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Anti-infective control in human bronchiolar epithelial cells by mucin phenotypic changes following uptake of N-acetyl-L-cysteine

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Short title: Anti-infective control in bronchiolar epithelium by NAC

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Abstract

PURPOSE: Aspiration pneumonia is infection of the respiratory tract resulting from accumulation of sputum on the larynx. N-acetyl-L-cysteine (NAC) might regulate mucin (MUC) expression and activate inherent anti-infective system in bronchiolar epithelial cells after cellular uptake, therefore, serve as the preventative agent for chronic lung disease including aspiration pneumonia. The purpose of this *in vitro* study was to evaluate the effect of uptake of NAC by human bronchiolar epithelial cells on bacterial infection and regulations of mucin expression in association with cellular redox status under co-culture with a representative pathogen for hospital- and community-acquired pneumonia, *Streptococcus pneumoniae*.

MATERIALS AND METHODS: Human bronchiolar epithelial cells preincubated with or without 20 mM NAC for 3 h were co-cultured with or without bacteria for 8 h and evaluated with respect to cellular redox balance, expressions of various types of MUC, proinflammatory cytokines and mediators and bacterial infection state by biochemical, genetic and immunofluorescent assays.

RESULTS: Markedly-increased intracellular reactive oxygen species and oxidized glutathione levels plus increased release and expression of proinflammatory cytokines and mediators were observed in cells co-cultured with bacteria. These bacteria-induced cellular redox disturbance and proinflammatory events were prevented and alleviated by pretreatment with NAC. Cells co-cultured with bacteria did not increase expression of anti-infective membranous MUC4 but exhibited increases in gel-forming MUC5AC expression and bacterial infection. However, NAC-pretreated cells avoided bacterial infection along with enhancement of MUC4, but not MUC5AC, expression.

CONCLUSION: Uptake of NAC by human bronchiolar epithelial cells prevented bacterial infection and upregulated membranous, but not gel-forming, MUC expression along with the increase of intracellular antioxidant level under co-culture conditions with *S. pneumonia*.

Keywords: Thiols, Airway inflammation, Antimicrobial, Antioxidant, COX-2, AP-1

Introduction

Aspiration pneumonia is widely prevalent in hospitalized patients and residents of nursing homes [1,2], and has become a major cause of death in elderly people, particularly for those with dysphagia or a reduction in the cough reflex [3]. This type of pulmonary syndrome is an infectious process caused by silent aspiration of sputum [4]. Sputum is mucus that contains food debris, exfoliated tissue, and/or pathological bacteria originating from the oropharyngeal region [3,5], such as *Streptococcus pneumonia*, which is a representative pathogen for hospital- or community-acquired pneumonia [6]. Mucus is mainly produced by bronchial epithelium and submucosal glands via production of inflammatory mediators in response to bacteria or chemical substances. Mucus serves as a slimy defensive barrier to trap and transport these foreign materials out of the airway [7]. However, hypersecretion and increased viscoelasticity of mucus beyond ciliary clearance cause bronchiolar obstruction and bacterial infection [7]. In addition to maintenance of good oral hygiene and improvements in swallowing/cough reflexes of elderly people under nursing care as basic approaches [8], development of an adjunctive approach to reduce accumulation of sputum in the pharyngeal region might be effective to prevent aspiration pneumonia [9].

Mucus consists of mucin (MUC) molecules, which are high molecular weight glycoproteins. There are two types of MUCs: secretory and membranous. Secretory MUCs, referred to as gel-forming MUCs, are the major components of mucus [10]. MUC5AC is a major gel-forming MUC and increases mucus viscoelasticity [11,12]. Increased expression of MUC5AC is associated with chronic inflammatory lung disease [12,13] despite its secretion in response to bacterial attachment to wash out them [14]. In contrast, membranous MUCs, such as MUC1 and MUC4, are membrane-tethered glycoproteins that protect cells from pathogenic invasion by acting as decoys that are shed from the cell surface in response to bacterial adherence [15,16]. This function suggests that membranous MUCs can compensate for gel-forming MUCs to prevent bacterial infection [17]. Expression of both

types of MUCs can be regulated by cellular redox status [18,19].

N-acetyl-L-cysteine (NAC) is an antioxidant amino acid derivative and membrane-permeable thiol compound with a small molecular weight (163. 19). NAC protects cells from oxidative stress by directly scavenging reactive oxygen species (ROS) and enhancing cellular antioxidant capability via supplementation of the major cellular antioxidant molecule, glutathione [20]. Previous studies have demonstrated that loss of viability and cellular dysfunction by infection with *Staphylococcus aureus* or *Streptococcus pyogenes* were prevented in gingival fibroblasts and osteoblasts on a collagenous scaffold containing NAC in a bacterial cell co-culture model [21]. Invasion of bacteria into cells is completely prevented on a scaffold containing NAC, in contrast to abundant bacteria within the cytoplasm on material without NAC [21]. This indicated that NAC can improve the cellular defense system to prevent bacterial invasion into cells. In addition, NAC may directly modulate certain transcriptional factors nuclear factor kappa B (NFκB) or activator protein-1 (AP-1) [20] which involve expressions of various types of MUC in epithelial cells [22-24]. Therefore, it has been hypothesized that cellular uptake of NAC prevents infection in association with regulations of MUC's expression in bronchiolar epithelium. Proving this hypothesis would provide important information for the development of external medications for the lower airway to prevent chronic inflammatory lung diseases, in particular, aspiration pneumonia.

The purpose of this *in vitro* study was to evaluate the effect of uptake of NAC by human bronchiolar epithelial cells on bacterial infection and regulations of MUC's expression in association with cellular redox status under co-culture with a pathogen.

Materials and methods

Prior culture of epithelial cells and bacteria

Primary human bronchiolar epithelial cells (HBECs) were purchased from Lonza (Walkersville, MD, USA). The cells were cultured in 40-mm polystyrene dishes in small airway epithelial cell growth medium (SAGM, Lonza). After the first passage, the cells were seeded at a density of 5×10^4 cells/cm^2 in a 24-well culture grade polystyrene plate with 0.5-ml SAGM and incubated at 37°C in a humidified incubator with 5% CO_2. As a model bacterium, the GTC261 strain of *Streptococcus pneumoniae* was employed. Bacteria were grown in a tightly capped 15-ml sterile plastic tube with 3-ml trypticase soy broth (Becton Dickinson, Franklin Lakes, New Jersey, USA) at 37°C statically overnight (16–18 h) before use in co-culture experiments with epithelial cells.

NAC pretreatment and bacterial co-culture

A 1-mol/l NAC stock solution (pH 7.2) was prepared by dissolving NAC powder (Sigma-Aldrich Japan, Tokyo, Japan) in HEPES buffer. At 12 h after seeding, the medium of the epithelial cell cultures was changed to 0.5-ml SAGM with or without 20 mM NAC. The cells were incubated for 3 h at 37°C in a humidified incubator with 5% CO_2. Then, the culture medium was changed to small airway epithelial cell basal medium (SABM, Lonza) with or without 10 μl of a bacterial suspension containing 2×10^5 cells to achieve a final concentration of 1×10^7 bacterial cells/ml. The multiplicity of infection (MOI) of the co-culture was approximately 2. The epithelial cell cultures were divided into four groups: untreated or NAC-pretreated HBECs with or without bacterial co-culture. The cultures were incubated for 8 h at 37°C in a humidified incubator with 5% CO_2 and then used in subsequent experiments.

Measurement of viable attached cells and cellular reactive oxygen species (ROS)

The number of attached cells and the amount of intracellular ROS generation was evaluated by calcein-AM (Dojindo Molecular Technologies, Rockville, MD) and 5-(and-6)-carboxy-20,70-dichlorofluorescein diacetate (carboxy-DCF-DA) (Invitrogen, MD, USA),

respectively. After discarding the supernatant from the cultures, the cells were gently rinsed twice with D-PBS. Then, the cells were incubated in a calcein-AM solution for 30 min at 37°C. After removing the solution, the cells were gently rinsed again with D-PBS. The fluorescence intensity of calcein or DCF-DA was measured using a fluorescence plate reader at 535 nm (excitation at 485 nm) against D-PBS as the blank.

Cellular glutathione (GSH) detection

The amount of total cellular glutathione was determined by measuring the concentration of 2-nitro-5-thiobenzoic acid resulting from a chromogenic reaction between DTNB and reduced glutathione in a glutathione recycling system. Oxidized glutathione (GSSG) was detected by masking reduced glutathione (GSH) with a masking reagent. The cells were washed twice with D-PBS and then lysed in 200 µl of a 0.1 M HCl solution by freeze/thawing. The lysate was then subjected to total glutathione and GSSG detection. The absorbance at 415 nm was measured using a plate reader. The intracellular concentrations were calculated based on a parallel glutathione standard curve. The amount of GSH was calculated by subtracting the amount of GSSG from the amount of total glutathione in the same lysate.

Cellular proinflammatory cytokine detection

The amount of proinflammatory cytokines, such as interleukin (IL)-1β, IL-6 and IL-8, in HBECs was quantified using the multiplex cytokine immunoassay system (Veritas, Tokyo, Japan). The cells were washed twice with D-PBS and lysed in 200-µl D-PBS by freeze/thawing. The lysate was incubated with beads coated with antibodies corresponding to the various cytokines according to the manufacturer’s instructions. The bead size and fluorescence were measured on the Luminex 200 system (Luminex Japan, Tokyo, Japan). Data were analyzed using a Master Plex QT (Hitachi Solutions, Tokyo, Japan).

Production of prostaglandin E2 (PGE2)

The amount of prostaglandin E2 in HBECs was measured using an immunochemical assay (Prostaglandin E2 EIA Kit; Cayman Chemical Company, MI, USA). This assay is based on competition for an anti-PGE2 monoclonal antibody between sample-derived PGE2 and a PGE2-acetylcholineesterase (AChE) conjugate (PGE2 tracer). The amount of PGE2 tracer remains constant while the concentration of PGE2 varies, and the amount of tracer that binds to the anti-PGE2 monoclonal antibody will be inversely proportional to the concentration of PGE2 in the sample. Cells were washed twice with D-PBS and lysed in 200-µl D-PBS by freeze/thawing. The lysate was then subjected to PGE2 detection. The absorbance at 415 nm was measured using a plate reader. The intracellular concentrations of PGE2 were calculated based on a parallel PGE2 standard curve.

RNA isolation and reverse transcriptase-polymerase chain reaction

Gene expression in HBECs was analyzed by a reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA from cells was extracted using TRIzol (Invitrogen, CA, USA) and an RNeasy purification column (Qiagen, Valencia, CA, USA). After DNAse I treatment, reverse transcription of 0.5 µg total RNA was performed using MMLV reverse transcriptase (Clontech, Carlsbad, CA, USA) in the presence of an oligo(dT) primer (Clontech). PCR was performed using EX Taq DNA polymerase (Takara Bio Inc., Shiga, Japan) to detect *IL6*, *IL8*, *MUC5AC*, *MUC1*, *MUC4*, and cyclooxygenase (COX)-2 (*PTGS2*) mRNA. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was employed as a housekeeping gene. The primers (synthesized by Sigma-Aldrich) are listed in Table 1. Preliminary PCRs were performed to determine the annealing temperature and optimal cycle number to yield a linear range of PCR amplification for each primer set. PCR products were visualized on 1.5% agarose gels by ethidium bromide staining. Band intensities were detected and

quantified under UV light. Band intensity was quantified under UV light and normalized with reference to GAPDH mRNA.

Immunofluorescent analysis of MUC5AC and MUC4

Cells were fixed with 4% formaldehyde for 10 min and then incubated in 0.1% PBS-Tween containing 1% bovine serum albumin, 10% normal goat serum, and 0.3 M glycine (Wako Pure Chemical Industries, Ltd., Osaka, Japan) for 1 h to permeabilize the cells and block non-specific protein–protein interactions. The cells were then incubated with anti-MUC5AC (ab3649, 0.4µl/ml), or MUC4 (ab60720, 0.2µl/ml) antibodies (Abcam Plc, Cambridge, UK) for 30 min at 22°C. The secondary antibody, DyLight 488 goat anti-mouse IgG (green) (Abcam), was applied for 30 min at 22°C. 4′-6-diamidino-2-phenylindole (DAPI) (blue) (Vector Laboratories, Inc., CA, USA) was present in the mounting agent to counterstain the cells. Cellular MUC expression was observed under a fluorescence photomicroscope (Axiophoto2, Carl Zeiss, Co., Ltd. Jena, Germany). To evaluate cellular MUC expression, three different fields of cells with typical morphological features were randomly selected for analysis. Expression of MUC5AC and MUC4 was analyzed by measuring the positive area and the number of cellular nuclei in the selected fields using image analysis software (ImageJ; National Institutes of Health, MD, USA). The MUC-positive area per unit nucleus was set as a representative value for each group. Five independent cultures of different cell batches were evaluated in each group.

Evaluation of bacterial colonization on and/or within cells

For this analysis only, the bacteria were pre-labeled with 5-cyano-2,3-ditolyl-2H-tetrazolium chloride (CTC, Dojindo Molecular Technologies, Inc., Gaithersburg, MD) before co-incubation with HBECs to prevent undesirable CTC accumulation in viable HBECs. After washing the culture once with D-PBS, the culture was incubated in calcein-AM solution as a selective dye for viable HBECs.

Subsequently, the stained culture was fixed in 10% formalin and covered with a slide glass applied with an encapsulating material without DAPI (VECTASHIELD Mounting Medium, Vector, Burlingame, CA). The stained culture was observed under a fluorescent photomicroscope (Axiophoto2, Carl Zeiss Co., Ltd. Jena, Germany).

Bacterial colonization on and/or within cells was also evaluated on the basis of the methodology previously described [21]. After 8 h co-incubation of HBECs with bacteria pre-labeled with CTC, unattached bacteria were removed by washing the culture twice with D-PBS. The cultures were incubated in 0.2-mL sterile distilled water (ddH_2O) per well for 30 min and pipetted repeatedly to destroy cell membranes and to generate cellular lysates containing colonizing bacteria. CTC quantity was measured using a fluorescence plate reader at 630 nm (excitation at 450 nm) against ddH_2O as a blank.

Statistical analysis

For immunofluorescent image analysis about MUC expression, five independent cultures of different cell batches were evaluated in each group (n = 5). The other culture experiments were performed four times for each assay (n = 4). All repeated data were analyzed by Bonferroni multiple comparisons after one-way analysis of variance or Student's t-test to determine the differences between each culture condition using statistical software (SPSS standard version 16; International Business Machines Corporation, NY, USA). Statistical significance was set at $P < 0.05$.

Results

Effects of pretreatment with NAC on HBEC cell death caused by bacteria

Calcein-based quantification showed that attached cells in HBEC cultures with bacterial co-incubation were half that of those present in cultures without bacterial co-incubation (Figure 1A)

($P < 0.05$). However, the value of cultures pretreated with NAC was higher than that of untreated cultures with bacterial co-incubation, despite not coming up to the values in the cultures without bacterial co-incubation.

Effects of pretreatment with NAC on redox status in HBECs in the presence of bacteria

Compared with cells without bacterial co-incubation, intracellular ROS levels in untreated cells were markedly increased by bacterial co-incubation (Figure 1B). Pretreatment with NAC prevented increases in ROS levels, even with bacterial co-incubation. The concentration of GSH per unit cell was increased by pretreatment with NAC, regardless of bacterial co-incubation (Figure 1C). Bacterial co-incubation did not change the concentration of GSH per unit cell (Figure 1C) but markedly increased the concentration of GSSG per unit cell in untreated cells (Figure 1D). Regardless of bacterial co-incubation, cells pretreated with NAC did not show a change in the concentration of GSSG per unit cell compared with untreated cells without bacterial co-incubation.

Effects of NAC on gene expression of proinflammatory cytokines and MUCs in HBECs

IL6 and *IL8* gene expression was upregulated in cells with bacterial co-incubation compared with that in untreated or NAC-pretreated cells without bacterial co-incubation (Figure 2A). *IL6* or *IL8* expression was markedly or slightly lower in cells pretreated with NAC with bacterial co-incubation, than that in untreated cells with bacterial co-incubation, respectively. *PTGS2* gene expression was steady under all of the culture conditions, but its expression was highest in untreated cells with bacterial co-incubation (Figure 2A). *MUC5AC* expression in untreated cells was upregulated by bacterial co-incubation in contrast with a little or no detection in untreated or NAC-pretreated cells without bacterial co-incubation. However, this upregulation by bacterial co-incubation was suppressed by pretreatment with NAC (Figure 2A). In contrast, *MUC4* expression was upregulated by pretreatment with NAC, regardless of bacterial co-incubation, in contrast to that in untreated cells

with or without the bacterial co-incubation. *MUC1* expression was increased by bacterial co-incubation, but not by pre-treatment with NAC.

Effects of pretreatment with NAC on the proinflammatory cytokine production of HBECs against bacteria

PGE2 synthesis was increased by 1.5-fold in untreated cells with bacterial co-incubation compared with that in cells without bacterial co-incubation (Figure 2B). However, PGE2 synthesis did not increase in the cells pretreated with NAC regardless of bacterial co-incubation. Bacterial co-incubation increased the production of proinflammatory cytokines including IL1β, IL6 and IL8 by more than 2-fold in untreated cells (Figure 2C). The bacteria-induced proinflammatory cytokine production was reduced by pretreatment with NAC. In particular, IL-1β per unit cell under bacterial co-incubation was reduced by pretreatment with NAC to the level observed in cells without bacterial co-incubation.

Effects of NAC on MUC5AC and MUC4 expression in HBECs

Immunofluorescent staining of MUC5AC demonstrated strong expression in untreated cells with bacterial co-incubation only (Figure 3A, upper left images). However, regardless of bacterial co-incubation, MUC5AC expression in cells pretreated with NAC was similar to that in untreated cells without bacterial co-incubation. In contrast, expression of MUC4 was higher in cells pretreated with NAC, regardless of bacterial co-incubation (Figure 3A, upper right images). Untreated cells also expressed MUC4 to some extent, although it appeared to be unrelated to bacterial co-incubation. MUC5AC expression per unit nucleus was 3-fold higher in untreated cells with bacterial co-incubation than in cells without bacterial co-incubation (Figure 3B). The expression of MUC5AC per unit nucleus in NAC-pretreated cells with or without bacterial co-incubation was equal to or less than that in untreated cells without bacterial co-incubation. MUC4 expression per unit nucleus was

more than 5-fold higher in NAC-pretreated cells than in the untreated cells, regardless of bacterial co-incubation (Figure 3C).

Effect of pretreatment with NAC on bacterial colonization on and/or within HBECs.

Fluorescent microscopic observation demonstrated that untreated cells with bacterial co-incubation were apparently rounded and exhibited intensive CTC accumulation, in contrast with the relatively flattened shape of NAC pre-treated cells combined with low CTC accumulation (Figure 4A). CTC fluorescent intensity in cell lysis containing pre-labeled bacteria on and/or within cells was higher in cultures without pre-treatment of NAC than in cultures pre-treated with NAC (Figure 4B).

Discussion

This study employed primary cell clusters obtained from human healthy bronchiolar epithelium. The cell cluster was obtained from a distal portion of normal human lung tissue in the 1-mm bronchiole area (2^6 to 2^{23} branches), the epithelium composition of which was reported as ciliated simple columnar (or cubic) epithelium containing Clara cells [25]. This cellular composition was slightly different from the intrapulmonary bronchial epithelium or the respiratory epithelium of the upper airways (respiratory portion of the nasal cavity, nasopharynx, larynx, trachea and both extra-pulmonary epithelium), the epithelium composition of which was a ciliated pseudostratified (or simple) columnar epithelium containing goblet cells. The present study employed the undifferentiated cells and culture system in order to ignore the histological differences between bronchial and bronchiolar epithelium. The gross-sectional area of the lower airway increases in size towards the alveoli. In addition, the progression of chronic inflammatory lung disease is associated with the disease state of the bronchiole [26,27]. Moreover, NAC can be applied as an inhalation aerosol [28] that is able to reach the bronchiole and alveoli. Based on these aspects, the present study was conducted on bronchiolar epithelium.

The ratio of bacteria to epithelial cells (MOI) was 2 in the present study. This value was within the MOI range of a previous study employing a co-culture model (MOI: 0.005–5) [29] in which the number of U2OS osteosarcoma cells co-cultured with *Staphylococcus epidermidis* for 48 h was reduced by approximately 90% in comparison with cells without bacterial co-incubation. Previously, a co-culture experiment demonstrated the anti-infective capability of NAC on the basis of bacteriostatic and antioxidant abilities in a bacteria–animal cell co-culture model [21]. Rat gingival fibroblastic and osteoblastic cells on a collagen scaffold with or without NAC-addition were co-cultured with *Staphylococcus aureus* or *Streptococcus pyogenes* at a MOI of 6.25. Co-incubation with *S. pneumoniae* killed half of the attached human bronchiolar epithelial cells in the present study. This result indicates that the co-culture protocol used in the present study was capable of providing sufficiently harsh conditions for epithelial cells survival.

In the present study, NAC treatment of HBECs was performed before bacterial co-incubation. This culture condition did not allow coexistence of NAC with bacteria, in contrast to a previous study using co-cultured tissue-forming cells with wound pathogens in the presence of NAC, which demonstrated the anti-infection capability of NAC [21]. Pretreatment with NAC prevented cellular proinflammatory cytokine production to bacterial exposure, as indicated by unchanged or slightly increased levels of proinflammatory cytokine expression and PGE2 synthesis in cells pretreated with NAC, even with bacterial co-incubation. Cells pretreated with NAC exhibited not only avoidance of intracellular ROS elevation, but also a marked increase in cellular GSH levels regardless of bacterial co-incubation. This finding could be supported by biochemical transformation of NAC into GSH within the cytoplasm after cellular uptake of NAC [30]. However, cellular GSH and GSSG levels did not change in cells pretreated with NAC even with bacterial co-incubation, indicating avoidance of bacteria-induced oxidative stress in cells pretreated with NAC. In addition, detection of CTC-labeled

bacteria was lower for cells pretreated with NAC than untreated cells. All of these observations indicated that uptake of NAC enhanced a cellular defensive system against infection along with antioxidant reinforcement.

The increase in cellular MUC production by bacterial co-incubation was alleviated by pretreatment with NAC. *MUC5AC* gene and protein expression did not increase in cells pretreated with NAC, even with bacterial co-incubation. Interestingly, regardless of bacterial co-incubation, *MUC4* gene and protein expression, a membranous MUC, was increased only in cells pretreated with NAC. The membranous types of MUC function as protective decoys for bacterial infection by shedding from the cell surface [15,16]. In contrast to gel-forming MUCs, membranous MUCs should not accumulate in sputum as a result of bacterial clearance. Therefore, NAC might reinforce the bacterial clearance system mediated by MUC4 without accumulation of sputum (Figure 5).

In this study, MUC4 expression in HBECs was enhanced by uptake of NAC regardless of bacterial co-incubation. In contrast, the expression of *MUC1*, another major cell-protective membranous MUC molecule, was not affected by pretreatment with NAC. Both MUC1 and MUC4 are ubiquitously expressed in tracheal epithelial cells. They have similarities in function including steric hindrance, and act as releasable decoys and tumor metastasis markers [15,24]. However, their similarity with regards to transcriptional regulation remains to be fully elucidated. It was reported that *MUC1* expression in cancer cells is upregulated by signal transduction and activator of transcription (STAT) or NFκB [24,31], whereas, the promoter region of *MUC4* has binding sites for various transcriptional factors including AP-1 as well as STAT and NFκB [22]. It has been suggested that thiol compound like NAC or GSH directly modulates AP-1 by affecting the cysteine residuals of them , [32,33] which is an important regulatory factor for osteoblastic differentiation [34]. Interestingly, NAC can enhance osteoblastic differentiation, and functions as an osteogenesis-enhancing molecule for bone

regeneration [35]. The biomolecular mechanism underlying NAC-mediated enhancement of MUC4 expression in HBECs should be of great interest for future research.

NAC might exert multiple unique effects that contribute to the prevention of chronic inflammatory lung diseases. NAC has bacteriostatic effects on various types of pathogens and functions as a mucolytic drug [36]. In contrast with anti-biotics or non-steroidal anti-inflammatory drugs, the routine use of NAC is not expected to be issues that are the emergence of antibiotic-resistant strains of oral-resident pathogenic bacteria such as *Streptococcus pneumoniae* or *Staphylococcus aureus* [37,38] or the occurrence of gastrointestinal mucosa ulcer complications [39]. Thus, the present study has implications for the development of a novel strategy for the treatment of chronic inflammatory lung diseases such as aspiration pneumonia. However, the article about the experimental model of infection with bacteria indicated that *S. pneumonia* formed biofilm on differentiated epithelium in a similar way to *in vivo*, but rapidly killed the undifferentiated cells instead of forming biofilms [40]. This suggested the limitation of this culture model. As well as the NAC's delivery method and protocol, the effects of uptake of NAC on differentiated bronchial and bronchiolar epithelial cells or other airway epithelial tissue such as subepithelial gland tissue or alveoli [41] should be investigated in future research.

Conclusion

Uptake of NAC by HBECs prevented bacterial infection and increased expression of membranous, but not gel-forming, MUC along with the increase of intracellular antioxidant level under co-culture conditions with *S. pneumonia*.

Acknowledgments

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References

[1] Teramoto S, Fukuchi Y, Sasaki H, Sato K, Sekizawa K, Matsuse T. High incidence of aspiration pneumonia in community- and hospital-acquired pneumonia in hospitalized patients: a multicenter, prospective study in Japan. J Am Geriatr Soc 2008;56(3):577-9.

[2] Marrie TJ, Durant H, Kwan C. Nursing home-acquired pneumonia. A case-control study. J Am Geriatr Soc 1986;34(10):697-702.

[3] DiBardino DM, Wunderink RG. Aspiration pneumonia: A review of modern trends. J Crit Care 2014.

[4] Marik PE. Aspiration pneumonitis and aspiration pneumonia. N Engl J Med 2001;344(9):665-71.

[5] El-Solh AA, Pietrantoni C, Bhat A, Okada M, Zambon J, Aquilina A, Berbary E. Colonization of dental plaques: a reservoir of respiratory pathogens for hospital-acquired pneumonia in institutionalized elders. Chest 2004;126(5):1575-82.

[6] File TM, Jr., Marrie TJ. Burden of community-acquired pneumonia in North American adults. Postgrad Med 2010;122(2):130-41.

[7] Voynow JA, Rubin BK. Mucins, mucus, and sputum. Chest 2009;135(2):505-12.

[8] van der Maarel-Wierink CD, Vanobbergen JN, Bronkhorst EM, Schols JM, de Baat C. Oral health care and aspiration pneumonia in frail older people: a systematic literature review. Gerodontology 2013;30(1):3-9.

[9] Elman LB, Dubin RM, Kelley M, McCluskey L. Management of oropharyngeal and tracheobronchial secretions in patients with neurologic disease. J Palliat Med 2005;8(6):1150-9.

[10] Sears PR, Davis CW, Chua M, Sheehan JK. Mucociliary interactions and mucus dynamics in ciliated human bronchial epithelial cell cultures. Am J Physiol Lung Cell Mol Physiol 2011;301(2):L181-6.

[11] Seagrave J, Albrecht HH, Hill DB, Rogers DF, Solomon G. Effects of guaifenesin, N-acetylcysteine, and ambroxol on MUC5AC and mucociliary transport in primary differentiated human tracheal-bronchial cells. Respir Res 2012;13:98.

[12] Thornton DJ, Rousseau K, McGuckin MA. Structure and function of the polymeric mucins in airways mucus. Annu Rev Physiol 2008;70:459-86.

[13] Fahy JV, Dickey BF. Airway mucus function and dysfunction. N Engl J Med 2010;363(23):2233-47.

[14] Shen H, Yoshida H, Yan F, Li W, Xu F, Huang H, Jono H, Li JD. Synergistic induction of MUC5AC mucin by nontypeable Haemophilus influenzae and Streptococcus pneumoniae. Biochem Biophys Res Commun 2008;365(4):795-800.

[15] Linden SK, Sheng YH, Every AL, Miles KM, Skoog EC, Florin TH, Sutton P, McGuckin MA. MUC1 limits Helicobacter pylori infection both by steric hindrance and by acting as a releasable decoy. PLoS Pathog 2009;5(10):e1000617.

Free Radic Res.

[16] Ruvoen-Clouet N, Mas E, Marionneau S, Guillon P, Lombardo D, Le Pendu J. Bile-salt-stimulated lipase and mucins from milk of 'secretor' mothers inhibit the binding of Norwalk virus capsids to their carbohydrate ligands. Biochem J 2006;393(Pt 3):627-34.

[17] Wang IJ, Yu CJ, Hu FR. Alteration of ocular surface mucins in MUC5AC-DTA transgenic mice. Mol Vis 2009;15:108-19.

[18] Barnes PJ. Mediators of chronic obstructive pulmonary disease. Pharmacol Rev 2004;56(4):515-48.

[19] Bavarva JH, Tae H, McIver L, Garner HR. Nicotine and oxidative stress induced exomic variations are concordant and overrepresented in cancer-associated genes. Oncotarget 2014;5(13):4788-98.

[20] Zafarullah M, Li WQ, Sylvester J, Ahmad M. Molecular mechanisms of N-acetylcysteine actions. Cell Mol Life Sci 2003;60(1):6-20.

[21] Yamada M, Ishihara K, Ogawa T, Sakurai K. The inhibition of infection by wound pathogens on scaffold in tissue-forming process using N-acetyl cysteine. Biomaterials 2011;32(33):8474-85.

[22] Chaturvedi P, Singh AP, Batra SK. Structure, evolution, and biology of the MUC4 mucin. FASEB J 2008;22(4):966-81.

[23] Li JD. Exploitation of host epithelial signaling networks by respiratory bacterial pathogens. J Pharmacol Sci 2003;91(1):1-7.

[24] Horm TM, Schroeder JA. MUC1 and metastatic cancer: expression, function and therapeutic targeting. Cell Adh Migr 2013;7(2):187-98.

[25] Ross MH, Pawlina W. Histology : a text and atlas : with correlated cell and molecular biology. Seventh edition. ed. Baltimore, MD: Lippincott Williams and Wilkins; 2015. p 662-697.

[26] Hogg JC, Chu F, Utokaparch S, Woods R, Elliott WM, Buzatu L, Cherniack RM, Rogers RM, Sciurba FC, Coxson HO and others. The nature of small-airway obstruction in chronic obstructive pulmonary disease. N Engl J Med 2004;350(26):2645-53.

[27] van den Berge M, ten Hacken NH, Cohen J, Douma WR, Postma DS. Small airway disease in asthma and COPD: clinical implications. Chest 2011;139(2):412-23.

[28] Kamin W, Schwabe A, Kramer I. Inhalation solutions: which one are allowed to be mixed? Physico-chemical compatibility of drug solutions in nebulizers. J Cyst Fibros 2006;5(4):205-13.

[29] Subbiahdoss G, Kuijer R, Grijpma DW, van der Mei HC, Busscher HJ. Microbial biofilm growth vs. tissue integration: "the race for the surface" experimentally studied. Acta Biomater 2009;5(5):1399-404.

[30] Millea PJ. N-acetylcysteine: multiple clinical applications. Am Fam Physician 2009;80(3):265-9.

[31] Mori Y, Akita K, Tanida S, Ishida A, Toda M, Inoue M, Yashiro M, Sawada T, Hirakawa K,

Free Radic Res.

Nakada H. MUC1 protein induces urokinase-type plasminogen activator (uPA) by forming a complex with NF-kappaB p65 transcription factor and binding to the uPA promoter, leading to enhanced invasiveness of cancer cells. J Biol Chem 2014;289(51):35193-204.

[32] Erlanson DA, Chytil M, Verdine GL. The leucine zipper domain controls the orientation of AP-1 in the NFAT.AP-1.DNA complex. Chem Biol 1996;3(12):981-91.

[33] Abate C, Patel L, Rauscher FJ, 3rd, Curran T. Redox regulation of fos and jun DNA-binding activity in vitro. Science 1990;249(4973):1157-61.

[34] Kim HJ, Lee MH, Park HS, Park MH, Lee SW, Kim SY, Choi JY, Shin HI, Ryoo HM. Erk pathway and activator protein 1 play crucial roles in FGF2-stimulated premature cranial suture closure. Dev Dyn 2003;227(3):335-46.

[35] Yamada M, Tsukimura N, Ikeda T, Sugita Y, Att W, Kojima N, Kubo K, Ueno T, Sakurai K, Ogawa T. N-acetyl cysteine as an osteogenesis-enhancing molecule for bone regeneration. Biomaterials 2013;34(26):6147-56.

[36] Riise GC, Qvarfordt I, Larsson S, Eliasson V, Andersson BA. Inhibitory effect of N-acetylcysteine on adherence of Streptococcus pneumoniae and Haemophilus influenzae to human oropharyngeal epithelial cells in vitro. Respiration 2000;67(5):552-8.

[37] Ojulong J, Mwambu TP, Joloba M, Bwanga F, Kaddu-Mulindwa DH. Relative prevalence of methicilline resistant Staphylococcus aureus and its susceptibility pattern in Mulago Hospital, Kampala, Uganda. Tanzan J Health Res 2009;11(3):149-53.

[38] Kaplan SL, Mason EO, Jr. Management of infections due to antibiotic-resistant Streptococcus pneumoniae. Clin Microbiol Rev 1998;11(4):628-44.

[39] Malfertheiner P, Chan FK, McColl KE. Peptic ulcer disease. Lancet 2009;374(9699):1449-61.

[40] Marks LR, Parameswaran GI, Hakansson AP. Pneumococcal interactions with epithelial cells are crucial for optimal biofilm formation and colonization in vitro and in vivo. Infect Immun 2012;80(8):2744-60.

[41] Mata M, Morcillo E, Gimeno C, Cortijo J. N-acetyl-L-cysteine (NAC) inhibit mucin synthesis and pro-inflammatory mediators in alveolar type II epithelial cells infected with influenza virus A and B and with respiratory syncytial virus (RSV). Biochem Pharmacol 2011;82(5):548-55.

Figure legends

Table 1. Primers used for RT-PCR analyses.

Figure 1. Calcein-based relative cell numbers (A) and levels of cellular ROS (A) , GSH (B), and GSSG (C) per unit cell of human bronchiolar epithelial cells with or without NAC pretreatment after co-incubation with *S. pneumoniae* for 8 h. Data are shown as mean ± SD (n = 4). **P* < 0.05 (Bonferroni correction).

Figure 2. Expression of *IL6*, *IL8*, *PTGS2*, *MUC5AC*, *MUC1* and *MUC4* genes analyzed by RT-PCR and results of the semi-quantification of band intensities (A) and PGE2 (B) per unit cell, and cellular proinflammatory cytokines (IL-1β, IL6 and IL8) (C) in human bronchiolar epithelial cells with or without NAC pretreatment after co-incubation with *S. pneumoniae* for 8 h. Data are shown as mean ± SD (n = 4). **P* < 0.05 (Bonferroni correction).

Figure 3. (A) Representative images of immunochemical fluorescent staining for MUC5AC (left) and MUC4 (right) (green) with DAPI counterstaining (Blue) in human bronchiolar epithelial cells with or without NAC pretreatment after co-incubation with *S. pneumoniae* for 8 h. Histogram of MUC5AC- (B) and MUC4- (C) detected area per unit nucleus in images presented in panel A are shown. Data represent mean ± SD (n = 5). **P* < 0.05 (Bonferroni correction).

Figure 4. (A) Representative fluorescent microscopic images of calcein-stained human bronchiolar epithelial cells (green) with or without NAC pretreatment after co-incubation with *S. pneumoniae* labeled with CTC (red) for 8 h. Appearance of attached viable cell colonies, CTC accumulation in the same cells and merged images (bar = 20 μm) are shown. (B) Quantification of CTC-positive bacteria on and/or within human bronchiolar epithelial cells with or without NAC pretreatment after

co-incubation with *S. pneumoniae* labeled with CTC (red) for 8 h. Data represent mean ± SD (n = 4). ** $P < 0.05$, significant difference (Student's t-test).

Figure 5. Proposed mechanism underlying enhancement anti-infective defense of human bronchiolar epithelial cells by uptake of NAC (left) in contradistinction to phenomenon in the cells without NAC (right). Cells taking NAC increases MUC4 synthesis as anti-infective defense, instead of MUC5AC hepersecreation (See "Discussion" section in main text for detailed description).

Table.1

	Sequence (5'-3')	Annealing Temperature	Numbers of Cycles	Size of Product (bp)
IL8	GAATGGGTTTGCTAGAATGTGATA	60.7	30	129
	CAGACTAGGGTTGCCAGATTTAAC			
IL6	CCCCCAGGAGAAGATTCCA	64.2	30	104
	GGCGCTTGTGGAGAAGGAG			
PTGS2	TCCAGTACCAAAATCGTATTGCT	61.2	30	370
	AGTGCTTCCAACTCTGCAGACAT			
MUC5AC	TCCGGCCTCATCTTCTCC	60.2	36	683
	ACTTGGGCACTGGTGCTG			
MUC1	GCACCCAGTCTCCTTTCTTC	60.7	30	430
	GGAAATGGCACATCACTCAC			
MUC4	CAGCCTCTGCCAGCACCTCACCTG	74.9	30	236
	GGAGGGGTTTGATGAAAACCTTGTCGTCTCTCC			
GAPDH	TGAAGGTCGGTGTCAACGGATTTGGC	67	27	983
	CATGTAGGCCATGAGGTCCACCAC			

Fig.1

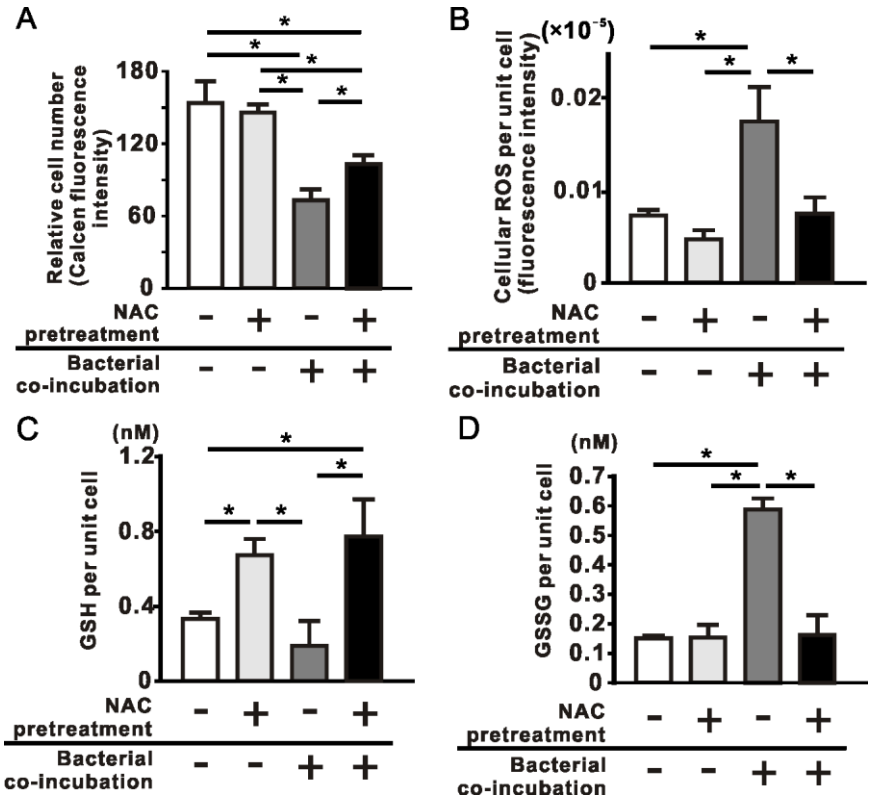

A
Relative cell number (Calcen fluorescence intensity)
180
120
60
0
NAC pretreatment
Bacterial co-incubation
B
Cellular ROS per unit cell (fluorescence intensity)
(×10⁻⁵)
0.02
0.01
0
C
GSH per unit cell
(nM)
1.2
0.8
0.4
0
D
GSSG per unit cell
(nM)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0

Fig.2

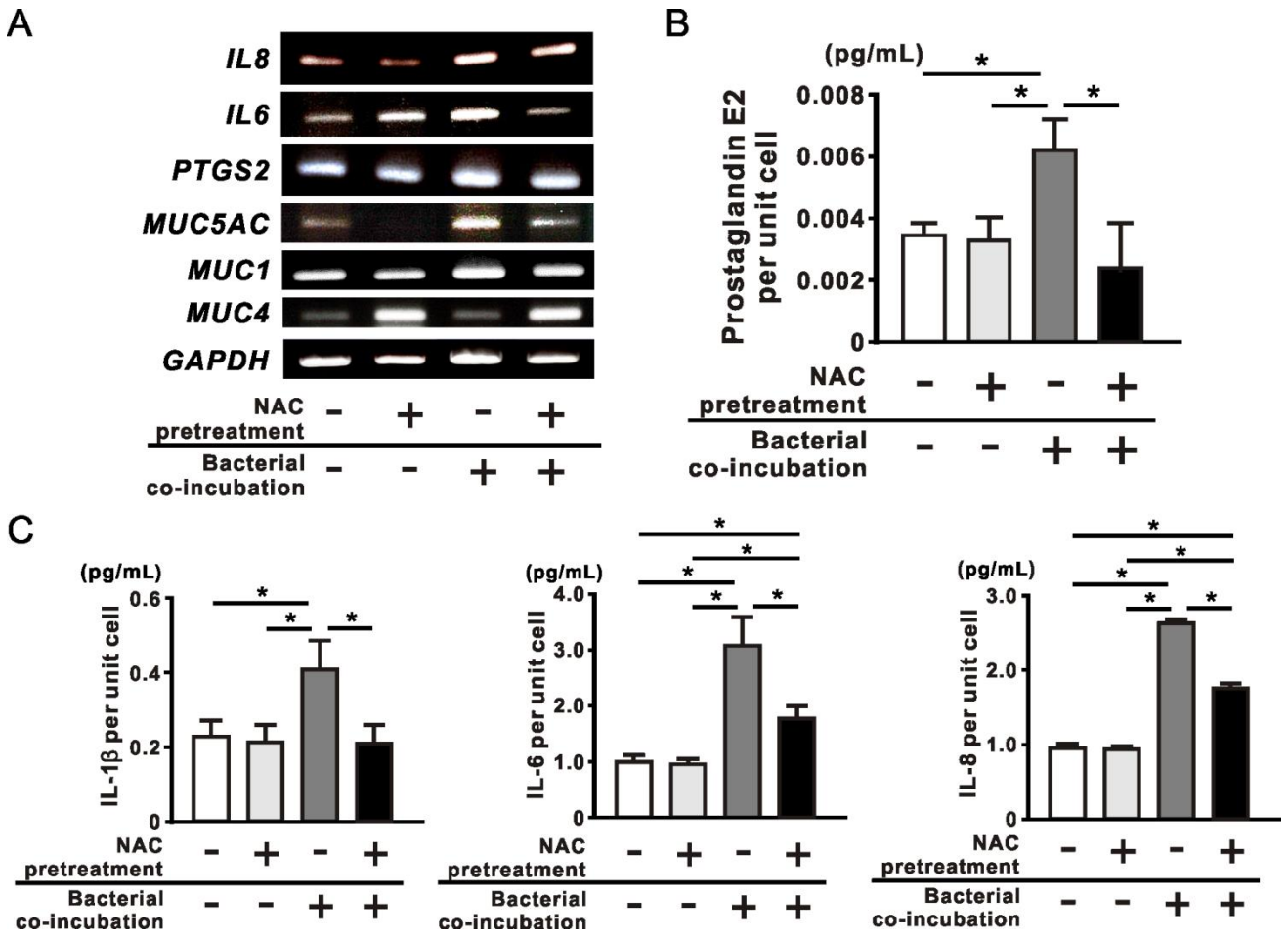

A
IL8
IL6
PTGS2
MUC5AC
MUC1
MUC4
GAPDH
NAC pretreatment - + - +
Bacterial co-incubation - - + +
B
(pg/mL)
Prostaglandin E2 per unit cell
0.008
0.006
0.004
0.002
0
NAC pretreatment - + - +
Bacterial co-incubation - - + +
C
(pg/mL)
IL-1β per unit cell
0.6
0.4
0.2
0
IL-6 per unit cell
4.0
3.0
2.0
1.0
0
IL-8 per unit cell
3.0
2.0
1.0
0
NAC pretreatment - + - +
Bacterial co-incubation - - + +

Fig.3

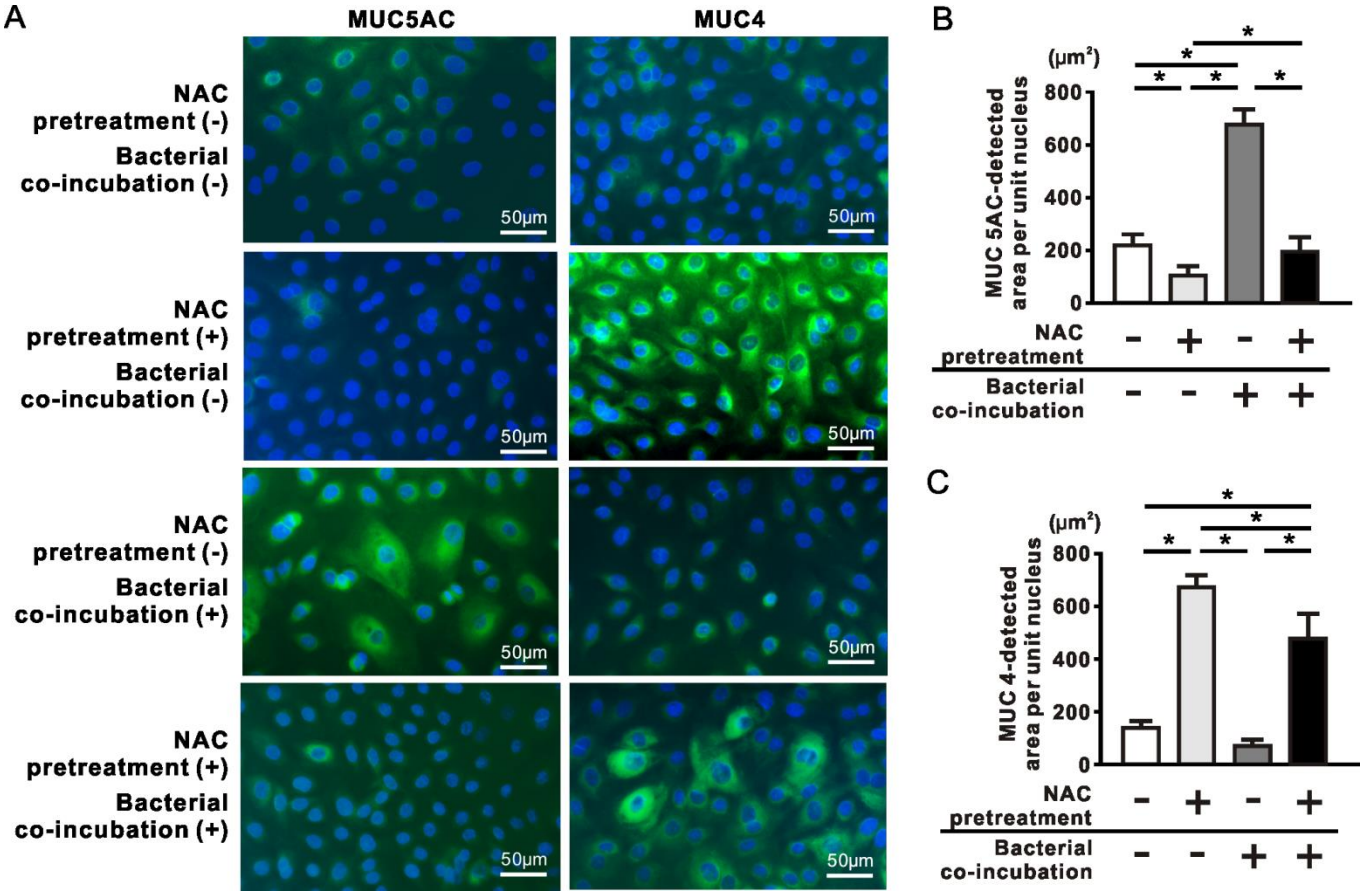

A
MUC5AC
MUC4
NAC pretreatment (-)
Bacterial co-incubation (-)
NAC pretreatment (+)
Bacterial co-incubation (-)
NAC pretreatment (-)
Bacterial co-incubation (+)
NAC pretreatment (+)
Bacterial co-incubation (+)
50μm
B
(μm²)
MUC 5AC-detected area per unit nucleus
800
600
400
200
0
NAC pretreatment
Bacterial co-incubation
C
(μm²)
MUC 4-detected area per unit nucleus
800
600
400
200
0
NAC pretreatment
Bacterial co-incubation

Fig.4

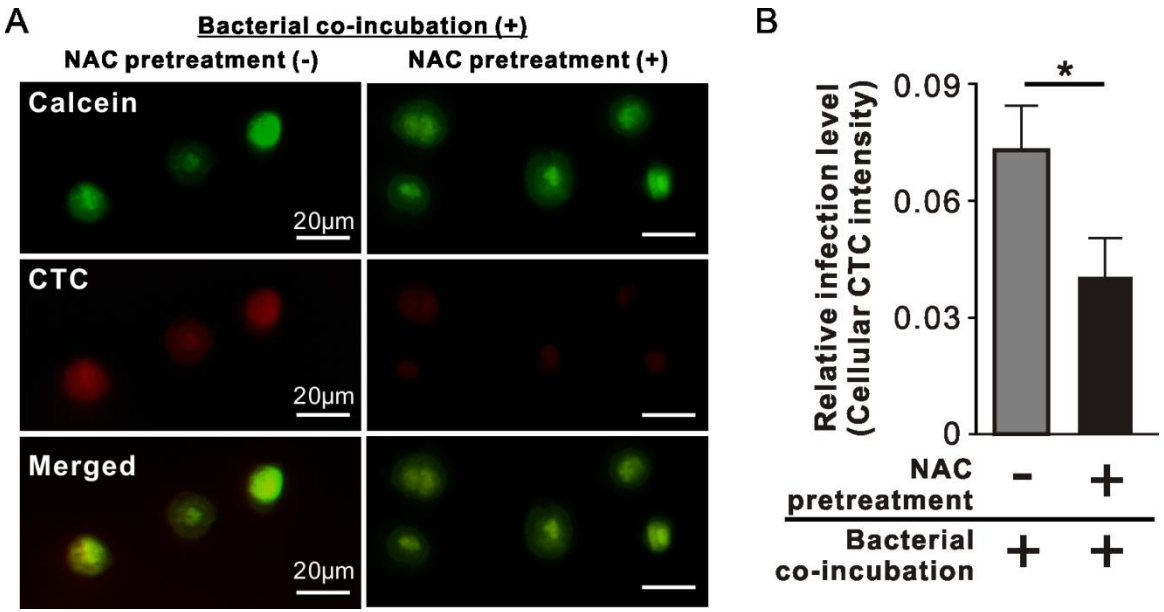
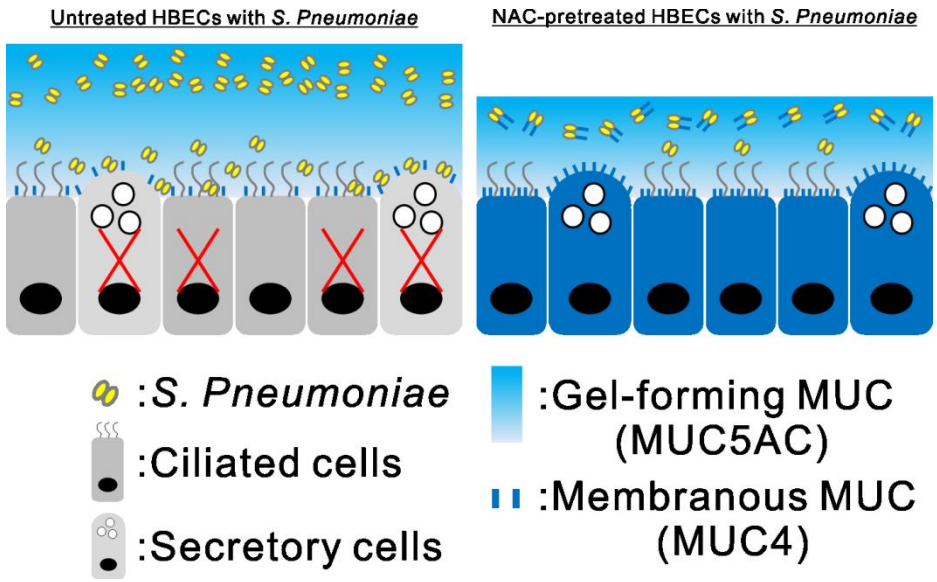

A
Bacterial co-incubation (+)
NAC pretreatment (-)
NAC pretreatment (+)
Calcein
CTC
Merged
20μm
20μm
20μm
B
Relative infection level
(Cellular CTC intensity)
0.09
0.06
0.03
0
*
NAC pretreatment
-
+
Bacterial co-incubation
+
+

Fig.5


Untreated HBECs with S. Pneumoniae
NAC-pretreated HBECs with S. Pneumoniae
:S. Pneumoniae
:Ciliated cells
:Secretory cells
:Gel-forming MUC (MUC5AC)
:Membranous MUC (MUC4)