

MODULATION OF AIRWAY MUCIN GLYCOSYLATION BY PSEUDOMONAS AERUGINOSA PYOCYANIN

BY

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THESIS

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ABSTRACT

Cystic fibrosis (CF) patients battle life-long pulmonary infections with the respiratory pathogen *Pseudomonas aeruginosa* (PA). A favorable niche for PA growth is provided by an overabundance of mucus in CF airways. When compared to that of non-CF individuals, mucus of CF airways has been found to be enriched in sialic acid, especially in the form of the tetra carbohydrate moiety sialyl-Lewis^x, which is a preferred binding receptor for PA. Furthermore, sialyl-Lewis^x levels have previously been shown to directly correlate with infection levels in CF patients. In this study, we examined the ability of PA virulence factors pyocyanin (PCN), LPS, flagella and alginate to modulate the levels of sialyl-Lewis^x modifications on airway mucins in a mouse model and in *in vitro* cell culture. We found PCN to be a potent inducer of sialyl-Lewis^x in both mouse airways and in the cultured airway epithelial cells. PCN increases the expression of ST3Gal-IV and C2/4GnT, two enzymes responsible for the stepwise synthesis of sialyl-Lewis^x through a TNF- α dependent phosphoinositol-specific phospholipase C (PI-PLC) pathway. Importantly, PA is better able to bind airway epithelial cells previously exposed to PCN. These results suggest 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.

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INTRODUCTION

Pulmonary bacterial infections with *Pseudomonas aeruginosa* (PA) are a critical clinical concern for patients living with cystic fibrosis (CF) (Boucher, 2002; Lyczak *et al.*, 2002), underscored by the finding that 97% of CF patients are colonized with PA by age three (Burns *et al.*, 2001). Pulmonary failure, as a sequela of acute exacerbations and tissue scarring in chronic infections, results in a large majority of mortality in CF patients (Boucher, 2002; Lyczak *et al.*, 2002). Previously understood contributing factors to PA pathogenesis in the CF lung involve overproduction of hyperviscous mucus and impeded mucociliary clearance of trapped bacteria in airways (Boucher, 2002). The ability of PA to initially and persistently colonize the lung is an important factor in determining the course and chronicity of infection (Carnoy *et al.*, 1994; Lamblin *et al.*, 2001).

Airway mucus is comprised of mucin glycoproteins and water. The structure of mucin glycoproteins contains a diverse population of carbohydrate chains that have been shown to be receptors for bacteria, and whose intraluminal location in the airway serves as a first line of interaction with microbes in the lung (Colomb *et al.*, 2012; Scharfman *et al.*, 1999; Delmotte *et al.*, 2002; Lamblin *et al.*, 2001). Mucins recovered from the lungs of CF patients have been shown to be enriched with the tetra carbohydrate structure sialyl-Lewis^x (Lo-Guidice *et al.*, 1994; Lamblin *et al.*, 1984; Davril *et al.*, 1999). Several studies have independently shown that PA binds these modified CF mucins with a higher affinity over control tissues and other carbohydrate moieties, respectively (Carnoy *et al.*, 1993; Carnoy *et al.*, 1994; Devaraj *et al.*, 1994, Scharfman *et al.*, 1999). It has been thoroughly established that the enzymes core 2 β 1, 6

N-acetylglucosaminyltransferase (C2/4GnT) and α 2, 3-sialyltransferase IV (ST3Gal-IV) are crucial for sialyl-Lewis^x synthesis, and are upregulated in conditions of pulmonary inflammation, which is a characteristic typical of the CF lung (Delmotte *et al.*, 2001; Delmotte *et al.*, 2002; Groux-Degroote *et al.*, 2008; Ishibashi *et al.*, 2005; Colomb *et al.*, 2012). Specifically, levels of sialyl-Lewis^x increase in response to treatment with TNF- α , IL-6 and IL-8. However, although the CF epithelium has been shown to be hyper reactive and to have a prolonged inflammatory response to stimuli when compared to tissue from non-CF patients (Muhlebach & Noah, 2002), several reports have shown that inflammation cannot initially occur in the absence of bacteria (Berger, 2002; Dakin *et al.*, 2002; Muhlebach & Noah, 2002). Therefore, increases in sialyl-Lewis^x cannot be exclusively attributed to tissue differences due to primary diseases. The further finding of a direct correlation between severity of infection and the levels of sialyl-Lewis^x on bronchial mucins (Davril *et al.*, 1999) underscores the importance of a bacterial etiology as an initial inciting factor in the modification of these mucins. Together, these findings warrant investigation in order to facilitate a deeper understanding of the effects of PA in relation to these changes in sialyl-Lewis^x levels.

LITERATURE REVIEW

Cystic fibrosis history and incidence

Cystic fibrosis is the most common life-limiting genetic disease among people of Caucasian heritage (O'Sullivan & Freedman, 2009). Cystic fibrosis became classified as an independent disease in 1938, at which time it was distinguished from a general disorder of malabsorption, known as celiac's disease (Andersen, 1938). This distinction was prompted by the finding of lesions in the pancreatic ducts on autopsy of a portion of infants who died from malnourishment, and prompted the condition to be termed "cystic fibrosis of the pancreas" (Andersen, 1938). Because many exocrine glands were found to be affected (di Sant Anese, 1956), and the observation of an autosomal recessive inheritance pattern was found (Andersen & Hodges, 1946), a fervent research effort began to search for a genetic origin of this disease. Because of the abnormally thick mucus that often was found to block airways, the initial search was focused on the mucus as the source of the defect (Davis, 2007). This changed in 1948, during a heat wave in New York, when a pediatrician noticed that the majority of the infants being presented to him for heat-related symptoms had CF (di Sant' Agnese *et al.*, 1953). He hypothesized that they were unable to tolerate the heat due to decreased cooling by sweating, and examined their sweat for abnormalities. He found that the Na and chloride present in the sweat of these patients was fivefold higher than normal. This led to investigation of the Na chloride channels for clues about the disease, and to a better way to test for CF disease, one that is still used today- the "sweat test" (Quinton *et al.*, 2012). The elevated Na and chloride in sweat is found in almost all individuals with CF, and almost none without it. This test also allows

for the identification of patients with milder symptoms, who may not have signs of pancreatic disease as seen with more severe cases of CF (Quinton *et al.*, 2012; Davis, 2007). It was not until 1983 that chloride transport was found to be the basic defect in the disease (Quinton, 1983). Shortly after, the CF gene was discovered in 1989 (Kerem *et al.*, 1989; Riordan *et al.*, 1989; Rommens *et al.*, 1989). Currently, “cystic fibrosis” is used to describe patients with a mutation in this gene, which is expressed in many types of epithelial cells. These cells are found in many organs, including the sweat ducts, airways, pancreatic ducts, intestine and biliary tree. The phenotype of this disease is one that will eventually develop into progressive, fatal lung disease (Davis, 2007).

The incidence of cystic fibrosis is highest in European- derived populations, with 1 in 2000-3000 births affected (Farrell, 2000), and a carrier rate of 1 in 26 (Romeo *et al.*, 1989). The incidence in North America is lower, with approximately 1 in 3,500 births affected (Kosorok *et al.*, 1996). The outlook for patients with CF today is much more promising than in the past. A model used in the United Kingdom predicts median survival ages of >50 years old for children born after the year 2000 (Dodge *et al.*, 2007).

Pathology of CF: primary genetic defect

The cystic fibrosis gene, termed the cystic fibrosis transmembrane conductance regulator (CFTR), was identified on the long arm of human chromosome 7 in 1989 (Rommens *et al.*, 1989; Kerem *et al.*, 1989; Riordan *et al.*, 1989). There are over 1,000 different recorded mutations in the CFTR gene to date (Cystic Fibrosis Gene Consortium). Mutations in this gene result in multi organ disease affecting populations of epithelial cells throughout the body

(Boucher, 2002). These effects translate to a variety of disease manifestations which reflect the many functions of the CFTR protein, and the range of possible phenotypes resulting from the CFTR defect. (Tsui, 1995). The vast majority (70%) of CFTR mutations are caused by a deletion of three base pairs that code for a phenylalanine at position 508, which is noted as $\Delta F508$ CFTR (Cystic Fibrosis Gene Consortium). This mutation confers abnormal folding in the CFTR protein during translation, which leads to recognition and labeling by chaperone proteins that direct the degradation of the CFTR protein prior to its transit to the apical plasma membrane. (Cheng *et al.*, 1990; Ward *et al.*, 1995).

The CFTR channel has been shown to be vital in the regulation of airway surface liquid (ASL) volume (Matsui *et al.*, 1998). The ASL is composed of two layers, the first being a thin (7 μ m), low density, pericilliary liquid (PCL), that surrounds the airway cilia, and provides a low-viscosity medium in which they can beat (Tarran *et al.*, 2001a). The second is a layer of mucus that sits on top of the PCL layer, which traps debris and pathogens, and is constantly being moved proximally via the beating action of the cilia to remove trapped foreign objects from the airway (Tarran *et al.*, 2001a; Tarran *et al.*; 2001b).

In normal individuals, the CFTR functions as a cAMP-mediated chloride channel, serving in electrolyte homeostasis at the luminal surface of the airway. Specifically, the CFTR can secrete chloride ions (Bear *et al.*, 1992; Rich *et al.*, 1990), which establish an osmotic gradient favorable for addition of water to the ASL (Boucher, 1994a; Boucher, 1994b, Boucher, 2002). The CFTR channel also provides tonic inhibition of the epithelial sodium channel (ENaC) (Stutts

et al., 1995), a sodium channel also found on the apical surface of airway epithelium. With the loss of CFTR in CF, the tonic inhibition on the ENaC is lost, leading to an unregulated increase in the absorption of sodium ions into the cell. To balance out the charge, chloride ions are absorbed through the paracellular pathway (Boucher, 1994a; Boucher, 1994b), leading to a net increase in absorption of sodium chloride (NaCl) into the epithelium (Jiang *et al.*, 1993; Matsui *et al.*, 1998; Tarran *et al.*, 2001b). The resulting osmotic gradient favors the increased absorption of water, which depletes the PCL, slows mucus clearance from the lung, and eventually causes mucus to directly contact and adhere to the surface of the cilia (Matsui *et al.*, 1998). The mucus layer can act as a reservoir to donate water to the PCL in order to compensate for the volume depletion (Tarran *et al.*, 2001a), although this changes its physical characteristics to be more viscous and concentrated (Boucher, 2002). With a non-functional CFTR, chloride ion release to reverse this gradient and add water back to the ASL is not possible (Knowles *et al.*, 1983). In addition, there is evidence that the mucus-producing mechanism of goblet cells has a very poor feedback mechanism, as there is continued production in the face of reduced clearance. This results in increased mucus layer depth, and leads to mucus plugging and bronchiectasis (Boucher, 2002). It is this mechanism of the CFTR's effect on ASL volume that is commonly agreed upon to be the link between the genetic mutation and the chronic lung disease that is characteristic of CF (Matsui *et al.* 1998; Tarran *et al.*, 2001a).

Pathology of CF: secondary bacterial infections

The primary defects in mucociliary clearance discussed earlier prime the CF lung for bacterial infection. Analysis has shown that bacterial defensins in the mucus layer are capable

of controlling bacterial growth and even decreasing CFU for up to 6hrs (Cole *et al.*, 1999), which is the normal amount of time for mucocilliary clearance in a non-CF airway (Wanner *et al.*, 1996). After this, the bacteria were able to overcome the defensins, and flourish into productive infections in the airway (Cole *et al.*, 1999), confirming the importance of the speed of mucus transport in the airway. Chronic cycles of bacterial infection with periods of acute exacerbation are considered the most challenging clinical feature of CF lung disease, and one that results in the death of 80-95% of CF patients (Lyczak *et al.*, 2002). These infections are commonly caused by opportunistic infections of normal flora of the respiratory tract (*H. influenza*, *S. aureus*), or common non-pathogenic environmental pathogens (*P. aeruginosa*). Bacterial infections with *H. influenza* and *S. aureus* are more prevalent early in the life of CF patients, but decline drastically with time (Strausbaugh & Davis, 2007; Lyczak, 2002). Infections with the gram negative *Pseudomonas aeruginosa* (PA) are by far the most common, and incidence dramatically increases with age (Lyczak, 2002; Strausbaugh & Davis, 2007).

Pseudomonas aeruginosa has been shown to thrive and form biofilms in the hypoxic conditions of mucus plaques in CF airways (Worlitzsch *et al.*, 2002), and in this way is well adapted for infection in the CF lung. Growth in biofilms and within mucus also provides PA with increased resistance to antimicrobial therapies (Drenkard, 2003). Resistance to antimicrobial agents by PA is also acquired through the ability of the pathogen to rapidly develop adaptation strategies to numerous classes of antimicrobials (Tseng *et al.*, 1972; Bryan *et al.*, 1972b). One of the most concerning clinical problems associated with PA infection is a large inflammatory response (Lyczak, 2002), which can progressively damage airways and lead to declined pulmonary function, and eventually, failure (Boucher, 2002; Emerson *et al.*, 2002). Although CF

epithelium has been shown to have an exaggerated and prolonged inflammatory response as compared to non-CF tissue (Muhlebach & Noah, 2002; DiMango *et al.*, 1995), other studies have shown that inflammation does not occur without an initial inciting bacterial stimulus (Burns, 2001). The inflammatory reaction, characterized by increased neutrophil count and IL-8 levels, has been shown to begin in young patients (<6mo.), even though infection levels are low (Khan *et al.*, 1995).

Patients with CF, after chronic colonization with PA, experience acute exacerbations with inflammation that require aggressive antibiotic therapy for control. Neutrophilic infiltration is the dominant inflammatory response in CF airways (Konstan *et al.*, 1994), and it is the neutrophils themselves which have been shown to perpetuate the inflammation and subsequent lung damage of CF (Khan *et al.*, 1995). This damage is caused by neutrophil release of damaging oxidants (Van Der Vliet *et al.*, 2000), and proteases. One of the most damaging proteases is elastase, which has been shown to degrade nearly all the structural proteins of the lung including elastin, collagen type IV, fibronectin and proteoglycan (Janoff *et al.*, 1979), contributing to parenchymal destruction and airway remodeling. The PA bacteria can also contribute to airway pathology through the effects of several secreted and non-secreted factors on airway epithelia. (Caldwell *et al.*, 2009; Lau *et al.*, 2005). Pyocyanin (PCN), a redox active phenazine toxin secreted from stationary phase PA, is found in CF airways in concentrations of 100uM (27ug/mL) (Wilson *et al.* 1988). PCN has been shown to be critical for PA infection in mouse airways (Lau *et al.*, 2004). Prolonged administration of purified PCN into mouse airways has been shown to cause goblet cell hyperplasia and metaplasia, airway fibrosis and alveolar airspace destruction, consistent with pathology found in chronically infected CF

airways (Caldwell *et al.*, 2009). Pyocyanin can freely cross cell membranes and exert an oxidative effect on cytosolic and mitochondrial components, inducing oxidative stress and interfering with airway epithelial cell metabolism (Rada & Leto, 2009). Pyocyanin also induces apoptosis in neutrophils, causing them to release oxidative granzyme material, further damaging epithelial cell tissues (Usher *et al.*, 2002). These mechanisms demonstrate PA's ability to cause inflammation and tissue injury in the lung, leading to the phenotypic characteristics of serious progressive lung disease in cystic fibrosis.

Mucin glycoproteins: structure and modifications in CF

Airway mucus is composed of water (97%) and high molecular weight glycoproteins called mucins (2%) (Fahy & Dickey, 2010). Mucins are synthesized in goblet cells and submucosal glands in the conducting airways (trachea, bronchi, and bronchioles) of the respiratory tree, and can either be secreted or membrane-tethered (Rose & Voynow, 2006; Fahy & Dickey, 2010). Mucin glycoprotein synthesis involves transcription in the nucleus of one of the 17 MUC genes in the human genome to encode a MUC mRNA, which is translated to a mucin protein backbone on ribosomes in the endoplasmic reticulum, and then post-translationally modified by the addition of O-glycosylations in the cis-golgi (Rose & Voynow, 2006; Thornton *et al.*, 2008, Rose, 1992). Two of the most abundant mucins found in human airways are the secreted, polymer-forming MUC5AC and MUC5B. (Rose & Voynow, 2006; Thornton *et al.*, 2008). Post translational modification leaves mucin glycoproteins heavily glycosylated, with carbohydrate side chains representing 50-90% of their total weight (Rose & Voynow, 2006). These side chains can bind liquids to increase the weight of the mucin several

hundred times. These well-hydrated mucins allow the mucus layer to serve as a lubricant for the mucous membrane as well as a liquid reservoir for the PCL that is located beneath it (Knowles & Boucher 2002, Rose, 1992). The structure of the MUC protein backbone includes an NH₂ terminal domain, a central region with regions of tandem repeats (TR) separated by cysteine-rich regions, and a COOH terminal domain (Thornton *et al.*, 2008; Rose & Voynow, 2006; Rose, 1992). The TR structures are rich in serine and threonine, which are attachment sites for the O-glycans (Hang & Bertozzi, 2005; Rose 1992). Therefore, the more TR regions that a mucin has, the more heavily glycosylated it will ultimately be (Rose & Voynow, 2006). For example, MUC5B contains a TR sequence with 16 serine or threonine sites for possible O-glycan attachment. This TR sequence is repeated 72 times in the central region of the mucin backbone, affording over 1,100 possible attachment sites (Rose & Voynow, 2006). The glycosylation structure is comprised of a core, backbone and terminal region (Hanisch 2001). Four main core structures exist (Brockhausen *et al.*, 1985), and all have a base *N*-acetylgalactosamine (GalNAc) in common, which is added onto a serine or threonine of the mucin peptide backbone through the action of *N*-acetylgalactosaminyl peptidase enzyme (Rose, 1992). Once this initial addition is in place, combinations of single-chain or branched conformation galactose (Gal) and *N*-acetylglucosamine (GlcNAc) are added by their respective glycosyltransferase enzymes to form one of the possible four main core conformations of the glycan (Rose, 1992, Brockhausen *et al.*, 1985). These core structures can be elongated by the addition of the repetitive disaccharide element Gal β 1-4GlcNAc to the core, which forms the backbone portion of the glycosylation chain (Hansich, 2001). The terminal regions of the glycosylations are complex, due to the addition of many possible structures (Hanisch, 2001). Terminal structures can be modified with

additions of *N*-acetylneuraminic acid (sialic acid), fucose, sulfate, or blood group determinants (Hanish, 2001; Rose, 1992). These terminal additions can affect the rheological properties of the mucus. Specifically, *N*-acetylneuraminic acid and sulfate confer a negative charge to the mucin, and fucose has hydrophobic properties (Rose & Voynow, 2006).

Terminal additions to mucins have been found to be altered in CF, with enrichment in sulfate and sialic acid (Lo-Guidice *et al.*, 1994). Enrichments in sulfate can be attributed to the CFTR primary genetic mutation, as CFTR-mediated increases in acidity within the cell increase the addition of sulfate to mucins (Zhang, 1995). Differences in sialic acid, however, cannot be easily linked to the CFTR mutation, and have rather been associated with increased inflammation in the CF lung with chronic infection (Lo-Guidice *et al.*, 1994; Lamblin *et al.*, 1984; Davril *et al.*, 1999). The sialic acid, most often, is found in the form of the tetra carbohydrate structure sialyl-Lewis^x (NeuAc α 2-3Gal β 1-4{Fuc α 1-3}GlcNAc-R). Sialyl-Lewis^x has been shown to be a preferential binding receptor for PA (Carnoy *et al.*, 1993; Carnoy *et al.*, 1994; Devaraj *et al.*, 1994; Scharfman *et al.*, 1999). Sialyl-Lewis^x, is formed in a stepwise fashion by many different glycosyl- and sialyltransferases (Delmotte *et al.*, 2002b; Chen, 2011). These enzymes have also been shown to be upregulated in conditions of inflammation. (Groux-Degroote *et al.*, 2008; Delmotte *et al.*, 2001; Delmotte *et al.*, 2002; Ishibashi *et al.*, 2005). Specifically, studies have shown increased levels of sialyl-Lewis^x in response to inflammatory cytokines IL-8, IL-6 and TNF- α . In contrast to the regulation of mucin production, which has been shown to be modulated through the EGFR pathway (Hao *et al.*, 2012; Ishibashi *et al.*, 2005) regulation of TNF- α -

dependent terminal addition of sialyl-Lewis^x has been found to be controlled through the phosphoinositol-specific phospholipase C (PI-PLC) pathway (Ishibashi *et al.*, 2005).

RESEARCH JUSTIFICATION

Cystic fibrosis is the most common life-limiting genetic disorder in populations of European heritage. Although the life expectancy for patients with CF has increased dramatically over the last 30 years, much of the therapy relies on aggressive antibiotic treatment of chronic pulmonary infections. The most common etiologic agent of these infections is *Pseudomonas aeruginosa* (PA), which can evade and/or develop resistance to last line antibiotic therapy options. The result is uncontrolled fulminant pulmonary inflammation, which leads to airway and parenchymal tissue destruction in the lung, and a subsequent pulmonary functional decline and failure. This pattern of pathogenesis necessitates better understanding of the mechanisms of PA airway infection. Previous work has established a link between PA pulmonary infection levels and increased expression of the preferential binding receptor sialyl-Lewis^x on airway mucins. Despite this, no work examining the effect of purified virulence factors on the expression of sialyl-Lewis^x has been completed. The study herein describes the effect of several purified virulence factors on the expression of sialyl-Lewis^x and its biosynthetic enzymes, and impact on PA binding efficiency to airway epithelial cells. A deeper understanding of the mechanisms of PA colonization and infection in the lung will afford new possibilities for treatment targets or further research with the aim to improve the outcome of chronic PA lung infection in CF patients.

RESULTS

Pyocyanin is a potent inducer of sialylomucins *in vivo*. We examined the ability of various purified PA components to induce changes in mucin glycosylation in chronic *in vivo* models of exposure. Recovered lung sections were stained using the high iron diamine-alcian blue (HID-AB) stain to detect sialylomucins and sulfomucins. Although all bacterial components were able to induce at least a low level of sialylomucin staining compared to the PBS control, chronic administration of PCN resulted in the most dramatic increase in sialylomucins (Fig. 1). For this reason, our study focused on the relationship between PCN and mucin sialylation. We observed no staining of sulfomucins in lung sections, despite a prominent presence in large colon control sections from the same animals (Fig. S1).

Pyocyanin induces sialyl-Lewis^x epitopes in bronchiolar epithelium *in vivo*. Previous studies have shown increased levels of sialic acid modifications to terminal mucin glycosylation chains in the form of sialylated Lewis^x epitopes (Davril *et al.*, 1999). Sialyl-Lewis^x has been shown to be a preferential binding receptor for PA (Sharfman *et al.*, 1999). We examined the effect of chronic PCN exposure on the levels of sialyl-Lewis^x epitopes on the surface of the bronchial mucosa. Lung sections from mice chronically administered PCN were used for immunohistochemical (IHC) analysis with a primary antibody against sialyl-Lewis^x. Chronic PCN administration significantly increases the sialyl-Lewis^x epitopes in both large and small airways by 10 and 35-fold, respectively, when compared with baseline levels in control lungs exposed to PBS (Fig. 2). These results confirm that PCN induces an increase in the amount of sialyl-Lewis^x present in bronchial mucosa *in vivo*.

Pyocyanin induces sialyl-Lewis^x in cultured NCI-H292 airway epithelial cells. In order to more closely examine the mechanism of PCN's effect on sialyl-Lewis^x additions to mucin glycosylations, we tested the effect of PCN administration on the levels of sialyl-Lewis^x in an *in vitro* cell culture model using the NCI-H292 airway epithelial carcinoma cells in western blotting analysis with a primary antibody against sialyl-Lewis^x. An increase in the level of the sialyl-Lewis^x signal was seen at 2.5 and 5.0 µg/mL concentrations after 24 hr (Fig. 3A). Furthermore, PCN (5.0 µg/mL) induces a time-dependent increase in sialyl-Lewis^x epitopes (Fig. 3B). To further confirm our findings, we performed immunofluorescence studies on NCI-H292 cells treated with PCN with the anti-sialyl-Lewis^x antibody. In the PCN treated cells, 3.8-fold higher sialyl-Lewis^x epitopes were seen, along with a change in cellular localization from perinuclear to diffuse cytoplasmic (Figs. 3C-D, S2). Taken together, these results confirmed the ability of PCN to induce sialyl-Lewis^x in NCI-H292 cells in concentration and time dependent manners.

Pyocyanin induces upregulation of C2/4GnT and ST3Gal-IV. Sialyl-Lewis^x is synthesized through a stepwise process involving many enzymes (Delmotte *et al.*, 2002). Previous work has shown that levels of sialyl-Lewis^x biosynthesis enzymes C2/4GnT and ST3Gal-IV are upregulated in response to inflammation (Delmotte *et al.*, 2001; Delmotte *et al.*, 2002b; Groux-Degroote *et al.*, 2008). We examined whether PCN could directly upregulate these enzymes by exposing H292 cells to PCN in a time dependent manner. PCN significantly increased the amounts of both C2/4GnT (Fig. 4A) and ST3Gal-IV (Fig. 4B) by 4 and 3-fold, respectively. These results indicate that PCN is capable of upregulating the expression of enzymes crucial for the biosynthesis of sialyl-Lewis^x.

PCN induces an increase in sialyl-Lewis^x through the PI-PLC pathway. Most recently, we have shown that PCN induces the expression of MUC5B AND MUC5AC mucins through activation of EGFR (Hao *et al.*, 2012). In contrast, EGFR has been shown to negatively regulate C2/4GnT and ST3Gal-IV (Beum *et al.*, 2003; Ishibashi *et al.*, 2005). Rather, these glycosyltransferases are induced through signaling in the phosphoinositol-specific phospholipase C (PI-PLC) pathway (Ishibashi *et al.*, 2005). To test whether PCN-induced upregulations in sialyl-Lewis^x and its biosynthetic enzymes C2/4GnT and ST3Gal-IV were also PI-PLC dependent, NCI-H292 cells were exposed to PCN either alone or in the presence of PI-PLC pathway inhibitor U-73122 in increasing concentrations. Our results showed that PCN upregulated PLC-γ2 (Fig. S3), a pathway component of PI-PLC, and that cells treated with U-73122 had a dose-dependent decrease in PCN-mediated upregulation in sialyl-Lewis^x, C2/4GnT, ST3Gal-IV (Fig. 5A-D). These results indicate that upregulation of these components by PCN is occurring through signaling of the PI-PLC pathway.

Pyocyanin increases binding affinity of *P.aeruginosa* to airway epithelial cells in vitro. Because sialyl-Lewis^x has been shown to be a preferential binding receptor for PA (Scharfman *et al.*, 1999), and our results indicate a PCN-mediated increase in sialyl-Lewis^x levels, we examined whether PA would be better able to bind to cells treated with PCN. Our results showed a 50% increase in binding efficiency of PAO1 in the PCN-treated cell group (Fig. 6). These results indicate that the modifications induced by PCN are able to facilitate the binding of PA in a more efficient manner.

DISCUSSION

PCN is a redox-active phenazine toxin found to be excreted in levels up to 100 μM (27 $\mu\text{g/mL}$) in PA-infected bronchiectatic airways (Wilson *et al.*, 1988). In experimental mouse models, PCN has been found to be important for chronic lung infection, and also to cause goblet cell hyperplasia, airway fibrosis, and airspace destruction within the lung, pathological findings mirroring those found in a CF lung chronically infected with PA (Caldwell *et al.*, 2009). Levels of sialyl-Lewis^x, a binding receptor for PA, have also been found to be upregulated in mucins from patients with CF (Morelle *et al.*, 2001; Shori *et al.*, 2001), but historically have been primarily attributed to a condition of increased inflammation in the lung (Lamblin *et al.*, 2001). Evidence suggesting the importance of bacterial infection in modulating levels of sialyl-Lewis^x include observations of a correlation between severity of infection and increased sialyl-Lewis^x expression (Davril *et al.*, 1999; Morelle *et al.*, 2001), as well as studies demonstrating an initial bacterial component required for inflammation in CF lungs (Berge, 2002; Dakin *et al.*, 2002; Muhleback *et al.*, 2002). We postulated that PA, or a factor thereof, may contribute to the initial stimulus in a pathway leading to increased sialyl-Lewis^x. In this study, we show that chronic PCN administration increases the expression of sialylated mucins in mouse airways through a TNF- α -PI-PLC pathway. No sulfated mucins were detected in mouse airways, despite strong reactions on positive control slides of large colon tissue (Fig. S1). This is not surprising because mucin sulfation in cystic fibrosis has been shown to be directly linked to the primary genetic defect in the epithelium (Zhang *et al.*, 1995), which is absent in our mouse model. Our study also showed a causal link between chronic PCN administration in mouse airways and increased levels of sialyl-Lewis^x (Fig. 2). This finding, as well as increased expression of C2/4GnT

and ST3Gal-IV, enzymes crucial for sialyl-Lewis^x biosynthesis, was also seen in NCI-H292 cells cultured *in vitro* (Figs. 3 and 4). Collectively, these results demonstrate the ability of PCN to modulate sialyl-Lewis^x synthesis.

Previous studies have shown a significant increase in C2/4GnT, ST3Gal-IV and sialyl-Lewis^x in response to TNF- α , IL-6 and IL-8 in models of airway cell culture and culture of bronchial explants (Delmotte *et al.*, 2002; Groux-Degroote *et al.*, 2008; Ishibashi *et al.*, 2005; Colomb *et al.*, 2012). Upregulation of sialyl-Lewis^x in response to TNF- α treatment was confirmed in our cell culture model (Fig. S4.) In agreement, PCN has been shown to induce production and release of TNF- α , IL-6 and IL-8 from bronchial airway epithelial cells (Denning *et al.*, 1998; Rada & Leto, 2011). Taken together, these results suggest PCN secreted from PA may increase sialyl-Lewis^x by inducing TNF- α to produce observed effects in sialyl-Lewis^x. Previous studies involving the effects of inflammatory cytokines on enzymes responsible for the synthesis of sialyl-Lewis^x demonstrate, in addition to showing an increased expression in C2/4GnT and ST3Gal-IV, an upregulation in the expression of the α 1-3 fucosyltransferase enzyme FucT-III/IV/VII (Delmotte *et al.*, 2002; Ishibashi *et al.*, 2005; Groux-Degroote *et al.*, 2008). Increased expression of FucT-III/IV/VII was not observed in response to PCN administration (data not shown). We hypothesize 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 pathway (Hao *et al.*, 2012; Rada & Leto, 2011), which has been shown to be inhibitory to these glycosyltransferases (Ishibashi *et al.*, 2005; Beum *et al.*, 2003). It is possible that duality of action exerted by the TNF- α -PI-PLC and EGFR pathways seen as a result of PCN administration offer an environment in which the individual signaling pressures of multiple pathways are

summed to equal a different overall signaling stimulus than that of pure TNF- α , as in previous studies, and consequently yield a shifted response from downstream elements. However, as PCN is present in appreciable quantities in the lungs of some CF patients (Wilson *et al.*, 1988), the simultaneous upregulation of both signaling pathways may reflect more closely the stimulus environment of the airway during infection. Further studies will be required to elucidate the interaction of PCN and FucT-III/IV/VII in the future.

Sialyl-Lewis^x has been previously shown to be a preferential binding receptor for PA (Sharfman *et al.*, 1999). Because PCN upregulates the amount of sialyl-Lewis^x in mouse airways and H292 cells, this suggests it is able to alter the binding environment during chronic lung infection by PA. This suggestion proposes a PA-mediated binding environment more favorable for subsequent colonization after aggressive antibiotic therapy that would eradicate most PA cells, but would leave in the airway mucins more equipped to bind remaining PA or new bacteria that enter the airways. Due to the reactivity of epithelium unique to the CF lung, it is also possible that these modified mucins may have continued production after the removal of a bacterial stimulus. In support of this idea, Muhleback *et al* described increased and prolonged inflammatory responses in CF lungs compared to non-CF lungs, which were also found in CF lungs devoid of bacterial infection, suggesting an inflammatory response in CF epithelium that is not only pronounced, but delayed in cessation after release of stimulation. In summary, PCN is able to modulate airway mucin glycosylation by the upregulation in levels of sialyl-Lewis^x. The importance of this carbohydrate moiety as a binding receptor for PA underscores the importance in increasing knowledge of the effects of PCN on airway mucins. This current finding

adds further supporting evidence to the importance of PCN during PA-mediated chronic pulmonary infection in cystic fibrosis.

MATERIALS AND METHODS

Chemicals and purified bacterial components. Chemically synthesized PCN was purchased from Sigma Aldrich (Sigma #R9532). Synthesized PCN is preferred to PCN purified from PA cultures to eliminate contaminants which may cause lung injury. PCN was resuspended in sterile water to a concentration of 0.25 µg/µL. PA LPS (Sigma) was instilled once daily intranasally at 2 µg/mouse for 7 days. Alginate was purified from the mucoid PA strain FRD1 and quantified as previously described (Hoffman *et al.*, 2005), with some modifications. Briefly, *P. aeruginosa* mucoid strain FRD1 was streaked on *Pseudomonas* isolation agar and grown at 37C overnight. A sterile tip was used to inoculate 250mL of LB/ 1%glycerol media and grown at 37C overnight, shaking at 250rpm. Cells from overnight growth were harvested by centrifugation at 10,000rpm on a Beckman JA-17 rotor. The resulting supernatant was transferred to a sterile container and heated at 80C for 30 minutes to inactivate enzymes. The alginate in the supernatant was precipitated by the addition of three volumes of ice-cold ethanol. Precipitated alginate, seen as a white clod, was harvested from the media with a sterile rod and was washed three times in sterile saline. The alginate was finally suspended in sterile saline and put to a shaker overnight to form a homogenous suspension. Alginate was stored at 4C until use. To quantify the concentration of alginate, a carbazole-borate assay was performed. Protocol from Knutson & Jeanes, 1968 was followed. Reagents: stock borate solution was made by dissolving 24.74g H₃BO₃ (boric acid) in 100mL 4M KOH (22.44g KOH in 100mL H₂O) to give 4M BO₃³⁻. H₂SO₄- borate reagent- 1.25mL stock borate solution was made to 50mL with concentrated H₂SO₄. Carbazole reagent: 0.1% solution prepared by dissolving carbazole in 100% EtOH. Alginic acid standards: alginic acid Na salt (Sigma #180947) was used

as a standard. 35mg/mL was used as a starting concentration, dilutions of 1:1, 1:10 and 1:100 were made for a standard curve. Assay procedure: 6mL of H₂SO₄-borate reagent was pipetted into each tube and equilibrated in an ice bath. Carefully, 0.7mL of test solution (sample or standard) was layered on top of the acid and allowed to equilibrate in an ice bath; the mixture was then vortexed for four seconds and equilibrated in an ice bath. Carbazole solution (0.2mL) was added and mixed, and the samples were heated in a 55C water bath for 20 min to allow color reaction to develop. 200ul of each sample or standard was transferred to a 96-well plate and absorbance was read at 450nm. Flagella were purified from PA strain PAO1 as previously described (Campodonico *et al.*, 2009). Briefly, 500ul of liquid overnight culture of *P. aeruginosa* strain PAO1 was inoculated into 250mL of tryptic soy broth, and grown at 37C with shaking at 200rpm. Cells were harvested at 10,000 RPM in a Beckman JA-14 rotor. The resulting cell pellet was resuspended in cold PBS. The resuspension was put into a blender and sheared for 35 seconds. Resuspension was centrifuged at 13,000rpm in a Beckman JA-14 rotor. Resulting supernatant was further centrifuged at 40,000g for 3 hours in a Beckman H class ultracentrifuge with SW-28 rotor. Resulting pellets were resuspended in 10ul sterile PBS, and protein concentration was quantified by BCA assay. Samples were stored at -20C until use. U73122 was purchased from Sigma Aldrich (#U6756) and resuspended to a concentration of 0.9 mg/mL according to manufacturer's instructions. TNF- α was purchased from R&D systems (#210-TA-010) and resuspended to 100 μ g/mL in sterile PBS with 0.1% BSA.

Mouse exposure and lung tissue analyses. C57BL6 mice (6-week old, n = 10) Harlan Sprague Dawley) 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 lungs of mice anaesthetized with isoflourane every 24 hours for 3 weeks or 7 days (LPS, alginate and flagella), respectively. Control mice were exposed to the same volume (50 µL) of sterile water. Time points were based on (for PCN and LPS) our previous studies demonstrating PCN and LPS-induced GCH in C57BL6 mice, where clear differences in lung pathology, cytokine and immune cells profiles can be detected between the treated and control mice (Caldwell *et al.*, 2009; Hao *et al.*, 2012, and unpublished observation), and in the case of alginate and flagella on previously published studies (Campodonico *et al.*, 2009; Hoffman *et al.*, 2005) as well as health status of mice. Lungs tissues were collected for histopathological analyses as we have previously described (Caldwell *et al.*, 2009; Hao *et al.*, 2012). Briefly, a cannula was inserted in the trachea, and the lung was instilled with 10% neutral buffered formalin at a constant pressure (25cm H₂O). The trachea was ligated, and the inflated lung was immersed in 10% neutral buffered formalin for 24 hours before embedding in paraffin wax. Paraffin embedded lung sections (4-8 µm thickness) were stained with high-iron diamine-alcian blue (HID-AB) or periodic acid Schiff's stain (PAS). For immunohistochemistry analyses, mouse lung sections were stained with primary antibody against sialyl-Lewis^x (BD Pharmingen #551344); and visualized using the Vector M.O.M. immunodection kit (Vector Laboratories) according to the protocol supplied by the manufacturer. This study was carried out in strict accordance to the protocol approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Illinois at Urbana-Champaign.

Cell Culture. The human lung mucoepidermoid carcinoma cell line NCI-H292 was purchased from the American Type Culture Collection (ATCC) (Manassas). NCI-H292 cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum (Sigma) in 5% CO₂. Epithelial cells that reached 70% confluency were serum starved for 24hrs before exposure to PCN, TNF- α or U73122.

Sialyl-Lewis^x, C2/4GnT, ST3Gal-IV and PLC- γ 2 analysis. NCI-H292 cells were stimulated with PCN (0.5, 2.5 and 5.0 μ g/mL) for indicated time intervals (0, 0.25, 0.5, 1, 2, 4, 8, 12 and 24 hr). Total protein was extracted with M-PER Mammalian Protein Extraction Reagent (Thermo Scientific #78501). Protein concentration was determined with the Pierce BCA Protein Assay Kit (Thermo Scientific #23227). The same amount of protein from each treatment group was used in western blotting analysis. For inhibitor studies, NCI-H292 cells were serum starved for 24 hr and then exposed to the PI-PLC inhibitor U73122 at concentrations of 2.5 and 10 μ M for 40 min before addition of PCN or the same volume of sterile PBS. These cells were harvested after 24 hr for western blotting analysis with antibodies against sialyl-Lewis^x (BD Pharmingen #551344), C2/4GnT, ST3Gal-IV and PLC- γ 2 (Santa Cruz Biotechnology #sc-161625, #sc-134041, #sc-407, respectively). The immune complexes were visualized using the ECL Western Blotting Detection System (Amersham Biosciences) and Hyblot CL (Denville Scientific) autoradiography films.

Immunofluorescence of sialyl-Lewis^x. NCI-H292 cells were exposed to PCN (5 μ g/mL) for 24 hr. Immunofluorescence analysis was performed using a primary anti-sialyl-Lewis^x antibody, followed by FITC-labeled secondary antibody (Alexa Flour[®] 488, Invitrogen). Slides were mounted using DAPI, and the subcellular localization of sialyl-Lewis^x was observed using a confocal fluorescence microscope (Zeiss Axiovert 200M).

Image Analysis. Densitometry analysis of Western blots, total fluorescence levels of IFA images and threshold analysis of IHC staining area was accomplished using the ImageJ software from NIH (<http://rsbweb.nih.gov/ij/>). Protocol for threshold analysis was as described (Vayrynen *et al.*, 2012).

PA binding assay. After reaching 70% confluency, NCI-H292 cells ($\sim 5 \times 10^5$) (n = 11 cell culture wells for each treatment) were serum starved for 24 hours before stimulation with 5 $\mu\text{g/mL}$ PCN or equal volume of sterile PBS. After 16 hr, the cells were infected with $\sim 5 \times 10^5$ PA strain PAO1 (MOI= 1:1). After 1 hr incubation, these wells were washed vigorously with 7 changes of 1 ml sterile PBS. The epithelial cells were collected in 1 mL sterile PBS, serial diluted and plated onto Pseudomonas Isolation Agar for colony enumeration.

FIGURES

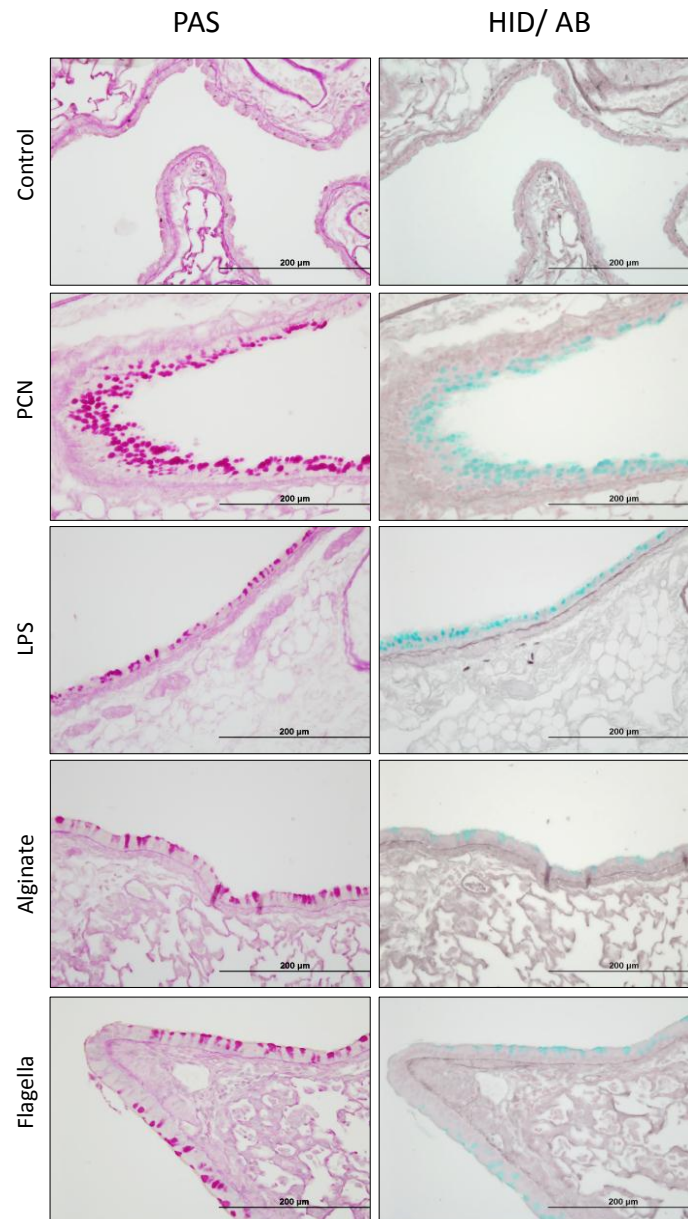


Fig. 1. PCN is a potent inducer of sialylomucins. Serial lung sections from mice (groups of 10) exposed to sterile H₂O control or various purified PA components were stained using PAS to detect goblet cell hyperplasia and HID/ AB technique to detect sialylo- and sulfomucins.

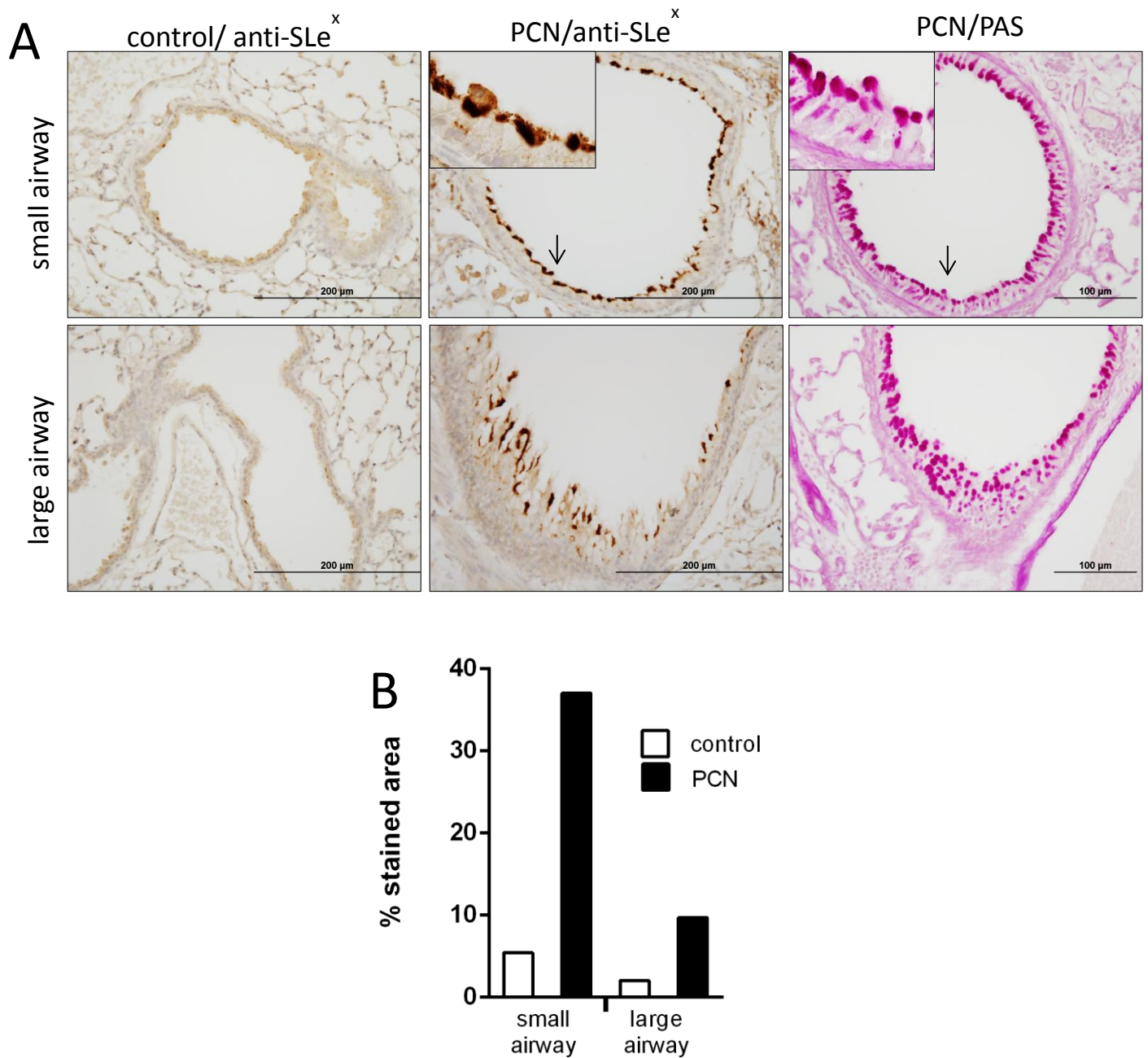


Fig. 2. PCN induces the expression of sialyl-Lewis^x epitopes in mouse airways. (A) IHC analyses of lung sections from mice exposed to PCN or same volume of sterile H₂O were stained with an anti-sialyl-Lewis^x antibody. Serial PCN-exposed sections were stained with PAS for the presence of goblet cell hyperplasia. (B) Quantification of percent sialyl-Lewis^x stained area in control versus PCN-treated large and small airways.

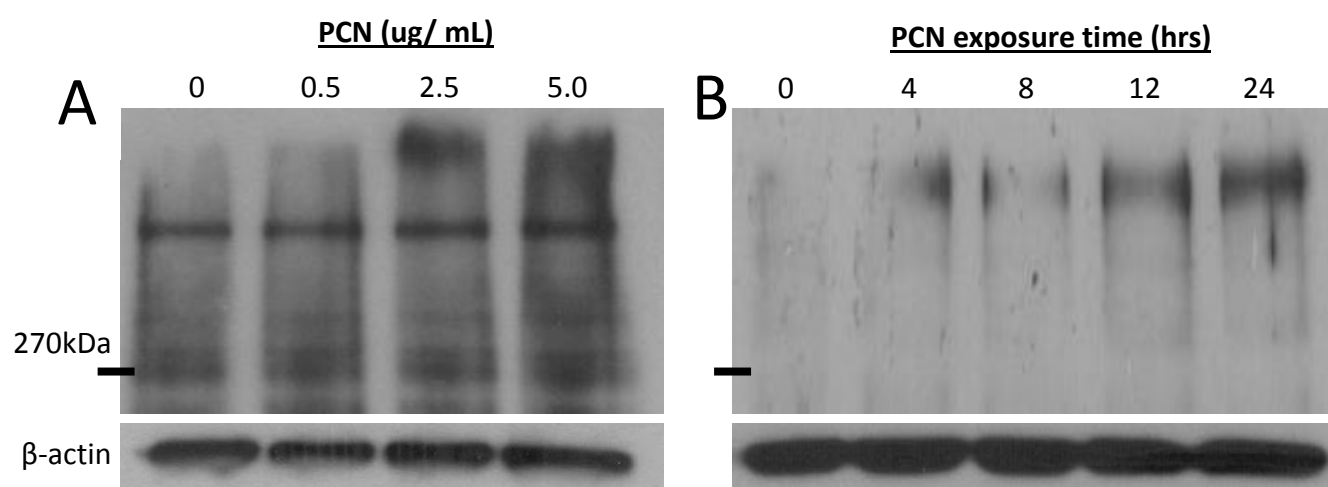


Fig. 3. PCN upregulates the expression of sialyl-Lewis^x in time and concentration-dependent manners. NCI-H292 cells were exposed to sterile H₂O (control) or indicated concentrations of PCN or time intervals. Total proteins were harvested and the expression of sialyl-Lewis^x was analyzed using specific antibody by Western blot (A and B). Membranes used in Western blot analysis were stripped and probed with antibody against β -actin for loading control.

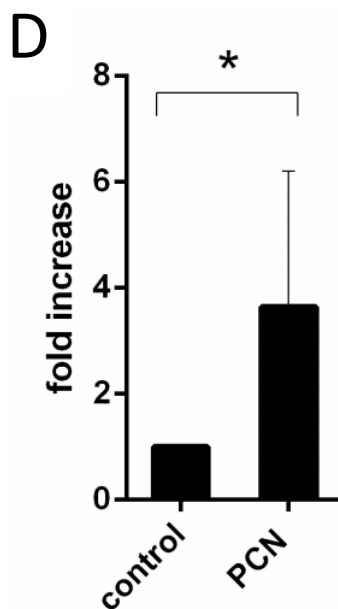
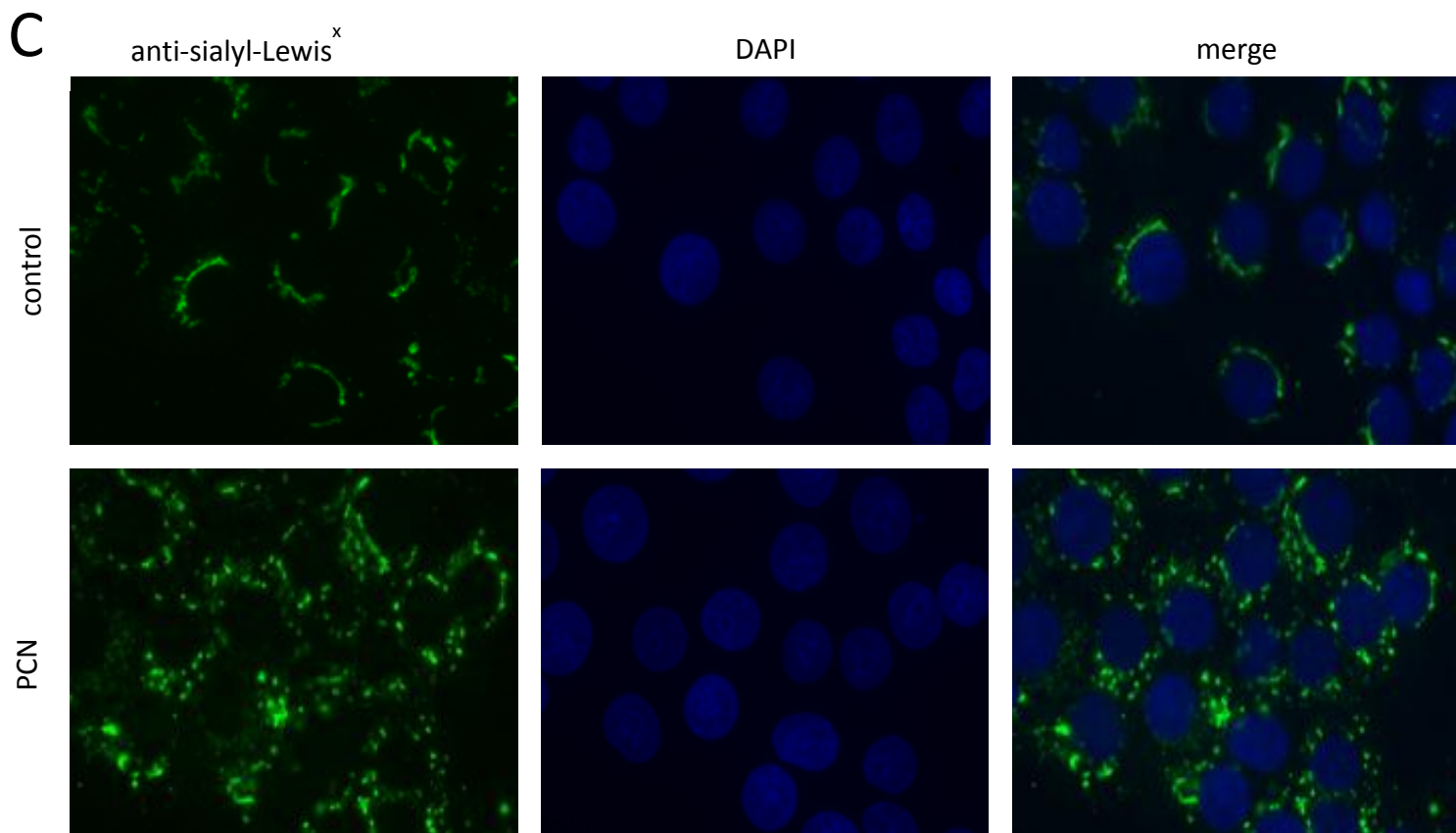


Fig. 3. (Cont) (C) H292 cells were treated with 5ug/mL PCN for 24 hours and then used for immunofluorescence microscopy with anti-sialyl-Lewis^x antibody. (D) Quantification of total fluorescence of control and PCN treated cells. Experiment was repeated 3 times with similar results. Analysis was accomplished by measuring total fluorescence in 10 representative high power fields from each treatment group in each individual experiment. * $p < 0.05$.

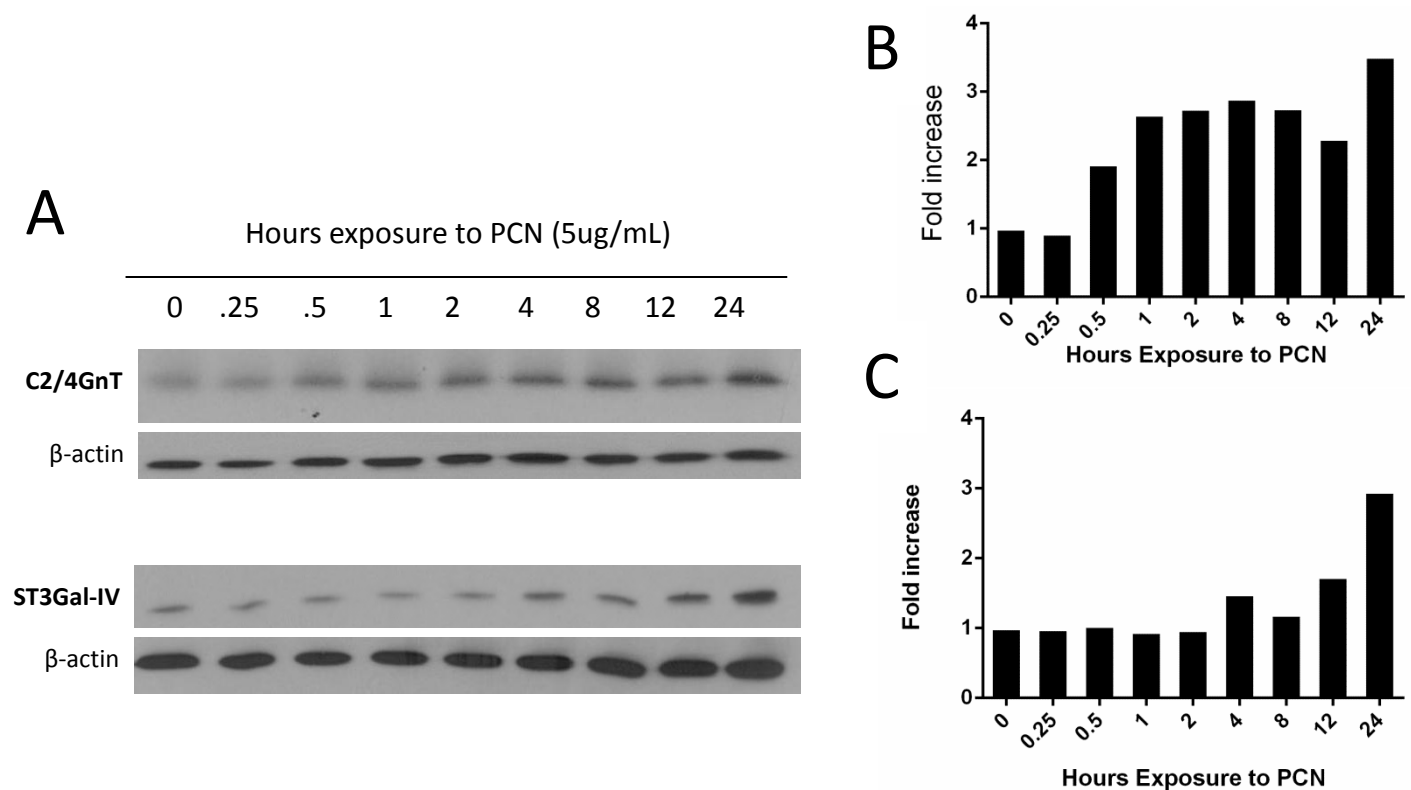


Fig. 4. PCN induces the expression of sialyl-Lewis^x biosynthetic enzymes. NCI-H292 cells were exposed to 5 μ g/mL PCN for the indicated time intervals. (A) The expression of C2/4GnT and ST3Gal-IV was analyzed by western blot. The membranes were stripped and probed with antibody against β -actin for loading controls. Experiments were repeated three times with similar results. Densitometry analysis of C2/4GnT (B) and ST3Gal-IV (C) from one of the representative western blots is shown.

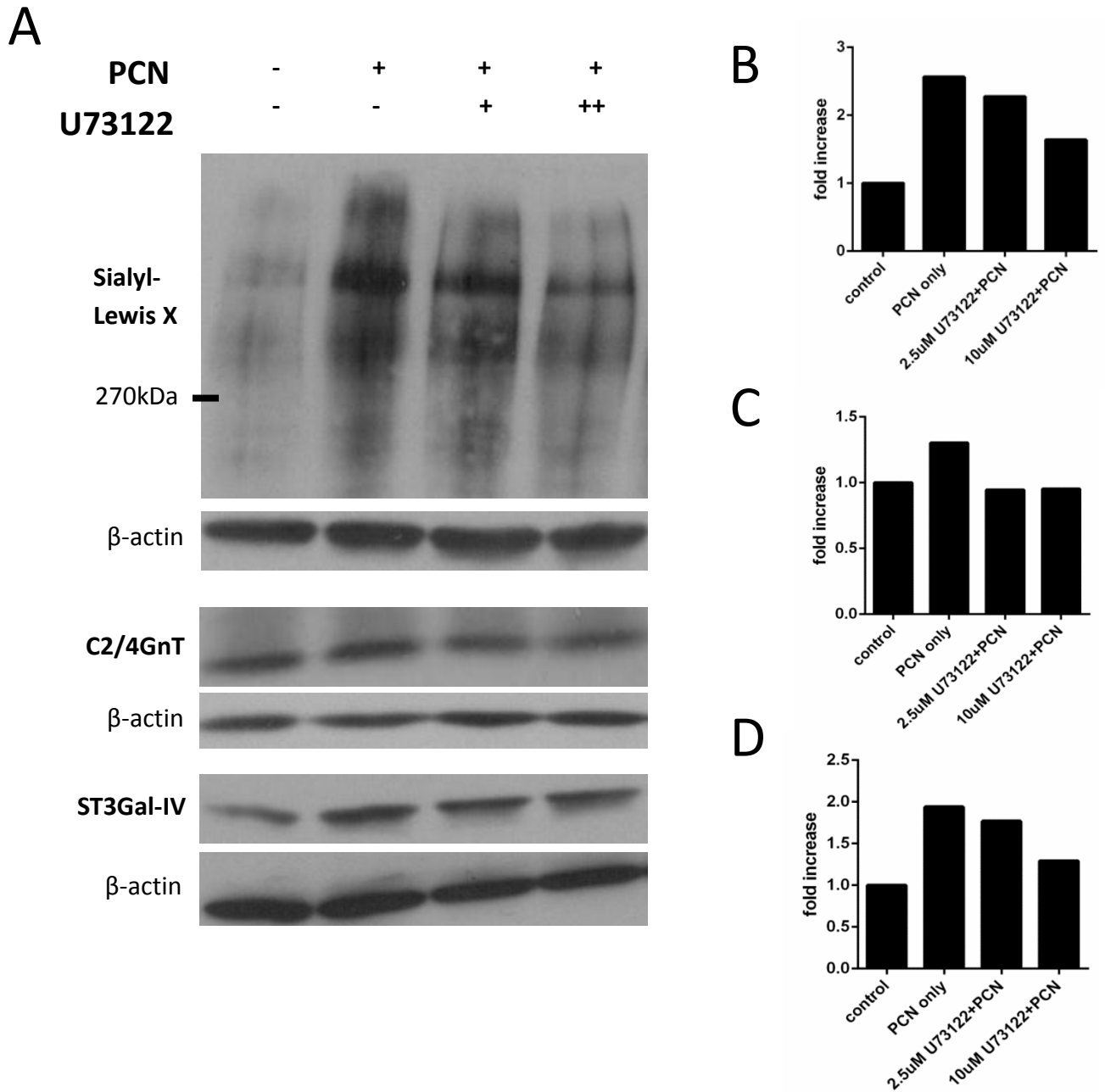


Fig. 5. PCN upregulates sialyl-Lewis^x through a phosphoinositol-specific phospholipase C (PI-PLC) pathway. NCI-H292 cells were exposed to PI-PLC inhibitor U73122 (2.5 or 10 μ M) for 40 minutes before the addition of sterile H₂O or PCN (5 μ g/mL) for 24 hr. Total cell lysates were used in Western blot analysis using antibodies against sialyl-Lewis^x, C2/4GnT or ST3Gal-IV (A). Membranes were stripped and probed with antibody against β -actin for loading control. Experiments were repeated three times with similar results. Densitometry data for sialyl-Lewis^x (B), C2/4GnT (C) and ST3Gal-IV (D) from one representative western blot is shown.

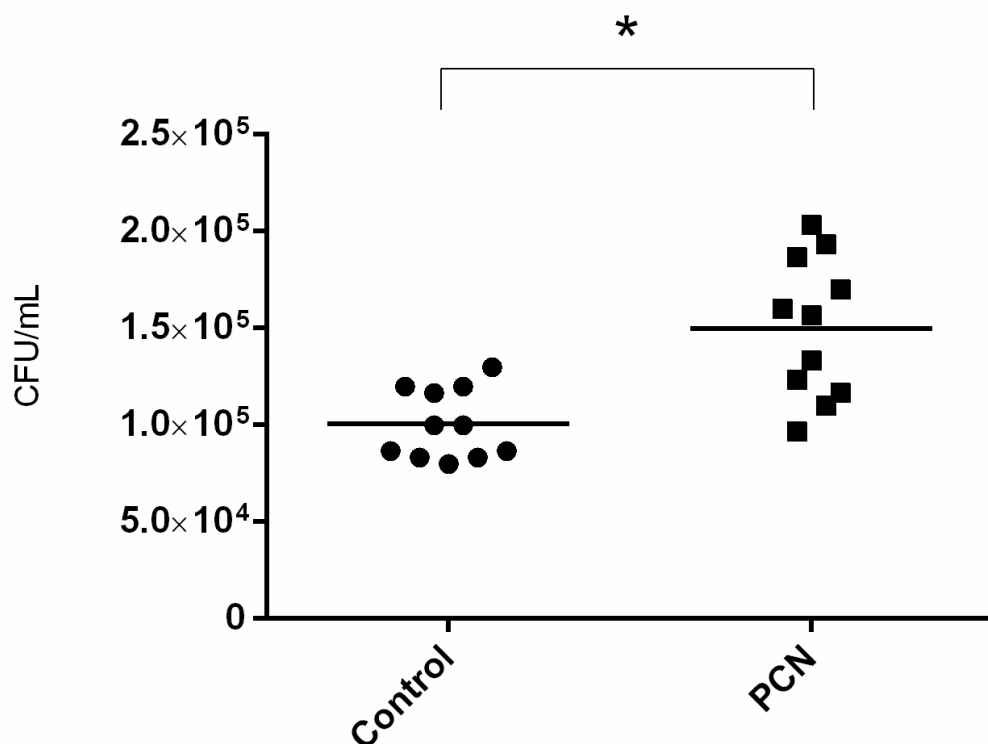


Fig. 6. Pyocyanin increases *P. aeruginosa* binding efficiency to NCI-H292 airway epithelial cells. PA strain PAO1 was added in a 1:1 ratio to wells (n = 11) of NCI-H292 cells treated with sterile H₂O or PCN (5. µg/ml) for 24 hr. After 60 min, cells were washed, and scraped off and plated on Pseudomonas isolation agar. Data represents colony forming units recovered from each group. **p* < 0.05.

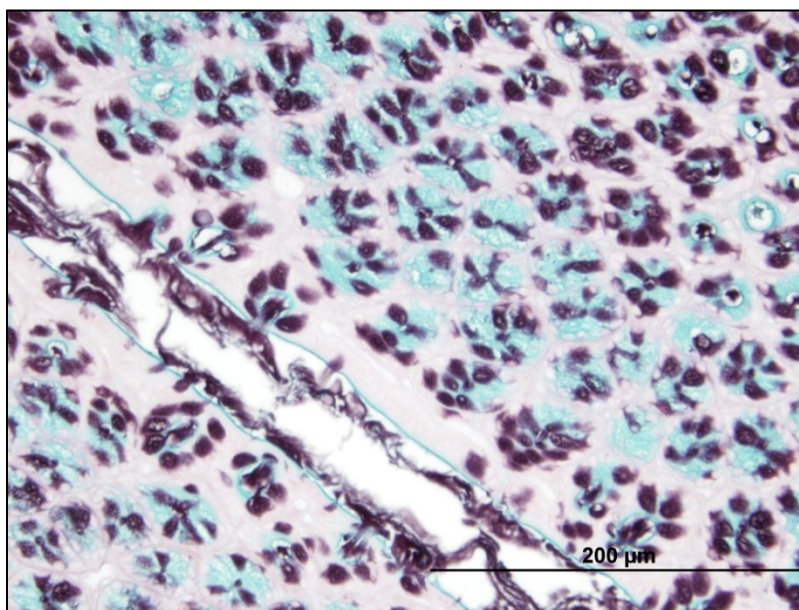


Fig. S1. Section of large colon used as a positive control tissue for the high iron diamine-alcian blue (HID-AB) staining.

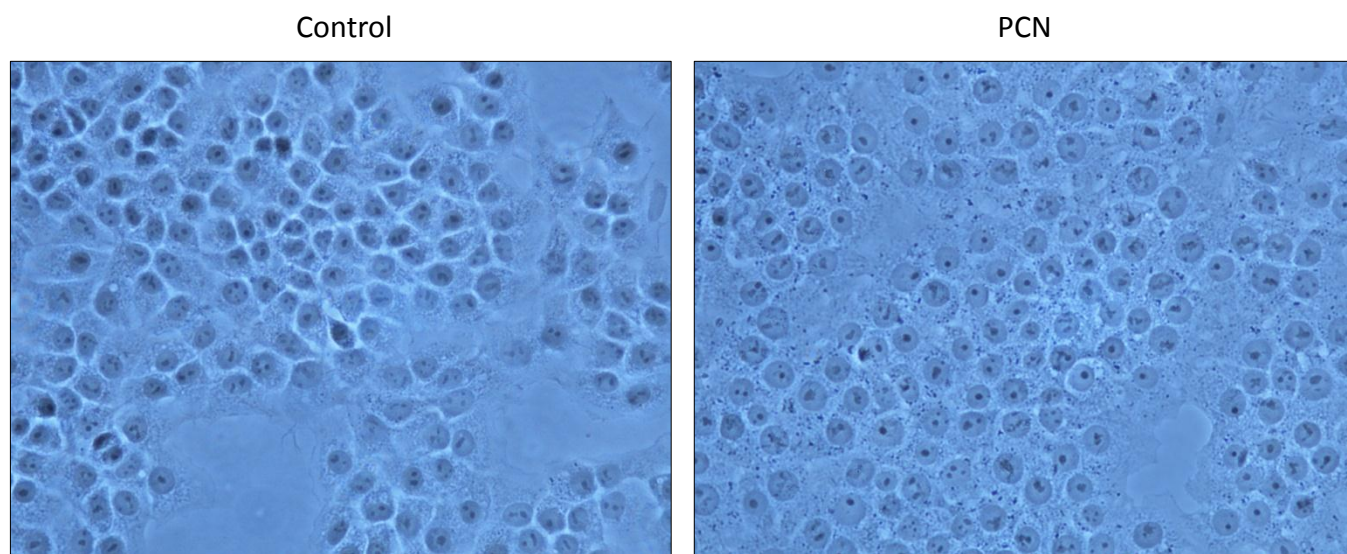


Fig. S2. Brightfield microscopy image of fixed NCI-H292 cells used for immunofluorescence assay to detect sialyl-Lewis^x.

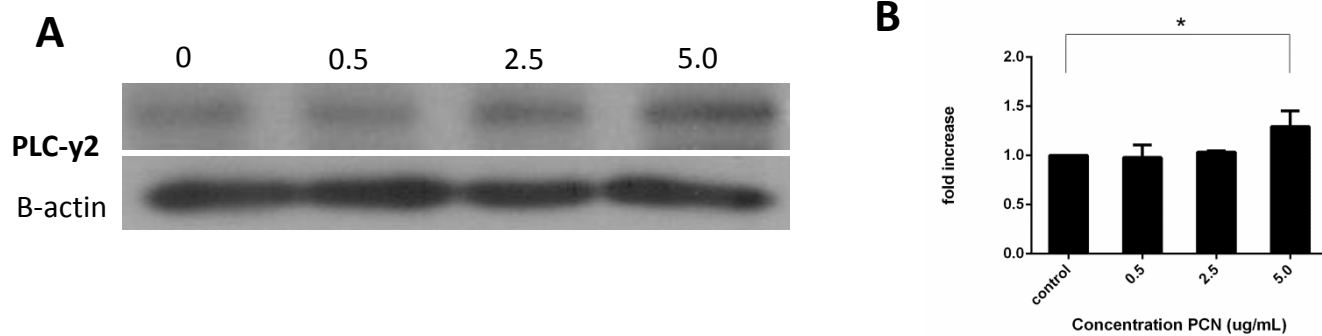


Fig. S3. A) Western blot of total protein from NCI-H292 cells treated with indicated concentrations of PCN, and probed with an antibody against PLC- γ 2. B) Densitometry analysis of A. *= $p < 0.05$

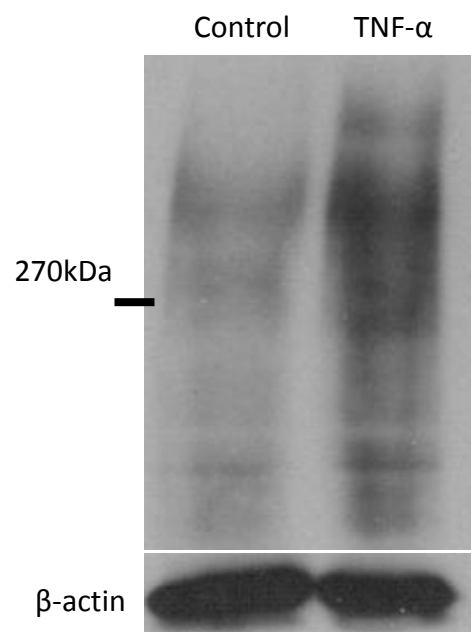


Fig. S4. H292 cells were treated with 20ng/mL TNF- α and extracted total protein was used in Western blot analysis with antibody against sialyl-Lewis^x. The same membrane was stripped and probed with antibody against β -actin for loading control.

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