccharide composed of galactose and glucose, instead of L4. As shown in Fig. 2A, neither LacNAc nor lactose exhibited any effects on flagellin-stimulated IL-8 production in NHBE cells. These results suggest that sulfates in L4 structure are required to suppress the flagellin-stimulated IL-8 production in NHBE cells. However, chondroitin-6-sulfate failed to show such inhibition (Fig. 2B). Thus, both the sulfates and the conformational structure of L4 are essential to elicit the anti-inflammatory effect.

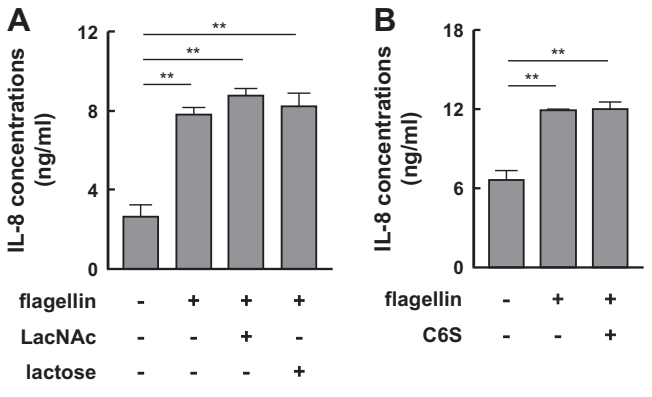

Fig. 2. Effects of LacNAc, lactose and chondroitin-6-sulfate on flagellin-stimulated IL-8 production in NHBE cells. (A) NHBE cells were treated with or without 1 mg/ml LacNAc or lactose, and 1 h later, flagellin was added. After 24 h, the concentrations of IL-8 in the media were measured by ELISA. Values: means ± SEM (n = 3 for each data). $^{**}P < 0.01$. (B) NHBE cells were treated with 1 mg/ml chondroitin-6-sulfate (C6S), and 1 h later, flagellin was added. After 24 h, the concentrations of IL-8 in the media were measured by ELISA. Values: means ± SEM (n = 3 for each data). $^{**}P < 0.01$.

3.3. L4 interacted directly with NHBE cells to suppress flagellin-stimulated IL-8 production

The suppressive effect of L4 on flagellin-stimulated IL-8 production in NHBE cells was gradually attenuated along with the reduction of L4 concentration (Fig. 3A). To evaluate that L4 directly acts on NHBE cells, we examined if the pretreatment of NHBE cells with L4 modulates the IL-8 production of cells stimulated with flagellin. As shown in Fig. 3B, when NHBE cells were treated with L4 prior to flagellin stimulation, IL-8 production was significantly suppressed although the effect was moderate compared to the simultaneous cell stimulation with L4 and flagellin. These results indicate that L4 interacts with NHBE cells and modulates IL-8 production stimulated with flagellin.

3.4. L4 inhibited EGFR phosphorylation in NHBE cells stimulated with flagellin

Since TLR agonists-stimulated IL-8 production is revealed to be mediated by EGFR phosphorylation [17], we examined if L4 inhibits flagellin-induced phosphorylation of EGFR. The level of EGFR phosphorylation was transiently increased 1 h after stimulation, and then gradually returned to the basal level without changing the expression level of EGFR (Fig. 4A), whereas L4 did not affect

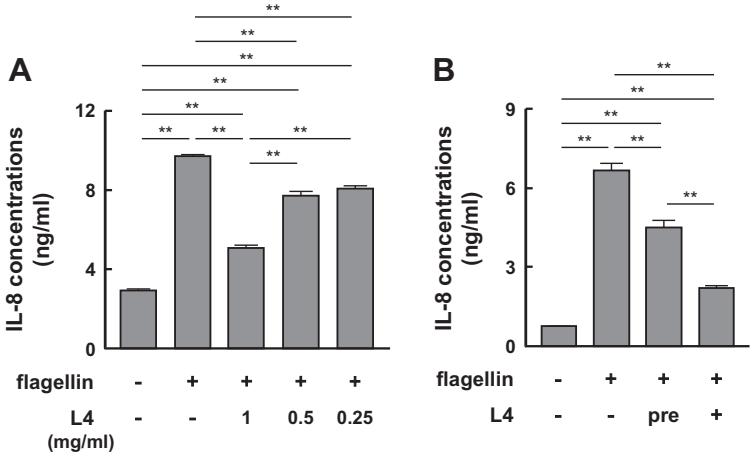

Fig. 3. Effects of pretreatment with L4 on flagellin-stimulated IL-8 production in NHBE cells. (A) The dose response of the inhibitory effects of L4 on flagellin-stimulated IL-8 production was determined. NHBE cells were treated with or without 1, 0.5 or 0.25 mg/ml L4, and 1 h later, flagellin was added. After 24 h, the concentrations of IL-8 in the media were measured by ELISA. Values: means ± SEM ($n = 3$ for each data). $^{**}P < 0.01$. (B) NHBE cells were treated with 1 mg/ml L4 for 24 h, and then the cells were washed twice with BEBM and stimulated with 50 ng/ml flagellin. After 24 h, the concentrations of IL-8 in the media were measured by ELISA. Values: means ± SEM ($n = 3$ for each data). $^{**}P < 0.01$.

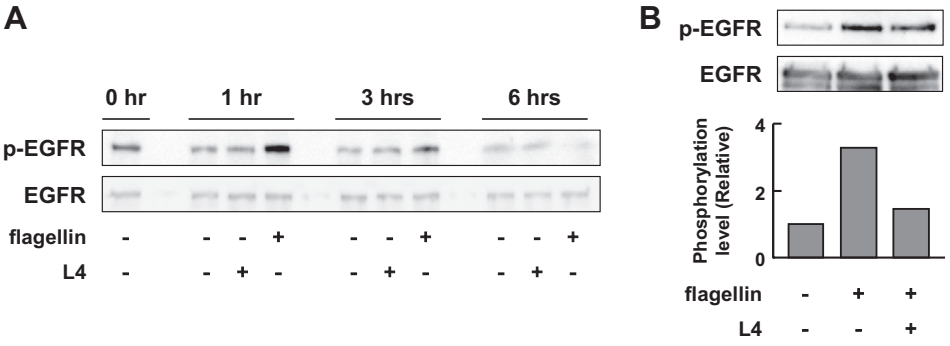

Fig. 4. Effects of L4 on flagellin-stimulated EGFR phosphorylation in NHBE cells. (A) NHBE cells were treated with or without 1 mg/ml L4 or 50 ng/ml flagellin for 6 h. Whole cell lysate was prepared in the presence of phosphatase inhibitors, and the levels of phospho-EGFR (p-EGFR) and total EGFR were analyzed by Western blotting after 0, 1, 3 and 6 h from the onset of stimulation. (B) NHBE cells were treated with or without 1 mg/ml L4, and 1 h later, flagellin was added to be a final concentration at 50 ng/ml. After 1 h, the levels of p-EGFR and total EGFR were analyzed by Western blotting.

either expression or phosphorylation levels of EGFR (Fig. 4A). When NHBE cells were co-treated with L4 and flagellin, the phosphorylation of EGFR was clearly suppressed (Fig. 4B). These results suggest that L4 inhibits flagellin/TLR5 signal transduction.

4. Discussion

In the lungs, GAGs are distributed in the interstice, sub-epithelial tissue, bronchial walls, and airway secretions. It has been reported that CS contributed to the insolubility of mucus, while HA regulated tissue kallikrein catalytic activity, due to the diversity of GAG structures. As for KS, although its polymers have been shown to inhibit MMP-2 and activate MMP-9 in corneal and dermal explant cultures [25], the biological significance of KS present in airway mucus has not been fully understood. In the present study, we found that KS disaccharide, L4, inhibited the induction of the neutrophil chemo attractant IL-8 in NHBE cells stimulated with TLR5 agonist flagellin, by suppressing the phosphorylation of EGFR. Moreover, this inhibition is largely dependent on the structure conformation of L4.

There are many reports on HA to show the apparent discrepancy between the length of sugar chains and their biological functions [28]. Because HA and KS are both long unbranched polysaccharides with high degrees of heterogeneity, it is possible that KS actions depend on its molecular size, the target cells or the parameters being investigated. L4 is a disaccharide with similar structure to lactosamine, 3Galβ1-4GlcNAcβ1, sulfated at the C6 of both hexose moieties. And it is the most abundant repeating disaccharide unit of KS [29]. Experimental studies using L4 suggest that it can be a simple model to clarify the biological function of KS, especially in lung. Our data also supported that different disaccharide construction and sulfate had different effect on the IL-8 production (Fig. 2). On the next step, we hope to clarify that how and to what extent the KS oligosaccharide structure can affect its biological function.

On the other hand, we demonstrated that L4 did not modulate IL-8 production in NHBE cells stimulated with most of the TLR agonists, except TLR5 agonist flagellin. TLR5 is localized on the cell surface and recognize a variety of bacterial products [30,31], suggesting that L4 associates with the molecules involved in the flagellin-TLR5-IL-8 production pathway and the effect is TLR5 specific.

Previously, it is reported that L4 suppressed the production of a pro-inflammatory cytokine IL-12 in macrophages stimulated with LPS [26]. Since NHBE cells were insensitive to LPS, we were unable

to reproduce such suppression. Our data suggest a possible interaction between L4 and flagellin/TLR5 and the association of L4 to the cell surface of NHBE cells subsequently prevents the interaction between flagellin and TLR5. However the mechanism is quite different from the case of macrophages, because the suppressed IL-8 production by L4 is mediated by the attenuation of EGFR phosphorylation, which is consistent with previous reports [17].

Flagellins of some bacterial species are known to be glycosylated [32–35], and *P. aeruginosa* flagellin glycosylation was reported to contribute to TLR5 recognition [33]. However, the flagellin used in this study (from *Salmonella typhimurium*) is a simple protein and thus the anti-inflammatory effect of L4 is unlikely to be mediated by simple competition between L4 and flagellin glycans over the common binding site on TLR5. Further study is required to elucidate the potential of L4 to interact with flagellin and/or TLR5.

There is little information on KS and its ligand proteins. Instead, another highly sulfate GAG, heparan sulfate/heparin have been studied extensively [36]. These studies showed that GAGs could bind and regulate a number of distinct proteins, including chemokines, cytokines, growth factors, and adhesion molecules [37–38]. The interaction with proteins involves van der Waals (VDW) forces, hydrogen bonds, hydrophobic interactions with the carbohydrate backbone, and also the strong ionic interactions. Using polymer of L4 or carbohydrate cluster of L4 will make it easy to figure out the interaction of L4 with the composition of flagellin-TLR5-EGFR-IL8 pathway.

P. aeruginosa infection of the airway is particularly involved in advanced COPD [6,7]. They stimulated the inflammatory responses in the airway epithelial cells and macrophages through the interaction between LPS and TLR4 or between flagellin and TLR5 [9]. Therefore, L4 may have the potential to attenuate airway inflammatory responses particularly in the advanced phase of COPD.

Acknowledgments

The present study was supported in part by the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO), and by a Grant-in-Aid for Scientific Research (A), 20249018, from the Japanese Ministry of Education, Culture, Sports, Science and Technology, Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.05.009.

References

[1] Global strategy for the diagnosis, management and prevention of COPD, Global Initiative for Chronic Obstructive Lung Disease (GOLD). Available from: <http://www.goldcopd.org/> 2013.
[2] J.C. Hogg, F. Chu, S. Utokaparch, R. Woods, W.M. Elliott, L. Buzatu, R.M. Cherniack, R.M. Rogers, F.C. Sciurba, H.O. Coxson, P.D. Pare, The nature of small-airway obstruction in chronic obstructive pulmonary disease, N. Engl. J. Med. 350 (2004) 2645–2653.
[3] T.F. Murphy, The many faces of *Pseudomonas aeruginosa* in chronic obstructive pulmonary disease, Clin. Infect. Dis. 47 (2008) 1534–1536.
[4] S. Sethi, T.F. Murphy, Infection in the pathogenesis and course of chronic obstructive pulmonary disease, N. Engl. J. Med. 359 (2008) 2355–2365.
[5] A. Rosell, E. Monsó, N. Soler, F. Torres, J. Angrill, G. Riise, R. Zalacaín, J. Morera, A. Torres, Microbiologic determinants of exacerbation in chronic obstructive pulmonary disease, Arch. Intern. Med. 165 (2005) 891–897.
[6] T.F. Murphy, A.L. Brauer, K. Eschberger, P. Lobbins, L. Grove, X. Cai, S. Sethi, *Pseudomonas aeruginosa* in chronic obstructive pulmonary disease, Am. J. Respir. Crit. Care Med. 177 (2008) 853–860.
[7] S. Sethi, A. Anzueto, D.J. Farrell, Antibiotic activity of telithromycin and comparators against bacterial pathogens isolated from 3,043 patients with acute exacerbation of chronic bronchitis, Ann. Clin. Microbiol. Antimicrob. 4 (2005) 5.
[8] H. Sarir, P.A. Henricks, A.H. van Houwelingen, F.P. Nijkamp, G. Folkerts, Cells, mediators and Toll-like receptors in COPD, Eur. J. Pharmacol. 585 (2008) 346–353.
[9] E. Raoust, V. Balloy, I. Garcia-Verdugo, L. Touqui, R. Ramphal, M. Chignard, *Pseudomonas aeruginosa* LPS or flagellin are sufficient to activate TLR-dependent signaling in murine alveolar macrophages and airway epithelial cells, PLoS One 4 (2009) e7259.
[10] R. Sorrentino, P.M. de Souza, S. Sriskandan, C. Duffin, M.J. Paul-Clark, J.A. Mitchell, Pattern recognition receptors and interleukin-8 mediate effects of Gram-positive and Gram-negative bacteria on lung epithelial cell function, Br. J. Pharmacol. 154 (2008) 864–871.
[11] D. Banerjee, O.A. Khair, D. Honeybourne, Impact of sputum bacteria on airway inflammation and health status in clinical stable COPD, Eur. Respir. J. 23 (2004) 685–691.
[12] P. Bresser, T.A. Out, L. van Alphen, H.M. Jansen, R. Lutter, Airway inflammation in nonobstructive and obstructive chronic bronchitis with chronic haemophilus influenzae airway infection. Comparison with noninfected patients with chronic obstructive pulmonary disease, Am. J. Respir. Crit. Care Med. 162 (2000) 947–952.
[13] S. Sethi, J. Maloney, L. Grove, C. Wrona, C.S. Berenson, Airway inflammation and bronchial bacterial colonization in chronic obstructive pulmonary disease, Am. J. Respir. Crit. Care Med. 173 (2006) 991–998.
[14] N. Soler, S. Ewig, A. Torres, X. Filella, J. Gonzalez, A. Zaubet, Airway inflammation and bronchial microbial patterns in patients with stable chronic obstructive pulmonary disease, Eur. Respir. J. 14 (1999) 1015–1022.
[15] J.L. Koff, M.X. Shao, S. Kim, I.F. Ueki, J.A. Nadel, *Pseudomonas* lipopolysaccharide accelerates wound repair via activation of a novel epithelial cell signaling cascade, J. Immunol. 177 (2006) 8693–8700.
[16] R. Shaykhiev, J. Behr, R. Bals, Microbial patterns signaling via Toll-like receptors 2 and 5 contribute to epithelial repair, growth and survival, PLoS One 3 (2008) e1393.
[17] J.L. Koff, M.X. Shao, I.F. Ueki, J.A. Nadel, Multiple TLRs activate EGFR via a signaling cascade to produce innate immune responses in airway epithelium, Am. J. Physiol. Lung Cell Mol. Physiol. 294 (2008) L1068–L1075.
[18] H.J. Choi, C.H. Seo, S.H. Park, H. Yang, K.H. Do, J. Kim, H.K. Kim, D.H. Chung, J.H. Ahn, Y. Moon, Involvement of epidermal growth factor receptor-linked signaling responses in *Pseudomonas fluorescens*-infected alveolar epithelial cells, Infect. Immun. 79 (2011) 1998–2005.
[19] C. Gao, T. Maeno, F. Ota, M. Ueno, H. Korekane, S. Takamatsu, K. Shirato, A. Matsumoto, S. Kobayashi, S. Kitazume, K. Ohtsubo, T. Betsuyaku, N. Taniguchi, Sensitivity of heterozygous α1, 6 fucosyltransferase knock out mice to cigarette smoke-induced emphysema: implication of aberrant TGF-β signaling and MMP gene expression, J. Biol. Chem. 287 (2012) 16699–16708.
[20] K. Kamio, T. Yoshida, C. Gao, T. Ishii, F. Ota, T. Motegi, S. Kobayashi, R. Fujinawa, K. Ohtsubo, S. Kitazume, T. Angata, A. Azuma, A. Gemma, M. Nishimura, T. Betsuyaku, K. Kida, N. Taniguchi, α1,6 Fucosyltransferase (Fut8) is implicated in vulnerability to elastase-induced emphysema in mice and a possible non-invasive predictive marker for disease progression and exacerbation in chronic obstructive pulmonary disease (COPD), Biochem. Biophys. Res. Commun. 424 (2012) 112–117.
[21] A. Asari, T. Kanemitsu, H. Kurihara, Oral administration of high molecular weight hyaluronan (900 kDa) controls immune system via Toll-like receptor 4 in the intestinal epithelium, J. Biol. Chem. 285 (2010) 24751–24758.
[22] S. Misra, B.P. Toole, S. Ghatak, Hyaluronan constitutively regulates activation of multiple receptor tyrosine kinases in epithelial and carcinoma cells, J. Biol. Chem. 281 (2006) 34936–34941.
[23] G.B. Johnson, G.J. Brunn, Y. Kodaira, J.L. Platt, Receptor-mediated monitoring of tissue well-being via detection of soluble heparan sulfate by Toll-like receptor 4, J. Immunol. 168 (2002) 5233–5239.
[24] M.E. Monzon, S.M. Casalino-Matsuda, R.M. Forteza, Identification of glycosaminoglycans in human airway secretions, Am. J. Respir. Cell Mol. Biol. 34 (2006) 135–141.
[25] N. Isnard, L. Robert, G. Renard, Effect of sulfated GAGs on the expression and activation of MMP-2 and MMP-9 in corneal and dermal explant cultures, Cell Biol. Int. 27 (2003) 779–784.
[26] H. Xu, H. Kurihara, T. Ito, H. Kikuchi, K. Yoshida, H. Yamanokuchi, A. Asari, The keratan sulfate disaccharide Gal(6S03) β1,4-GlcNAc(6S03) modulates interleukin 12 production by macrophages in murine Thy-1 type autoimmune disease, J. Biol. Chem. 280 (2005) 20879–20886.
[27] M. Kubota, K. Yoshida, A. Tawada, M. Ohashi, Characterization of oligosaccharides of the lactosamine series derived from Keratan sulfates by tandem mass spectrometry, Eur. J. Mass. Spectrom. 6 (2000) 193–203.
[28] G.M. Campo, A. Avenoso, S. Campo, A. D'Ascola, G. Nastasi, A. Calatroni, Molecular size hyaluronan differently modulates toll-like receptor-4 in LPS-induced inflammation in mouse chondrocytes, Biochimie 92 (2010) 204–215.
[29] J.L. Funderburgh, Keratan sulfate: structure, biosynthesis, and function, Glycobiology 10 (2000) 951–958.
[30] G.M. Barton, J.C. Kagan, A cell biological view of Toll-like receptor function: regulation through compartmentalization, Nat. Rev. Immunol. 9 (2009) 535–542.
[31] T. Kawai, S. Akira, The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors, Nat. Immunol. 11 (2010) 373–384.
[32] K. Takeuchi, F. Taguchi, Y. Inagaki, K. Toyoda, T. Shiraishi, Y. Ichinose, Flagellin glycosylation island in *Pseudomonas syringae* pv. glycinea and its role in host specificity, J. Bacteriol. 185 (2003) 6658–6665.

[33] A. Verma, S.K. Arora, S.K. Kuravi, R. Ramphal, Roles of specific amino acids in the N terminus of *Pseudomonas aeruginosa* flagellin and of flagellin glycosylation in the innate immune response, Infect. Immun. 73 (2005) 8237–8246.

[34] C.D. Brimer, T.C. Montie, Cloning and comparison of *fliC* genes and identification of glycosylation in the flagellin of *Pseudomonas aeruginosa* a-type strains, J. Bacteriol. 180 (1998) 3209–3217.

[35] K.D. Smith, E. Andersen-Nissen, F. Hayashi, K. Strobe, M.A. Bergman, S.L. Barrett, B.T. Cookson, A. Aderem, Toll-like receptor 5 recognizes a conserved site on flagellin required for protofilament formation and bacterial motility, Nat. Immunol. 4 (2003) 1247–1253.

[36] I. Capila, R.J. Linhardt, Heparin–protein interactions, Angew. Chem. Int. Ed. Engl. 41 (2002) 390–412.

[37] R.L. Jackson, S.J. Busch, A.D. Cardin, Glycosaminoglycans: molecular properties, protein interactions, and role in physiological processes, Physiol. Rev 71 (1991) 481–539.

[38] L. Haglund, V. Tillgren, P. Onnerfjord, D. Heinegard, The C-terminal peptide of chondroadherin modulates cellular activity by selectively binding to heparan sulfate chains, J. Biol. Chem. 288 (2013) 995–1008.