**Pseudomonas aeruginosa** pyocyanin modulates mucin glycosylation with sialyl-Lewis\(^x\) to increase binding to airway epithelial cells

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Cystic fibrosis (CF) patients battle life-long pulmonary infections with the respiratory pathogen *Pseudomonas aeruginosa* (PA). An overabundance of mucus in CF airways provides a favorable niche for PA growth. When compared with that of non-CF individuals, mucus of CF airways is enriched in sialyl-Lewis\(^x\), a preferred binding receptor for PA. Notably, the levels of sialyl-Lewis\(^x\) directly correlate with infection severity in CF patients. However, the mechanism by which PA causes increased sialylation remains uncharacterized. In this study, we examined the ability of PA virulence factors to modulate sialyl-Lewis\(^x\) modification in airway mucins. We found pyocyanin (PCN) to be a potent inducer of sialyl-Lewis\(^x\) in both mouse airways and in primary and immortalized CF and non-CF human airway epithelial cells. PCN increased the expression of C2/4GnT and ST3Gal-IV, two of the glycosyltransferases responsible for the stepwise biosynthesis of sialyl-Lewis\(^x\), through a tumor necrosis factor (TNF)-\(\alpha\)-mediated phosphoinositol-specific phospholipase C (PI-PLC)-dependent pathway. Furthermore, PA bound more efficiently to airway epithelial cells pre-exposed to PCN in a flagellar cap-dependent manner. Importantly, antibodies against sialyl-Lewis\(^x\) and anti-TNF-\(\alpha\) attenuated PA binding. These results indicate that PA secretes PCN to induce a favorable environment for chronic colonization of CF lungs by increasing the glycosylation of airway mucins with sialyl-Lewis\(^x\).

**INTRODUCTION**

Pulmonary infections with *Pseudomonas aeruginosa* (PA) are a critical clinical concern for patients with cystic fibrosis (CF),\(^1,2\) with 95% of individuals colonized with the pathogen by the age of 3.\(^3\) Pulmonary failure, a sequela of acute exacerbations and tissue scarring in chronic infections, results in high morbidity and mortality in CF patients.\(^1,2\) Previously understood factors contributing to PA colonization in the CF airways include overproduction of hyperviscous mucus and impeded mucociliary clearance of trapped microbes.\(^1\) Mucin glycoproteins are major components of airway mucus that contain on their structure a diverse population of carbohydrate chains that have been shown to be receptors for bacteria. Their intraluminal location in the airway serves as a first line of interaction with microbes in the lung.\(^4-8\) Mucins recovered from CF airways are enriched with the tetracarbohydrate moiety sialyl-Lewis\(^x\).\(^9-11\) Through its flagellar cap, PA binds sialyl-Lewis\(^x\)-glycosylated CF mucins with a higher affinity than other carbohydrate moieties over control lung tissues.\(^4,7,12,13\) The enzymes core 2/core 4 beta-1,6-N-acetylglucosaminyltransferase (C2/4GnT) and \(\alpha\)2,3-sialyltransferase IV (ST3Gal-IV), which are crucial for sialyl-Lewis\(^x\) synthesis, are upregulated during pulmonary inflammation, especially in CF.\(^6,8,14-16\) Specifically, exposure to tumor necrosis factor (TNF)-\(\alpha\), interleukin (IL)-6, and IL-8 increases the level of sialyl-Lewis\(^x\) on mucins.\(^13-17\) Although controversy remains, increasing evidence suggests that the CF epithelium is pro-inflammatory primed, and chronic bacterial infection causes a prolonged inflammatory response when...
compared with other diseased airways. The further finding of a direct correlation between severity of CF infection and the levels of sialyl-Lewis<sup>x</sup> glycosylation on airway mucins underlines the importance of bacterial etiology as an inciting factor in the modification of these mucins. Together, these findings warrant further investigation on the effects of PA virulence in relation to changes in sialyl-Lewis<sup>x</sup> levels.

RESULTS

Pyocyanin is a potent inducer of sialomucins

We evaluated the ability of various purified PA components to induce changes in mucin glycosylation during chronic exposure in mouse lungs. Recovered lung sections were stained with Periodic acid-Schiff to determine the presence of goblet cell hyperplasia and metaplasia (GCHM) and mucin hypersecretion, and by the high-iron diamine-alcian blue to detect sialomucins (blue) and sulfomucins (brown). Although all PA components were able to induce higher expression of sialomucins when compared with the phosphate-buffered saline (PBS), pyocyanin (PCN) caused the most dramatic increase (Figure 1). Interestingly, no sulfomucins were detected in mouse airways, despite their prominent presence in colon sections from the same animals (Figure 1).

PCN induces sialyl-Lewis<sup>x</sup> epitopes in the mouse airway epithelium

PCN is a redox-active tricyclic toxin that has been recovered in varying concentrations from trace quantities to 100 μM (27 μg ml<sup>-1</sup> sputum) in pulmonary secretions of CF and non-CF bronchiectatic patients infected by PA, and its concentrations are inversely correlated with the lung function of CF patients. We and others have shown that PCN is a potent inducer of GCHM and mucin hypersecretion by inactivating FOXA2, a key transcription repressor of GCHM and mucus biosynthesis. Because PCN also induces mucin sialylation, the remainder of this study focused on PCN-mediated mucin sialylation. We examined the effect of chronic PCN exposure on the levels of sialyl-Lewis<sup>x</sup> epitopes on mucins secreted by mouse bronchial mucosa. Periodic acid-Schiff staining indicated that PCN induced GCHM and mucin hypersecretion in mouse airways (Figure 2a). Immunohistochemical analyses demonstrated that chronic PCN administration significantly increased the expression of mucins harboring sialyl-Lewis<sup>x</sup> epitopes in both large and small airways by 10- and 35-fold, respectively, when compared with control lungs (Figure 2a,b). To examine whether secreted mucins were sialylated, we performed bronchoalveolar lavage, and examined mucin sialylation in recovered BAL fluid. At 1-week and 3-week post exposure, PCN increased the amounts of secreted sialomucins by 3.3- and 5.1-fold, respectively, in comparison with control mice exposed to PBS (Figure 2c,d).

These results indicate that PCN induces an increase in the amount of sialyl-Lewis<sup>x</sup> present on cell-associated and secreted mucins in bronchial mucosa in vivo.

PCN induces sialyl-Lewis<sup>x</sup> in time- and concentration-dependent manners

To examine the mechanism of PCN-induced mucin sialylation, we tested the amenability of NCI-H292 cells on the expression of sialyl-Lewis<sup>x</sup> in vitro during exposure to the toxin. After 24 h, PCN at 2.5, 5.0, and 10 μg ml<sup>-1</sup> concentrations significantly increased the expression of sialyl-Lewis<sup>x</sup> by 2.7-, 5.6-, and 6.4-fold, respectively (Figure 3a and Supplementary Figure S1a online). Furthermore, PCN (5.0 μg ml<sup>-1</sup>) induced an increase in sialyl-Lewis<sup>x</sup> at 24 h post exposure (Figure 3b and Supplementary Figure S1b), a time point where significant amounts of synthesized mucins are secreted. To further confirm these findings, an immunofluorescence assay (IFA) was used to examine the induction of sialyl-Lewis<sup>x</sup> by PCN. In the PCN (5.0 μg ml<sup>-1</sup>, 24 h)-treated NCI-H292 cells, 3.3-fold...
Higher sialyl-Lewis\textsuperscript{x} epitopes were seen, along with a change in cellular localization from predominantly perinuclear to diffused cytoplasmic (Figure 3c,d). Collectively, these results indicate that PCN induces the expression of sialyl-Lewis\textsuperscript{x} in NCI-H292 cells in concentration- and time-dependent manners.

PCN induces the sialyl-Lewis\textsuperscript{x} glycosylation on MUC5AC mucin in primary human bronchial epithelial cells

Normal primary human bronchial epithelial (NHBE) cells cultured in the air–liquid interface (ALI) have emerged as a powerful tool for the study of airway biology. We have shown that PCN induces the secretion of major airway mucins, MUC5B and MUC5AC, in NHBE cells\textsuperscript{23,24} Thus, we determined whether a clinically relevant concentration of PCN (12.5 \text{\mu g ml\textsuperscript{-1}}) could induce the modification of MUC5AC with sialyl-Lewis\textsuperscript{x} in ALI culture of NHBE cells. The amount of MUC5AC increased by 65% within 24 h (Figure 4a,b).

Significantly, PCN induced a 380% increase in the sialylation of MUC5AC when compared with PBS (Figure 4b).

To determine whether PCN-induced specific sialylation on MUC5AC, we immunoprecipitated MUC5AC from NCI-H292 cells previously exposed to PCN and examined the sialylation of the mucin with sialyl-Lewis\textsuperscript{x}-specific antibodies. Sialylated MUC5AC was detected (Figure 4c) on the western blot. Because MUC5AC is only one of the multiple mucins (e.g., MUC5B, MUC2, etc) secreted by airway cells, not surprisingly, the amount of sialylated MUC5AC was less when compared with total amounts of sialylated mucins in total cell extracts. Collectively, these results indicate that PCN is a potent inducer of mucin sialylation in airway epithelial cells.

PCN induces upregulation of C2/4GnT and ST3Gal-IV

Sialyl-Lewis\textsuperscript{x} is synthesized through stepwise processes involving many enzymes.\textsuperscript{8} Previous work has shown that levels of sialyl-Lewis\textsuperscript{x} biosynthesis glycosyltransferases C2/4GnT and
ST3Gal-IV are upregulated in response to inflammation.\textsuperscript{6,8,14,15} We examined whether PCN could directly upregulate these enzymes in the NCI-H292 cells in a time-dependent manner. PCN significantly increased the amounts of both C2/4GnT and ST3Gal-IV by 2.4- and 3.0-fold, respectively, at 24 h post exposure (Figure 5a,b). These results indicate that PCN is capable of upregulating the expression of enzymes crucial for the biosynthesis of sialyl-Lewisx.

PCN induces an increase in sialyl-Lewisx through the PI-PLC pathway

Previously, it has been shown that TNF-\textgreek{a} could increase the expression of C2/4GnT and ST3Gal-IV through the induction of phosphoinositol-specific phospholipase C (PI-PLC) signaling pathway.\textsuperscript{5,8,15,16} In addition, IL-6 and IL-8 may also have a role.\textsuperscript{17} In contrast, epidermal growth factor receptor (EGFR) signaling negatively regulates C2/4GnT and ST3Gal-IV.\textsuperscript{15,26} Importantly, PCN induces production and release of TNF-\textgreek{a}, IL-6, and IL-8 from bronchial airway epithelial cells.\textsuperscript{27} Furthermore, we and others have shown that PCN induces the expression of dominant airway mucins MUC5B and MUC5AC through activation of EGFR.\textsuperscript{23,25} We used two complementary approaches to examine the upregulation of PI-PLC dependency C2/4GnT and ST3Gal-IV induced by PCN. First, we showed that PCN could upregulate the expression of PLC-\textgreek{g}2, a component of the PI-PLC signaling pathway (Figure 6a). As expected, TNF-\textgreek{a} induced the expression of sialyl-Lewisx (Supplementary Figure S2). Next, we examined whether U-73122, a PI-PLC pathway inhibitor, could disrupt the induction of sialyl-Lewisx biosynthesis by PCN. In the absence of U-73122, PCN significantly increased the levels of sialyl-Lewisx, C2/4GnT, and ST3Gal-IV (Figure 6b–d). In contrast, addition of U-73122 caused a dose-dependent decrease in PCN-mediated upregulation in sialyl-Lewisx, C2/4GnT, and ST3Gal-IV (Figure 6b–d). Collectively, these results demonstrate that upregulation of sialyl-Lewisx biosynthesis by PCN is dependent upon the PI-PLC signaling pathway.

PCN induces the expression of sialyl-Lewisx in the immortalized CF airway epithelial IB3-1 cells

Mucins recovered from CF airways are enriched with the tetracarbohydrate moiety sialyl-Lewisx.\textsuperscript{9–11} We examined whether PCN could induce sialyl-Lewisx glycosylation on

Figure 3  Pyocyanin (PCN) upregulates the expression of sialyl-Lewisx in time- and concentration-dependent manners. (a, b) NCI-H292 cells were exposed to phosphate-buffered saline (PBS) (control) or indicated concentrations of PCN or at predetermined time intervals (with 5 \textmu g ml\textsuperscript{-1} PCN). Total proteins were separated on an agarose-acrylamide gel. The expression of sialyl-Lewisx was analyzed using specific antibody using western blots. \textbeta-actin was used as loading controls. Densitometry analysis of the sialyl-Lewisx expression can be found in Supplementary Figure S1. (c) Separate sets of cells from above were stained for sialyl-Lewisx with antibodies and visualized with Alexa Fluor488-conjugated secondary antibody (green color). (d) Quantification of total fluorescence of control and PCN-treated cells. The mean \pm s.e. of total fluorescence in 10 representative high-power fields from each treatment group in one typical experiment is shown. * \texttextsuperscript{P}<0.05 when compared against PBS control using the parametric Student’s \textit{t}-test.

ST3Gal-IV are upregulated in response to inflammation.\textsuperscript{6,8,14,15} We examined whether PCN could directly upregulate these enzymes in the NCI-H292 cells in a time-dependent manner. PCN significantly increased the amounts of both C2/4GnT and ST3Gal-IV by 2.4- and 3.0-fold, respectively, at 24 h post exposure (Figure 5a,b). These results indicate that PCN is capable of upregulating the expression of enzymes crucial for the biosynthesis of sialyl-Lewisx.

PCN induces an increase in sialyl-Lewisx through the PI-PLC pathway

Previously, it has been shown that TNF-\textgreek{a} could increase the expression of C2/4GnT and ST3Gal-IV through the induction of phosphoinositol-specific phospholipase C (PI-PLC) signaling pathway.\textsuperscript{5,8,15,16} In addition, IL-6 and IL-8 may also have a role.\textsuperscript{17} In contrast, epidermal growth factor receptor (EGFR) signaling negatively regulates C2/4GnT and ST3Gal-IV.\textsuperscript{15,26} Importantly, PCN induces production and release of TNF-\textgreek{a}, IL-6, and IL-8 from bronchial airway epithelial cells.\textsuperscript{27} Furthermore, we and others have shown that PCN induces the expression of dominant airway mucins MUC5B and MUC5AC through activation of EGFR.\textsuperscript{23,25} We used two complementary approaches to examine the upregulation of PI-PLC dependency C2/4GnT and ST3Gal-IV induced by PCN. First, we showed that PCN could upregulate the expression of PLC-\textgreek{g}2, a component of the PI-PLC signaling pathway (Figure 6a). As expected, TNF-\textgreek{a} induced the expression of sialyl-Lewisx (Supplementary Figure S2). Next, we examined whether U-73122, a PI-PLC pathway inhibitor, could disrupt the induction of sialyl-Lewisx biosynthesis by PCN. In the absence of U-73122, PCN significantly increased the levels of sialyl-Lewisx, C2/4GnT, and ST3Gal-IV (Figure 6b–d). In contrast, addition of U-73122 caused a dose-dependent decrease in PCN-mediated upregulation in sialyl-Lewisx, C2/4GnT, and ST3Gal-IV (Figure 6b–d). Collectively, these results demonstrate that upregulation of sialyl-Lewisx biosynthesis by PCN is dependent upon the PI-PLC signaling pathway.
mucins produced by the CF IB3-1 cells. In contrast to NCI-H292 cells, we found that PCN at the concentration of 10 μg ml⁻¹ and at the time point of 48 h was most optimal in inducing the glycosylation of mucins with sialyl-Lewisx. IFA analyses indicated that at 5.0 and 10.0 μg ml⁻¹ PCN concentrations, the expression of sialyl-Lewisx was increased by 4.8- and 13.4-fold, respectively, when compared against the PBS controls (Figure 7). The IFA results were corroborated using western blot analysis, which showed an increased expression of sialyl-Lewisx (Figure 8a), as well as the glycosyltransferases C2/4GnT (Figure 8b) and ST3Gal-IV (Figure 8c). Collectively, these results confirmed the ability of PCN to induce sialyl-Lewisx in the cystic fibrosis transmembrane conductance regulator-deficient IB3-1 cells.

PCN increases binding affinity of P. aeruginosa to immortalized CF and non-CF airway epithelial cells
Because our results demonstrated that PCN increases mucin glycosylation with sialyl-Lewisx, we examined whether PA would be better able to bind to NCI-H292 and IB3-1 cells previously treated with PCN. The binding of PA strain PAO1 to PCN-treated NCI-H292 and IB3-1 cells was 3- and 2.3-fold higher than the control cells (Figure 9a,b). In addition, the binding was partially dependent on the expression of flagellar cap protein FliD. PCN-mediated increase in binding affinity to NCI-H292 and IB3-1 cells was abolished in the flagellar cap-deficient ΔfliD mutant (Figure 9a,b). In addition, the basal levels of PAO1 and ΔfliD cells bound to IB3-1 cells were higher than NCI-H292 cells in the absence of PCN induction.

Next, we examined the binding of PA CF clinical isolates to NCI-H292 and IB3-1 cells. CF1 is non-mucoid, motile, and produces PCN. CF2 is non-mucoid, non-motile, and PCN-deficient. CF26 is mucoid, non-motile, and produces low levels of PCN. CF32 is mucoid, non-motile, and PCN-deficient (Supplementary Figure S3a,b). Binding experiments showed that only CF1 was able to bind to airway cells, consistent with the requirement of flagellum for attachment to sialylated mucins. In addition, binding to NCI-H292 and IB3-1 cells pretreated with PCN increased by 1.95- and 1.75-fold, respectively (Figure 9c,d). Collectively, these results indicate that the elevated mucin sialylation induced by PCN facilitates the binding of PA in a more efficient manner.

Blocking of sialyl-Lewisx and TNF-α with antibodies attenuates the binding of P. aeruginosa to airway epithelial cells pre-exposed to PCN
Blocking the binding of PA-sialylated mucins may serve as an adjunctive therapy against chronic colonization and infection within CF airways. We examined whether treatment of NCI-H292 and IB3-1 cells with antibodies against sialyl-Lewisx and anti-TNF-α could reduce the binding of PA to NCI-H292 and IB3-1 cells. Blocking with anti-sialyl-Lewisx antibodies decreased the binding of PAO1 and CF1 to NCI-H292 cells by 2.88- and 3.01-fold, respectively (Figure 10a), and by 3.26- and 4.2-fold, respectively, to IB3-1 cells (Figure 10b), when compared against cells pretreated with an irrelevant human IgG. Blocking with anti-sialyl-TNF-α antibodies decreased the binding of PAO1 and CF1 to NCI-H292 cells by 1.78- and 1.75-fold, respectively (Figure 10c); by 1.70- and 1.68-fold, respectively, to IB3-1 cells (Figure 10d). Collectively, these results suggest that blocking of sialylated mucins as well as...
signaling pathway-regulating mucin sialylation may inhibit PA binding and reduce infection in CF airways.

**DISCUSSION**

PCN is a redox-active phenazine toxin found to be excreted in levels up to 100 μM in PA-infected bronchiectatic airways.\(^{20,21}\) PCN is important for chronic lung infection.\(^{22}\) Chronic instillation of PCN causes GCHM, fibrosis, and airspace destruction, pathological features mirroring those found in CF lungs chronically infected with PA.\(^{22}\) Levels of sialyl-Lewis\(^x\), which acts as a binding receptor for PA,\(^{7,13}\) are also upregulated in CF mucins.\(^{5,9–12,28,29}\) Historically, this has been attributed to increased inflammation in the lung.\(^{5}\) Evidence suggesting the importance of bacterial infection in modulating levels of sialyl-Lewis\(^x\) includes observations of correlation between severity of infection and increased sialyl-Lewis\(^x\) expression,\(^{11,28}\) as well as studies demonstrating an initial bacterial component required for inflammation in CF lungs.\(^{18,19}\) We postulated that virulence factors of PA may be the initial stimuli leading to increased sialyl-Lewis\(^x\). In this study, we show that chronic PCN administration increases the expression of sialomucins in mouse airways through a TNF-α-PI-PLC pathway. Little or no sulfomucins were detected in mouse airways, despite strong expression in colon tissues from the same animals. This is not surprising because mucin sulfation in CF has been directly linked to the loss of cystic fibrosis transmembrane conductance regulator function,\(^{30}\) which is absent in our mouse model and may be independent from the bacteria-mediated inflammation. Our study suggests a causal link between chronic PCN administration in mouse airways and increased levels of sialyl-Lewis\(^x\). Furthermore, increased expression of sialyl-Lewis\(^x\) was also seen in ALI culture of NHBE cells, and in immortalized mucoepidermoid NCI-H292 cells. Moreover, PCN also increased the expression of sialyl-Lewis\(^x\) in the CF cell line IB3-1, which was accompanied by elevated binding of PA. Importantly, antibody-based blocking of TNF-α and sialylated mucins reduce the binding of PA to airway cells exposed to PCN. Collectively, these results demonstrate the ability of PCN to augment sialyl-Lewis\(^x\) expression in the CF airway epithelial cells.

The expression of sialyl-Lewis\(^x\) increases significantly in response to stimulation by TNF-α, IL-6, and IL-8 in both immortalized airway cells and bronchiol explants.\(^{6,8,15,16}\) In agreement, PCN induces the production of TNF-α, IL-6, and IL-8 from airway epithelial cells.\(^{27}\) Taken together, this suggests that PCN may induce the secretion of TNF-α to increase sialyl-Lewis\(^x\) in the airways. Previous studies involving the effects of inflammatory cytokines on glycosyltransferases responsible for the synthesis of sialyl-Lewis\(^x\) demonstrate, in addition to C2/4GnT and ST3Gal-IV, an upregulation of the α1-3 fucosyltransferases FucT-III/IV/VII.\(^{8,15,16}\) However, increased expression of FucT-III/IV/VII was not observed in response to PCN administration (data not shown). We hypothesize that this may be due to higher sensitivity of FucT-III/IV/VII than C2/4GnT and ST3Gal-IV to repression by the PCN-activated EGFR signaling,\(^{23,25}\) which is inhibitory to these glycosyltransferases.\(^{15,26}\) It is likely that the amount of sialyl-Lewis\(^x\) glycosylation is the net result of PCN-mediated induction of the positively acting TNF-α-PI-PLC signaling vs. the negatively acting EGFR signaling. Because anti-TNF-α antibodies were less efficient than anti-sialyl-Lewis\(^x\) antibodies in reducing the binding of PA (Figure 10), it is likely that additional signaling pathways also may be involved in

**Figure 5** Pyocyanin (PCN) induces the expression of sialyl-Lewis\(^x\) glycosyltransferases. NCI-H292 cells were exposed to 5 μg ml\(^{-1}\) PCN for the indicated time intervals. (a, b) The expression of C2/4GnT and ST3Gal-IV was analyzed with western blots. β-actin was used as loading controls. Representative western blots from one of the three independent experiments are shown. Densitometry analyses of C2/4GnT and ST3Gal-IV represent the mean ± s.e. from three independent western blot experiments. Statistical significance comparisons among various time points were determined by using the one-way ANOVA analysis (P<0.05). *P<0.05 when compared against time 0 h by using the Tukey’s test.
upregulating mucin sialylation. As we have mentioned earlier, FOXA2 is a key transcriptional repressor of mucin biosynthesis that maintains the airway mucus at healthy baseline levels,23,24,31 by inhibiting the function of SPDEF, an IL-4/IL-13-STAT6-dependent transcriptional activator of mucin biosynthesis and glycosyltransferases involved in mucin modification. Thus far, the sole evidence of SPDEF-activating sialyl/sulfo/fucosyltransferases was derived from transcriptome analysis.32 Previously, we have shown that PCN induces GCHM and mucin biosynthesis by inducing the IL-4/IL-13-STAT6 signaling. Thus, blocking both TNF-α-PI-PLC and IL-4/IL-13-STAT6 signaling may yield more robust inhibition of PA binding. Further studies will elucidate the aforementioned issues. In addition, it will be of interest to determine whether the levels of PCN within CF sputa positively correlate with the levels of sialomucins.

Sialyl-Lewis^x^ has been previously shown to be a preferential binding receptor for PA. Because PCN upregulates the amount of sialyl-Lewis^x^, this suggests that PA is able to modulate a favorable host environment to facilitate chronic lung infection by secreting PCN. Our observation may have clinical implications. For example, after aggressive antibiotic therapy that eradicates most PA cells, the airway mucins rich in sialyl-Lewis^x^ would be well suited for binding by the residual PA or new bacteria that entered the airways. It is also possible that the production of sialomucins may be continued after the removal

**Figure 6** Pyocyanin (PCN) upregulates sialyl-Lewis^x^ through the phosphoinositol-specific phospholipase C (PI-PLC) pathway. (a) PCN induced the expression of PI-PLC signaling pathway effector PLC-γ2 in NCI-H292 cells exposed to increasing concentrations of PCN. (b–d) NCI-H292 cells were pre-exposed to PI-PLC inhibitor U73122 for 40 min before the addition of phosphate-buffered saline (PBS) or PCN (5 μg ml^−1^) for 24 h. Total cell lysates were used in western blot analysis using antibodies against (a) PLC-γ2, (b) sialyl-Lewis^x^, (c) C2/4GnT, or (d) ST3Gal-IV. For sialyl-Lewis^x^ western blot (b), proteins were separated on an agarose-acrylamide gel. β-actin was used for loading controls. Experiments were repeated three times. Representative western blots are shown. Densitometry analyses represent the mean ± s.e. of western blots from three independent experiments. *P < 0.05 against PBS control, or in the presence or absence of U73122 by using the one-way analysis of variance (ANOVA) analysis. #P < 0.05 against control using the Tukey’s test.
of the majority of PA. In support of this idea, Muhlebach et al. described increased and prolonged inflammatory responses in PA and other bacteria-infected CF lungs compared with non-CF lungs, suggesting that an inflammatory response in the CF epithelium is not only pronounced, but delayed in cessation. Thus, adjunctive treatment involving blocking the binding of remainder PA to sialylated mucins may help to decrease recurring infection in CF.

In summary, PCN modulates airway mucin glycosylation by upregulating the levels of sialyl-LewisX. The importance of this carbohydrate moiety as a binding receptor for PA underscores the importance of increasing our knowledge of the effects of PCN on airway mucins during PA-mediated chronic pulmonary infection in CF.

METHODS

Chemicals and purified bacterial components. Chemically synthesized PCN (#R9532) and PA LPS (#L7018) were purchased from Sigma (St. Louis, MO). Alginate was purified from the mucoid PA strain FDR1 (ref. 32) and quantified as previously described.34 Flagella were purified from PA strain PAO1 as previously described.35 U73122 was purchased from Sigma-Aldrich (St. Louis, MO) (#U6756). TNF-α was purchased from R&D Systems (Minneapolis, MN) (#210-TA-010).

P. aeruginosa cultures. PA strains PAO1 and its isogenic ΔfliD mutant were generous gifts from Professor Reuben Ramphal (University of Florida). CF clinical PA isolates CF1, CF2, CF26, and CF32 were collected while Dr Lau was on staff at the University of Cincinnati College of Medicine under the Institutional Review Board #04-7-16-2. All PA strains were cultured in plain Luria-Bertani broth (Fisher Scientific, Grand Island, NY) with the exception of ΔfliD, which was grown in Luria-Bertani containing 75 μg ml⁻¹ gentamicin (Life Technologies, Grand Island, NY) at 37 °C overnight. They were then stored at −80 °C in 25% glycerol (Sigma-Aldrich). Before each experiment, bacteria were cultured from frozen stocks in 5 ml Luria-Bertani broth to stationary phase to optical density at 600 nm ~ 3.0 by using a spectrophotometer, Genesys 10 UV (Thermo Scientific). Bacteria were then washed 3 × with sterile PBS and diluted to appropriate concentrations for binding experiments.

Mucoidy phenotypes of CF clinical PA strains were determined by streaking the frozen stocks onto the Pseudomonas Isolation Agar plates. Motility of PA strains were determined by inoculating 1 μl of overnight bacterial culture (optical density 600 nm 3.0) onto the Brain Heart Infusion broth supplemented with 0.4% agar. Bacterial plates were incubated at 37 °C with 5% CO₂ for 48–72 h for observation.

Mouse lung exposure, tissue analyses, and BAL. Animal studies were carried out in strict accordance to the protocol approved by the Institutional Animal Care and Use Committee at the University of Illinois at Urbana-Champaign. C57BL6 mice (6-week old, n = 10) were housed in positively ventilated microisolator cages with automatic recirculating water, located in a room with laminar, high-efficiency particle accumulation-filtered air. The animals received autoclaved food, water, and bedding. PCN (25 μg), LPS (2 μg), alginate (90 μg), and flagella (2 μg) were intranasally inoculated into the mice anesthetized with isoflurane once daily for 3 weeks (PCN) or 7 days (LPS, alginate, and flagella). Control mice were exposed to the same volume (50 μl) of PBS for 3 weeks. Time points were based on our previous studies demonstrating PCN and LPS-induced GCHM in C57BL6 mice, where clear differences in lung pathology, cytokine, and immune cell profiles can be detected between the treated and control mice, as well as the health status of mice. Paraffin-embedded lung sections (4–5 μm thickness) were stained with high iron diamine-alcian blue blue or periodic acid-Schiff, or for immunohistochemical and IFA analyses with the primary antibodies against sialyl-LewisX (BD Pharmaning, San Jose, CA, #551344) or MUC5AC (Santa Cruz Biotechnology, Dallas, TX, #sc-21701).

To further confirm the expression of sialyl-LewisX in mouse airways, the lungs of PCN-exposed mice (n = 3) were BAL. The trachea was exposed and intubated with a 1.7-mm outer diameter polyethylene
catheter, followed by instillation of PBS in three successive 1-ml aliquots. Proteins from the first lavages (15 mg) were used for western blot analysis to determine the glycosylation of mucins with sialyl-LewisX by using anti-sialyl-LewisX antibodies.

**Cell cultures.** The human lung mucoepidermoid carcinoma cell line NCI-H292 (ATCCRL-1848) were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (Sigma) and 5% CO2. Epithelial cells that reached 70% confluency were serum-starved for 24 h before exposure to PCN, TNF-α, or U73122. The CF cell line IB3-1 (ΔF508/W1282X) was purchased from ATCC (Manassas, VA) (CRL-2777). IB3-1 cells were cultured in LHC-8 medium (Gibco, Grand Island, NY) on culture plates coated with a solution containing 35 mg ml⁻¹ bovine collagen (Advanced-BioMatrix, Carlsbad, CA), 1 mg ml⁻¹ human fibronectin (Advanced-BioMatrix), and 1 mg ml⁻¹ BSA (Sigma-Aldrich) supplemented with 10% fetal bovine serum in 5% CO2. For IFA analysis, NCI-H292 cells were exposed to PCN (5 μg ml⁻¹) for 24 h. IB3-1 cells were exposed to PCN (10 μg ml⁻¹) for 48 h. IFA analyses were performed using a primary anti-sialyl-LewisX, followed by Alexa Fluor488-conjugated secondary antibodies (Invitrogen, Grand Island, NY). Slides were mounted using 4,6-diamidino-2-phenylindole, and the subcellular localization of sialyl-LewisX was observed using a fluorescence microscope.

NHBE cells were purchased from Lonza (Walkersville, MD, USA) and cultured as we have previously described. Briefly, cells were thawed and passaged in 5% CO2 at 37°C using the bronchial epithelial growth medium supplemented with growth factors supplied in the SingleQuot kit (Lonza). Cells at passage 3 were trypsinized and seeded onto the Costar Transwells inserts with 0.4-μm pore size (Corning, NY) at a density of 1.5 × 10⁵ cells cm⁻² in media comprising 50% BEBM and 50% DMEM-F12 low-glucose (Invitrogen) supplemented with the growth factors provided in the SingleQuot kits and retinoic acid (50 nM). Once the cells reached confluency (~7 days after seeding, examined by transepithelial electrical resistance measurements (data not shown)), they were switched to an ALI for additional 2 weeks to achieve mucociliary differentiation. NHBE cells were exposed to PCN (12.5 μg ml⁻¹) for 24 h and stained with anti-MUC5AC and anti-sialyl-LewisX antibodies, and were visualized with Alexa Fluor488-conjugated secondary antibody (green color) and Alexa Fluor647-conjugated secondary antibody (red color), respectively.

**Figure 8** Pyocyanin (PCN) upregulates the expression of sialyl-LewisX in cystic fibrosis (CF) airway epithelial cells. IB3-1 cells were exposed to phosphate-buffered saline (PBS; control) or indicated concentrations of PCN or time intervals (with 5 μg ml⁻¹ PCN). (a) The expression of sialyl-LewisX was analyzed using specific antibody with western blots. Total protein extracts were separated on an agarose-acrylamide gel. (b, c) The expression of C2/4GnT (b) and ST3Gal-IV (c) was analyzed with western blot analysis. β-actin was used as loading controls. Representative western blots from one of the three independent experiments are shown. Densitometry analyses represent the mean ± s.e. from three independent western blot experiments. *P<0.05 when compared sialyl-LewisX (a) and C2/4GnT (b) and ST3Gal-IV (c) expression among the group by using the one-way analysis of variance (ANOVA) analysis. *P<0.05 when compared each sample against PBS control (a) or Time 0 h (b) by using the Tukey’s test.
under a confocal microscope. Nuclei were stained with 4,6-diamidino-2-phenylindole (blue color). The percentage of MUC5AC and sialyl-LewisX expression was calculated based on the fluorescence signal of MUC5AC or sialyl-LewisX divided by the total signal of 4,6-diamidino-2-phenylindole.

Protein extraction, immunoprecipitation, and western blot analysis. NCI-H292 cells were stimulated with PCN (0.5, 2.5, and 5.0 μg ml⁻¹) for the indicated time intervals. Total protein was extracted with M-PER Mammalian Protein Extraction Reagent (Thermo Scientific #78501), quantified with the Pierce BCA Protein Assay Kit (Thermo Scientific # 23227), and used in western blot analysis. For inhibitor studies, NCI-H292 cells were serum-starved for 24 h and then exposed to the PI-PLC inhibitor U73122 at concentrations of 2.5, 10, or 40 μM for 40 min before addition of PCN (5 μg ml⁻¹) or the same volume of PBS. These cells were harvested after 24 h. For immunoprecipitation, 600 μg of total protein extract was incubated with 2 μg of anti-TNF-α antibodies (Santa Cruz Biotechnology #sc-20118). For the analysis of sialyl-LewisX, total protein was separated on 0.5–6% agarose–acrylamide gradient gels as previously described.36 For the analysis of C2/4GnT, St3Gal-IV, and PLC-y2 expression, total protein was separated on 8% polyacrylamide gels. Western blot analyses were performed with antibodies against sialyl-LewisX, C2/4GnT, St3Gal-IV, and PLC-y2 (Santa Cruz Biotechnology #sc-161625, #sc-134041, and #sc-407). The immune complexes were visualized using the ECL Western Blotting Detection System (Amersham Biosciences, Pittsburgh, PA) and Hyblot CL (Denville Scientific, Holliston, MA) autoradiography films.

Image analysis. Densitometry analysis of western blots, total fluorescence levels of IFA images, and threshold analysis of immunohistochemical staining area were accomplished using the ImageJ software from NIH (http://rsbweb.nih.gov/ij/). Protocol for threshold analysis was as described.37 Quantitative analyses of IFA images on ALI cultures of NHBE cells were performed with the AxioVision Rel. 4.8 software (Carl Zeiss MicroImaging, LLC, Thornwood, NY). The percentage of positively stained cells was calculated against the total number of cells (4,6-diamidino-2-phenylindole-stained) within individual sections in the stained area.

PA-binding assay. After reaching 70% confluency, NCI-H292 and IB3-1 cells (~5 × 10⁵ per well, n = 12 wells) were serum-starved for 24 h before stimulation with PCN or equal volume of PBS. NCI-H292 cells were exposed to 5 μg ml⁻¹ PCN for 24 h, whereas IB3-1 cells were exposed to 10 μg ml⁻¹ PCN for 48 h. After exposure, the cells were washed with PBS three times, and incubated in sterile PBS and infected with PA strain PAO1, the isogenic flagellar cap-deficient mutant ΔfliD (multiplicity of infection = 1:1), or the clinical isolates CF1, CF2, CF26, and CF32. After 1 h of incubation, these wells were washed vigorously with three changes of 1 ml PBS. The epithelial cells were collected in 1 ml PBS, serially diluted, and plated onto Pseudomonas Isolation Agar for colonies enumeration. Similar results were obtained from three independent experiments. Results from a typical experiment are shown.

For the antibody-mediated blocking of sialomucins, following PCN exposure, NCI-H292 and IB3-1 cells were treated with anti-sialyl-LewisX antibodies (15 μg per well) for 2 h before the binding assay. For
Pseudomonas isolation agar. (washed to remove unattached PAO1, lysed, serially diluted, and plated on ratio to NCI-H292 or IB3-1 cells (n well) was used as controls. PA strains PAO1 or CF1 were added in a 1:1 simultaneously with PCN for the same duration. Human IgG (15 mg per airway cells pre-blocked with human IgG. PAO1-IgG and CF1-IgG: binding of bacteria to airway cells pre-blocked with anti-sialyl-LewisX antibodies (15 mg per well) were added 2 h before the binding assay. To block TNF-α, anti-TNF-α antibodies (15 mg per well) were added simultaneously with PCN for the same duration. Human IgG (15 mg per well) was used as controls. PA strains PAO1 or CF1 were added in a 1:1 ratio to NCI-H292 or IB3-1 cells (n = 12 wells). After 1 h, airway cells were washed to remove unattached PAO1, lysed, serially diluted, and plated on Pseudomonas isolation agar. (a, b) Binding of PAO1 and CF1 to NCI-H292 and IB3-1 cells pre-treated with anti-sialyl-LewisX antibodies or human IgG, respectively. (c, d) Binding of PAO1 and CF1 to NCI-H292 and IB3-1 cells pre-treated with anti-TNF-α antibodies or human IgG, respectively. Data represent colony-forming unit (CFU) recovered from each group. *P < 0.05 when compared bacterial binding among various treatments by using the one-way analysis of variance (ANOVA) analysis. **P < 0.05 when compared bacterial binding in each sample against human IgG controls by using the Tukey’s test. PAO1-IgG and CF1-IgG: binding of bacteria to airway cells pre-blocked with human IgG. PAO1-α-SLX and CF1-α-SLX: binding of bacteria to airway cells pre-blocked with anti-sialyl-LewisX antibodies. PAO1-α-TNF-α and CF1-α-TNF-α: binding of bacteria to airway cells pre-blocked with anti-TNF-α antibodies.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at http://www.nature.com/mi

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DISCLOSURE
The authors declare no conflict of interest.

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