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Reactive oxygen species mediate inflammatory cytokine release and EGFR-dependent mucin secretion in airway epithelial cells exposed to *Pseudomonas* pyocyanin

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Abstract

Despite the long-appreciated *in vivo* role of the redox-active virulence factor pyocyanin in *Pseudomonas* airway infections and the importance of airway epithelial cells in combating bacterial pathogens, little is known about pyocyanin's effect on airway epithelial cells. We find that exposure of bronchiolar epithelial cells to pyocyanin results in MUC2/MUC5AC induction and mucin secretion through release of inflammatory cytokines and growth factors (IL-1β, IL-6, HB-EGF, TGFα, TNFα) that activate the epidermal growth factor receptor pathway. These changes are all mediated by reactive oxygen species produced by pyocyanin. Microarray analysis identified 286 pyocyanin-induced genes in airway epithelial cells, including many of the inflammatory mediators elevated in cystic fibrosis (G-CSF, GM-CSF, CXCL1, SAA, IL-23) and several novel pyocyanin-responsive genes of potential importance in the infection process (IL-24, CXCL2, CXCL3, CCL20, CXCR4). This comprehensive study uncovers numerous details of pyocyanin's proinflammatory action and establishes airway epithelial cells as key responders to this microbial toxin.

Keywords

pyocyanin; cystic fibrosis; cytokines; mucin; *Pseudomonas*

Introduction

Pseudomonas aeruginosa is an opportunistic pathogen that rarely infects healthy individuals but causes infections in immunocompromised or mechanically ventilated patients or in

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patients with malignancies. *Pseudomonas aeruginosa* infects the lungs of patients with chronic obstructive pulmonary disease (COPD), chronic bronchiectasis and cystic fibrosis (CF)[1–3]. 80% of CF patients are colonized with *P. aeruginosa*.[4] The pathogenicity of *P. aeruginosa* is due to its wide range of virulence factors.[5] One of the major virulence factors is the redox-active toxin, pyocyanin.[6] Pyocyanin is crucial for full *in vivo* virulence of *Pseudomonas* in plants, *Caenorhabditis elegans* and *Drosophila melanogaster*.[7–9] Pyocyanin is essential for acute and chronic lung infections in mice.[10] Chronic administration of purified pyocyanin into mouse airways causes CF disease phenotype.[11] Hypervirulence of *P. aeruginosa* in humans has been associated with pyocyanin overproduction.[12] Most of the CF isolates of *P. aeruginosa* secrete high amounts of pyocyanin in culture[11, 13] and concentrations up to 129.4 μM have been detected in sputum of CF and bronchiectasis patients.[13]

Tracheobronchiolar epithelial cells (TBEC) play a crucial role in initiation of the host's immune response to bacteria by detecting the first colonizers and by attracting phagocytes through cytokine and chemokine release. Healthy airways can eradicate *P. aeruginosa*, but in the lungs of CF or bronchiectasis patients these mechanisms fail and *Pseudomonas* frequently establishes infection.[2, 14] TBECs play an important role in the host's failure to clear this opportunistic pathogen. In CF, reduced expression of the cystic fibrosis transmembrane conductance regulator (CFTR) anion transporter in the apical plasma membrane of TBECs leads to thicker mucus, lower airway surface liquid volume and higher osmolarity, which enable establishment of *Pseudomonas* infection.[3] In both acute and chronic respiratory infections *Pseudomonas* exists as biofilms[15], which are well-protected from immune defenses. *Pseudomonas*-infected airways are characterized as a dysregulated hyperinflammatory environment, with high concentrations of inflammatory cytokines, mucus overproduction and high numbers of infiltrated and activated neutrophils that significantly contribute to disease severity.[3] On one hand, this is due to the reported hyperinflammatory state of CF bronchiolar ECs[16] while, on the other, numerous late-phase *Pseudomonas* virulence factors have been implicated in contributing to maintenance of the hyperinflammatory conditions in CF airways.[3]

Epithelial cells are the first to be exposed to pyocyanin in *Pseudomonas*-infected airways. Pyocyanin is a secreted, membrane-permeable, heterocyclic compound that directly oxidizes intracellular pools of NAD(P)H and glutathione (GSH) and produces superoxide and downstream reactive oxygen species (ROS) that expose the host cell to oxidative stress.[17] The redox-active nature of pyocyanin is thought to be responsible for most of its effects described on host cells.[6, 17] Although numerous studies established the *in vivo* relevance of pyocyanin in *Pseudomonas* airway infections, only a handful described mechanisms by which pyocyanin affects airway epithelial cells: inhibition of ciliary beat frequency and catalase expression, inactivation of α1 protease inhibitor and release of IL-8.[17] In order to better understand the role of airway epithelial cells in pyocyanin toxicity, we explored details of pyocyanin's action on TBECs to capture acute responses to pyocyanin without any influencing effects of the neighboring tissues or infiltrating inflammatory cells.

Using pyocyanin at concentrations below average toxin levels detected in patients' airways[13] (< 10 μM), we found TBECs respond with inflammatory cytokine release,

activation of epidermal growth factor receptor (EGFR) signaling and mucin secretion. All these proinflammatory effects of pyocyanin on TBECs recapitulate many aspects of the CF lung disease phenotype and are due to intracellular reactive oxygen species production. The toxin-initiated oxidative stress in TBECs also triggers robust changes in transcription of several inflammatory mediators that were reported at elevated levels in CF lungs. We describe additional pyocyanin-inducible genes whose relevance in *Pseudomonas* airway infections has not yet been reported. All together, our work contributes significantly to understanding of the complex inflammatory environment in *Pseudomonas* respiratory infections by describing several novel effects of pyocyanin on the airway epithelium.

Results

Pyocyanin induces mucin synthesis in bronchiolar epithelial cells

Mucin production is a basic innate immune mechanism of the airways to remove entrapped bacteria. Mucus overproduction is a hallmark of chronic respiratory diseases including bronchiectasis and CF.[18] Several factors (environmental pollutants, bacteria, viruses) can trigger mucin production.[19] Although chronic administration of pyocyanin to mouse lungs over 12 weeks was shown to cause Goblet cell hyperplasia, no reports describe short-term effects of pyocyanin on mucin production in TBECs.[11] Treatment of epithelial cells with pyocyanin induced mucin synthesis detected by Periodic acid-Schiff (PAS) staining (Figure 1a). Quantitation of released mucins revealed a significant induction of mucin production by pyocyanin (1.4 mg/mL in pyocyanin-treated cells versus 0.5 mg/mL in untreated cells [Figure 1b]).

Pyocyanin induces MUC5AC and MUC2 in bronchiolar epithelial cells

Twenty mucin genes are known in the human genome, of which 12 are expressed in the airway epithelium; MUC5AC, MUC5B and MUC2 are the major components of the airway mucus.[18], [19] To determine which mucins are responsible for pyocyanin-induced mucin production, we measured gene expression changes of all 12 airway-expressed mucin genes following pyocyanin exposure. MUC5AC showed the highest induction (133.7-fold) and MUC2 scored second highest (31.4-fold), in contrast to only 2.6-fold induction of MUC5B (Figure 1c). Therefore, we focused primarily on MUC5AC and secondarily on MUC2 throughout this study. Quantitative real-time PCR (Figure 1d,e), qualitative RT-PCR (Figure 1c, gel pictures) and MUC5AC ELISA (Figure 1f) showed time- and dose-dependent induction of MUC5AC and MUC2 mRNA and proteins by pyocyanin in TBECs. Secretion of MUC5AC appeared only 24 hrs after pyocyanin addition (Figure 1f). Western blot analysis of concentrated supernatants of pyocyanin-induced cells revealed increased amounts of secreted MUC5AC (band above 500 kD) (Figure 1g). We examined other airway epithelial cells for pyocyanin-induced mucin production. BEAS-2B, IB3-1 and 16HBE14o- cells are non-mucoid epithelial cells and pyocyanin failed to induce MUC2 and MUC5AC transcription (Figure 1h). To exclude the possibility that contaminating bacterial components in the pyocyanin preparations induce mucins as well, we compared pyocyanin preparations from different *P. aeruginosa* strains and culturing conditions. The mucin-inducing effect of pyocyanin did not depend on the source of the toxin; 10 μM pyocyanin prepared from three different wild-type strains (PAO1, PA14, PA10145; 24 hr cultures)

induced MUC2 and MUC5AC in a source-independent manner (Figure 1i). Lipopolysaccharide (LPS), flagellum or pilus were not responsible for mucin induction since pyocyanin preparations from PAO1 strains lacking each of these virulence factors induced MUC2 and MUC5AC to the same extent as that from wild-type bacteria (Figure 1i). Equivalent extracts prepared from supernatants of pyocyanin-deficient strains (PA14 PhzM, PAO1 Phz1/2 or PA 15442; 24 hrs.) or from 4 hr cultures of the wild-type strains (when pyocyanin production is still absent) were without any effect (Figure 1i). These data confirm induction of MUC5AC and MUC2 by pyocyanin and not by other bacterial components.

Reactive oxygen species mediate mucin production induced by pyocyanin

Pyocyanin is a redox-active toxin causing oxidative stress in the host cell.[17] Superoxide anions are detected in TBECs exposed to pyocyanin (Figure 2a). Superoxide anions produced in the intracellular space can dismutate to hydrogen peroxide (H_2O_2), which can leak from cells. H_2O_2 is detected in both the intracellular and extracellular space of pyocyanin-exposed cells (Figure 2b). Since the H_2O_2 scavenger catalase does not enter the cell, intracellular H_2O_2 is resistant, whereas extracellular H_2O_2 is sensitive to catalase treatment (Figure 2b). Oxidative stress has been shown to trigger mucin production in TBECs.[20] To study ROS-dependence of pyocyanin-mediated mucin production, we treated epithelial cells with antioxidants (N-acetyl cysteine (NAC), glutathione (GSH), catalase (cat) and superoxide dismutase (SOD)) before addition of the toxin. Intracellular ROS scavengers NAC and GSH blocked MUC2 and MUC5AC induction, whereas extracellular antioxidants (cat+SOD) were without effect (Figure 2c). The oxidized form of GSH (GSSG), which has no antioxidant properties, had no effect either (Figure 2c). The same pattern of effects of these antioxidants occurs for MUC5AC release (Figure 2d). We conclude that intracellular, but not extracellular ROS, mediate pyocyanin-initiated MUC2 and MUC5AC induction.

Two members of the ROS-producing family of NADPH oxidases, Nox4 and Duox1, have been implicated in mucin synthesis in airway epithelial cells.[21, 22] None of the five NADPH oxidases detected were induced by pyocyanin (Figure 2e). Nox1, Duox1 and Duox2 transcript levels remained unchanged whereas Nox2 and Nox4 levels dropped with pyocyanin exposure (Figure 2e). Thus, induction of NADPH oxidases does not account for pyocyanin-induced ROS production.

Pyocyanin activates the epidermal growth factor receptor pathway

Mucin production can be triggered by interactions of bacteria with TBECs, as well as by different inflammatory/immune response mediators.[18] One of the major signaling pathways that mediate mucin production involves the epidermal growth factor receptor (EGFR).[23, 24] Since oxidative stress was shown to activate EGFR and mucin secretion through MEK1/2, we tested if a specific EGFR inhibitor (AG1478) or MEK1/2 inhibitors (U0126, PD98059) affect pyocyanin-mediated mucin induction.[21, 25] These inhibitors blocked induction of MUC2 and MUC5AC (Figure 3a,b), whereas a platelet-derived growth factor receptor inhibitor (AG1295) or an inactive analogue of AG1295 and AG1478 (AG9) had no effect (Figure 3a,b), suggesting that pyocyanin induces mucin secretion entirely through EGFR.

We tested if pyocyanin promotes proliferation of TBECs, since EGFR signalling is known to induce epithelial cell division.[26] EdU labeling revealed higher numbers of actively dividing epithelial cells following pyocyanin treatment relative to untreated cultures (Figure 3c, e), and total cell numbers were also elevated (Figure 3c, d). Extracellular-signal-regulated kinase (ERK) is a well-characterized kinase down-stream from EGFR.[26] We detected strong ERK phosphorylation 24 hrs post-exposure, confirming the activation of EGFR-MEK1/2-ERK pathway in pyocyanin-treated TBECs (Figure 3f).

Activation of EGFR can be ligand-dependent or –independent.[26] A variety of ligands can bind and activate EGFR, whereas reactive oxygen species can cause ligand-independent EGFR activation.[26] To see if pyocyanin-mediated MUC2/MUC5AC induction through EGFR requires *de novo* protein synthesis of potential EGFR ligands, we treated epithelial cells with the protein synthesis inhibitor cycloheximide (CHXM). CHXM completely inhibited MUC2 and MUC5AC induction by pyocyanin suggesting EGFR is not directly activated by oxidative stress but may act through synthesis of EGFR ligands (Figure 3a).

Pyocyanin induces transcription of inflammatory cytokines and EGFR ligands

Several inflammatory cytokines (Interleukin (IL)-1α, -1β, -4, -5, -6, -9, -13, -17, TNFα) and growth factors (EGF, HB-EGF, TGFα) are known to trigger MUC2 or MUC5AC production in airway epithelial cells.[19] EGF family ligands are important growth factors initiating growth and mucociliary differentiation of airway epithelial cells: amphiregulin- and heparin-bound EGF (HB-EGF), epidermal growth factor (EGF), tissue growth factor-alpha (TGF-α), betacellulin, epigen, epiregulin and neuregulins.[19] All EGF family members are synthesized as transmembrane protein proligands present on the cell surface and are cleaved by surface proteases leading to release of the active ligand.[27] A majority of the proteases responsible for this process called ectodomain shedding belong to the ADAM family of metalloproteases.[27] ADAM17 (TACE) has been suggested as the major ADAM mediating MUC5AC production in TBECs.[28] EGF ligands bind to ERBB family receptors (EGFR, ERBB2, ERBB3, ERBB4) and activate downstream signaling.[26]

Many of these genes associated with MUC5AC induction in airway epithelial cells demonstrated pyocyanin-induced transcriptional changes on human whole-genome microarrays (Figure 4a). Five cytokines (IL-1α, IL-1β, IL-6, IL-8, TNF-α) and two EGFR ligands (TGFα, HB-EGF) were strongly up-regulated by pyocyanin (Figure 4a). Induction of IL-8 by pyocyanin in TBECs was already shown[29]. We also detected 39-fold increase in IL-8 mRNA levels upon pyocyanin exposure by real-time PCR. Increased expression of IL-1α (23-fold), IL-1β (12-fold), IL-6 (391-fold), TNFα (26-fold), TGFα (4-fold) and HB-EGF (30-fold) was confirmed by quantitative real-time PCR (Figure 4b, c). Significantly enhanced amounts of IL-1β, TGFα, TNFα and IL-6 were detected by ELISA in the supernatant of pyocyanin-treated cells compared to untreated cells (Figure 4d). Pyocyanin resulted in a 5.2-fold increase in IL-6 secretion of TBECs (Figure 4d). Pyocyanin-mediated induction of IL-1α, IL-1β, IL-6, TNF-α, TGF-α and HB-EGF in TBECs has not been reported to date.

On the other hand, ADAM metalloproteases, MMP9, ERBB receptors, IL-17 family cytokines or MEK1 were not induced by pyocyanin (Figure 4a, S2). Th2 cytokines (IL-4,

_5, _9,_13) do not mediate the effect of pyocyanin (Figure 4a). Neither increased expression (Figure 4a) nor activation of STAT6 (Figure S1), the major transcription factor in IL-4/13 signaling, was elicited by pyocyanin. Pyocyanin failed to induce transcription and release of the major EGFR ligand, EGF (Figure S2, 4d). Thus, we identified IL-1α, IL-1β, IL-4, IL-6, IL-8, TNFα, TGFα and HB-EGF as potential candidates for mediating pyocyanin-triggered MUC5AC release.

IL-1β, TGFα and TNFα mediate pyocyanin-induced MUC5AC release

We found that inflammatory mediators such as TGFα, TNFα and IL-1β were strong inducers of MUC5AC release, whereas the response to IL-6 was weak (Figure 4e). ATP, which has been shown to trigger mucin production in TBECs[19], produced weak MUC5AC release (Figure 4e). Although induced in TBECs exposed to pyocyanin, IL-1α, IL-4, and IL-8 do not initiate mucin secretion and thus probably do not mediate the toxin's effects (Figure 4e). In contrast, EGF is an excellent stimulus of mucin production in these cells (Figure 4e), although it is not released upon exposure to pyocyanin (Figure 4a, d) and does not appear to participate in pyocyanin-mediated mucin production. MUC5AC induction by IL-1β, TGFα, TNFα or IL-6 was completely blocked by the EGFR inhibitor (AG1478), but not by the control inhibitor analogue (AG9) (Figure 4e). Thus, IL-1β, IL-6, TGFα and TNFα appear to signal upstream of EGFR to induce release of MUC5AC.

We used neutralizing antibodies against IL-1 β, IL-6, TGFα and TNFα to confirm their involvement in the process. All of these antibodies exhibited inhibitory effects on pyocyanin-induced MUC5AC release, although inhibition by anti-IL-6 did not reach significant levels (Figure 4f). Elimination of extracellular ATP by apyrase had no effect (Figure 4f), consistent with its low potency in inducing mucin release (Figure 4e). Since TNFα was shown to increase EGFR expression, we tested whether TNFα potentiates pyocyanin-induced MUC5AC release. Concentrations of TNFα found in supernatants of pyocyanin-treated epithelial cells (< 0.5 ng/mL, Figure 4d) increased both EGFR expression and pyocyanin-induced MUC5AC release (Figure 4g). These results support a mechanism in which pyocyanin acts through multiple autocrine/paracrine signals (IL-1β, TGFα, TNFα) that activate EGFR, leading to mucin secretion in TBECs.

Gene expression changes in response to pyocyanin

Comparison of genome-wide expression profiles in pyocyanin-exposed and unexposed cells revealed robust changes (Figure S3). Conservative thresholds of 4-fold change and p-value < 10^{-3} yielded 268 genes (unique Entrez IDs) modified by pyocyanin (Figure S3), including some previously reported genes mediating mucin synthesis (pyocyanin up-regulated genes, Table S1; down-regulated genes, Table S2). Gene annotation-based clustering of pyocyanin-responsive genes revealed 11 clusters containing at least five genes (Figure 5). One major group of biological functions (clusters 6, 7, 8, 11) were related to cell migration and responses to stimuli (inflammatory/wound response, chemotaxis, cell migration, wound healing) (Table 1 and Table S3). Other groups (clusters 1 and 2) contain genes involved in cell proliferation and positive regulation of gene expression. A third group of functions were associated with apoptosis (clusters 3, 9, 10). Cluster 8 subsumes many of the functions involved in stress responses, including responses to oxidative stress and reactive oxygen

species (Table S3). Ingenuity Pathway Analysis (IPA v.7.6) identified and ranked signaling networks with the highest statistical associations among pyocyanin-responsive genes. Network #1 was identified as genes associated with TNF–α (Figure S4), while the second highest involves genes associated with NF-κB (Figure S5). These data uncover strong transcriptional relationships in airway epithelial cells in response to pyocyanin, many of which can be primary or secondary consequences of exposure to reactive oxygen species.

Pyocyanin induces epithelial transcription of inflammatory mediators reported at elevated levels in CF patients

To gain insight into the relevance of pyocyanin's effects on TBECs in contributing to the inflammatory conditions of *Pseudomonas*-infected airways, we asked whether pyocyanin-induced transcriptional changes detected by microarray are consistent with inflammatory mediators known to be elevated in CF patients. Correlations with genes up-regulated in CF would suggest a novel mechanism in which pyocyanin-induced epithelial release would account for their gross increased levels in CF patients. From a survey of medical literature (reports strictly on human subjects, no animal model or cell culture studies) we assembled a list of 24 inflammatory mediators elevated in CF patients (sputum, serum or bronchoalveolar lavage fluid [BAL]) (Table S4). We subdivided these genes into two groups based on the focus of the study: (1) Four papers compared cytokine and chemokine levels between CF patients with or without *P. aeruginosa* infection (Table S4, List A). These reports showed 10 inflammatory mediators at significantly elevated levels in CF patients due to *Pseudomonas* infection: chemokine (C-C motif) ligand 17 (CCL17), chemokine (C-X-C motif) ligand 11 (CXCL11), IL-1β, IL-4, IL-6, IL-8, IL-13, granulocyte colony stimulating factor (G-CSF, CSF3), LTB4 and TNF-α. (2) The second group reported additional CF-associated inflammatory mediators whose correlation with *Pseudomonas* infection was not specified (Table S4, List B). This list comprised 14 genes detected at increased levels in CF patients: Chemokine (C-X-C motif) ligand 1 (CXCL1), IL-23, Serum-amyloid (SAA) 1 and 2, S100A8, S100A9, S100A12, IL-1 receptor antagonist (IL-1RN), granulocyte-monocyte colony stimulating factor (GM-CSF, CSF2), IL-17F, C-reactive protein, MCP-1 and MIP-1β. Our microarray data confirmed by real-time PCR shows that 6 of 10 PA-associated inflammatory mediators (G-CSF, IL-6, IL-1β, IL-8, TNFα, LTB4) and 6 of 14 additional CF-associated genes (GM-CSF, CXCL1, IL-23, IL-1Ra, SAA1, SAA2) were up-regulated in TBECs by pyocyanin (Figure 6a–c). This suggests that pyocyanin's effect on bronchiolar epithelial cells can be a major contributor to the initiation and maintainance of the hyperinflammatory state in CF airways.

Pyocyanin induces epithelial transcription of other genes involved in innate immunity and response to oxidative stress

Among the pyocyanin-upregulated genes we identified several interleukins and chemokines known to play important roles in the innate or adaptive immune system (Table 2). IL-11, -19 and -20 showed modest changes in transcription, whereas IL-24 showed robust up-regulation (Table 2). Beyond interleukins, pyocyanin strongly induced transcription of one IL-receptor (Interleukin 1 receptor, type 2 (IL1R2)), three chemokines (CXCL2, CXCL3, CCL20) and one chemokine receptor (CXCR4) (Table 2).

Pyocyanin imposes oxidative stress on cells by generating intracellular ROS. We identified 10 genes related to oxidative stress induced by pyocyanin (Table 2). Real-time PCR confirmed induction of mitochondrial superoxide dismutase (SOD2) by pyocyanin (8.5-fold) whereas transcript levels of SOD1 (1.01+/−0.23 fold, n=3) and SOD3 (0.82+/− 0.20, n=3) remained unchanged. Catalase activity was reported to be inhibited by pyocyanin.[30] We found slightly decreased catalase transcript levels upon pyocyanin exposure (0.63+/−0.21 fold, n=3). Growth differentiation factor-15 (GDF-15), a stress-induced cytokine, was strongly up-regulated by pyocyanin (fold increases: microarray, 70.0 fold; real-time PCR, 256.1 fold (n=3)).

Discussion

Obstruction of the airways due to mucus overproduction is characteristic of several chronic respiratory diseases (CF, asthma, COPD).[18] In addition to increased mucus production in CF respiratory epithelial cells, *P. aeruginosa* also elicits mucus secretion.[18] *Pseudomonas* culture supernatants, LPS and homoserine-lactone have been shown to induce MUC5AC or MUC2 in airway epithelial cells.[28], [31], [32] We show for the first time that TBECs respond to pyocyanin with acute mucin secretion (MUC5AC release and MUC2 induction). We hypothesize that this mechanism contributes to mucus overproduction seen in *Pseudomonas*-infected airways (Figure 7). We found that intracellular production of ROS is responsible for pyocyanin-induced mucin secretion. Although oxidative stress of different origins (bolus H_2O_2, Nox4, Duox1) was already associated with MUC5AC production, our observations are the first describing ROS-initiated transcription of MUC2.[21], [22], [33]

Several different surface stimuli converge by signaling through EGFR and activate common innate immune mechanisms. [24], [26], [34] We show evidence that MUC2 and MUC5AC induction by pyocyanin occurs entirely through activation of the EGFR signaling pathway (EGFR-MEK1/2-ERK) and that pyocyanin also promotes epithelial cell proliferation via EGFR (Figure 7). Pyocyanin was shown recently to induce Goblet cell hyperplasia in mouse lungs *in vivo*.[11] A two-step process was proposed as the mechanism for *in vivo* trans-differentiation of Goblet cells from ciliated epithelial cells.[23] First, activation of EGFR signaling was suggested to promote cell proliferation in ciliated airway epithelial cells, allowing the second, subsequent initiation of Goblet cell differentiation.[23] Our data support this model by showing a prominent role of ligand-activated (autocrine) EGFR signaling in response to pyocyanin.

Inhibiting *de novo* protein synthesis blocked pyocyanin-induced transcription of MUC2 and MUC5AC, suggesting EGFR is activated predominantly in a ligand-dependent manner, although we cannot rule out concurrent ligand-independent activation, such as ROS-mediated inhibition of intracellular phosphatases (Figure 7.).[26] Such ligand-independent EGFR activation could involve excess intracellular ROS produced by pyocyanin, since we observed inhibitory effects of intracellular antioxidants on mucin induction and secretion (Figure 2c, d). We identified several secreted cytokines and EGFR ligands that mediate pyocyanin-induced MUC5AC release: IL-1β, IL-6, TGF-α, TNF-α and HB-EGF. These inflammatory mediators are known to trigger MUC5AC production in TBECs, and we found they were released upon exposure to pyocyanin.[18] To our knowledge, this in the first

demonstration that MUC5AC release induced by IL-1β or IL-6 is EGFR-dependent. The exact mechanism is unknown but transactivation of EGFR by IL-1β or IL-6 is a possibility, since EGFR transactivation by various stimuli was already reported.[24], [33], [35] Our work describes for the first time increased transcription of two EGFR ligands, TGF-α and HB-EGF, by pyocyanin. TGFα shedding by ADAM17 was previously shown to be stimulated by ROS.[22] Pyocyanin-induced increases in MUC2 transcription could be mediated by TNF-α, IL-1β, TGF-α and HB-EGF, since these stimuli are known inducers of MUC2 transcription and we showed they are released from airway cells by pyocyanin.[35–38] Thus, we propose that a complex network of autocrine signals mediate pyocyanin-induced mucin production in TBECs (Figure 7).

Pyocyanin has been suggested to have proinflammatory effects on airway epithelial cells but the underlying mechanisms are poorly understood.[6] Previously, airway epithelial cells were shown to produce IL-8 in response to pyocyanin.[29] Beyond confirming increased IL-8 expression, we detected enhanced production of additional inflammatory cytokines: IL-1β, IL-6 and TNF-α, all of which were detected at elevated levels in CF patients and were associated with *Pseudomonas* infection (Figure 6a). They are induced by oxidative stress, their down-stream signaling involves ROS, and they are regulated by the transcription factor NF-κB.[39], [40] Pyocyanin-responsive genes were significantly associated with NF-κB (network #2, Figure S5), suggesting ROS-dependent activation of NF-κB is important in the release of these cytokines.

TNFα is a multifunctional proinflammatory cytokine. Increased concentrations of TNFα have been reported in CF BAL, and TNFα levels in CF sputum are negatively correlated with pulmonary function (Table S4). IPA analysis of our microarray findings identified TNFα-associated genes as the most statistically significant network among pyocyanin-responsive genes (Figure S4). Pyocyanin-induced epithelial TNFα release can induce several secondary genes (including MUC5AC, MUC2, GM-CSF). TNFα is a potent neutrophil priming and activating agent.[24] Pyocyanin-induced epithelial release of IL-1β is important in mediating mucus overproduction and can contribute to initiation of inflammatory responses. Upon exposure to pyocyanin, airway epithelial cells experienced more than a 5-fold increase in IL-6 secretion. IL-6 has both pro- and anti-inflammatory properties.[39] Increased IL-6 release by pyocyanin could contribute to the acute phase reaction and mucus overproduction but at the same time it could limit the extent of the inflammatory response by inducing the transcription of the natural IL-1 inhibitor, IL-1R antagonist (IL-1Ra) and by reducing IL-1β production.[39] Indeed, increase in IL-6 transcript levels is delayed compared to that of IL-1β mRNA levels (Figure 4b) and IL-1R antagonist transcript (IL1RN) levels are also enhanced (Figure 6b, c).

Pseudomonas-infected CF patients have higher serum G-CSF levels than CF patients without *Pseudomonas* infection (Table S4). Airway epithelial cells produce G-CSF and GM-CSF when exposed to *P. aeruginosa*.[41] We show for the first time that pyocyanin induces robust increases in G-CSF and in GM-CSF transcription in TBECs. G-CSF could contribute significantly to CF disease severity since it is a potent neutrophil chemoattractant (Figure 7), promotes neutrophil survival by inhibiting apoptosis and increases neutrophil release from

the bone marrow.[42] GM-CSF is important in the pulmonary immune system, as it also promotes neutrophil maturation, activation and survival.[41, 43]

Leukotriene B4 (LTB4) is a potent neutrophil chemoattractant seen at elevated levels in CF patients' sputum, saliva and BAL (Table S4). LTB4 is generated from arachidonic acid by the enzyme lipooxygenase. TBECs are capable of synthesizing LTB4.[44] Our microarray findings detected increased expression of ALOX5, the gene coding lipooxygenase, in pyocyanin-exposed TBECs (Figure 6a). This suggests that increased epithelial LTB4 production by pyocyanin could contribute to neutrophil recruitment in *Pseudomonas*-infected airways.

We found several chemokines (CXCL1, CXCL2, CXCL3, CXCL8, CCL20) that were induced by pyocyanin in epithelial cells (Figure 6b, Table 2). CXCL1 expression was increased the most. Increased CXCL1 levels have been associated with CF (Table S4). The CXC-chemokines (CXCL1-3 and CXCL8 (IL-8)) attract neutrophils; their increased epithelial expression could contribute to neutrophil infiltration in CF airways. CCL20 is strongly chemotactic for lymphocytes, weakly for neutrophils.[45] CXCR4 and CCL20 expression were reported in human airway epithelial cells; their increase by pyocyanin could play a role in enhanced lymphocyte recruitment (Figure 7).[45]

Pyocyanin also boosted transcription of the acute phase proteins, serum amyloid 1 and 2 (SAA) (Figure 6b). Increased levels of SAAs were detected in CF patients' serum (Table S4). SAA release was detected in airway epithelial cells.[46] Pyocyanin-induced epithelial SAA transcription could be mediated by IL-1β, IL-6 or TNFα since these proinflammatory mediators are known to induce SAA secretion in the liver.[46]

Table 2 presents four interleukins (IL-11, 19, 20 and 24) found to be up-regulated by pyocyanin in TBECs that have not been previously associated with CF and their function in *Pseudomonas* respiratory infections has not been elucidated. The robust induction of IL-24 is especially remarkable. Pyocyanin-induced expression of IL-23 in TBECs might be important since IL-23 has been associated with CF (Figure 6b) and was shown to mediate inflammatory responses in mouse lung infected with *P. aeruginosa*.[47] The lower part of Table 2 lists pyocyanin-modified genes associated with oxidative stress, consistent with its well-known pro-oxidant effects. Three genes (CRYAB, SCARA3, SNCA) were down-regulated whereas seven (DHRS2, DDIT3, DUSP1, HMOX1, SOD2, S100A7 and PTGS2) were up-regulated (Table 2).

Pseudomonas aeruginosa requires pyocyanin for full virulence in a variety of infection models. The majority of *Pseudomonas* strains infecting human airways secrete this toxin. Despite its *in vivo* significance, little is known about how this toxin affects airway epithelial cells. Our work uncovered numerous novel aspects of pyocyanin's proinflammatory action on the respiratory epithelium, as many of the characteristic features of CF lung disease (mucus overproduction, proinflammatory cytokine release and induction of inflammatory mediators involved in leukocyte recruitment) could be reproduced by exposing bronchiolar epithelial cells to pyocyanin. Our data consolidates the crucial role of airway epithelial cells in initiation and maintenance of the airway's immune and inflammatory response to

Pseudomonas aeruginosa and emphasizes the importance of the complex consequences of oxidative stress caused by this remarkable bacterial toxin.

Materials and Methods

Airway epithelial cells

The human mucoid bronchial epithelial cell line, H292, was purchased from ATCC. Cells were grown in RPMI-1640 medium (Invitrogen) containing 10% FBS (Hyclone), 1% penicillin-streptomycin, 1% L-Glutamine, 1% sodium-pyruvate and 1% HEPES (Invitrogen). The immortalized human bronchiolar epithelial cells, 16HBE14o- were obtained from Dr. Gruenert.[48] Cells were cultured on 6-well tissue culture plates coated with fibronectin coating solution (1% BSA, 0.03 mg/mL bovine collagen and 0.01 mg/mL human fibronectin) in MEM medium containing L-Glutamine, glucose, NaHCO3, 10% FBS and 1% penicillin-streptomycin (Invitrogen). IB3-1 cells and BEAS-2B cells were purchased from ATCC. IB3-1 cells were maintained in MEM medium containing L-Glutamine, glucose, 10% FBS and 1% penicillin-streptomycin (Invitrogen). BEAS-2B cells were cultured in LHC-8 medium containing L-Glutamine, 10% FBS and 1% penicillin-streptomycin (Invitrogen).

Measurement of reactive oxygen species production

Attached H292 cells were incubated by different concentrations of pyocyanin (0–70 μM) for 2 hrs. Intracellular superoxide production was measured by fluorescence of oxidized form of dihydroethidine (DHE, 1 μM; Invitrogen; exc: 485 nm, emission: 590 nm) using a fluorescent microscope (Leica). Intracellular hydrogen peroxide production was followed by DCFDA oxidation (20 μM; Invitrogen, excitation: 485 nm, emission: 535 nm) in a Fluoroskan plate reader. Extracellular hydrogen peroxide was followed by measuring resorufin (exc: 530 nm, emission: 590 nm) formation from Amplex Red by horseradish peroxidase (20 U/mL, HRP) in a Fluoroskan plate reader.

Sample preparation for microarrays

H292 cells were treated with or without 8 μM pyocyanin for 48 hrs in serum-free RPMI medium. RNA was isolated as described (Suppl. Methods) and an additional clean-up step was introduced using Qiagen RNeasy RNA isolation kit. The prepared RNAs were run on an RNA gel to ascertain the quality of the preparations. RNA was isolated in four independent experiments, collected and subjected to microarray analysis simultaneously.

Statistical analysis of microarray data

Signal intensity estimates ("medianSignal") from Feature Extraction were converted to $\log_2$ scale. Two-channel data within each array was LOESS normalized[s24] and median normalized between arrays. Log_2 ratios and p-values were generated for the contrast between pyocyanin-treated and untreated samples via a mixed-effects model ANOVA with arrays as a random effect. The significance level was calculated by FDR[s23] at 0.05 which equated to a raw p-value of $10^{-1.80}$. Thresholds for probes used in further analysis were selected at |log2 ratio| > 2 (four-fold difference in gene expression) and a stringent p-value of $< 10^{-3}$ (see Figure S4). This set comprises 389 probes representing 302 unique transcripts

and 268 unique Entrez gene identifiers. JMP-Genomics v4.1 software was used for data processing and statistical analysis. The microarray data have been deposited in NCBI's Gene Expression Omnibus and are accessible through GEO Series accession number GSE22430 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE22430).

Functional annotation cluster analysis

268 Entrez gene identifiers were submitted to the Functional Annotation Clustering Tool at the DAVID website (http://david.abcc.ncifcrf.gov:8080/).[s25, s26] In our analysis, DAVID served to initially assign functional annotation from the "GOTERM_BP_FAT" category to each gene, and to identify statistically significant associations to sets of genes both at the level of individual functional terms and for clusters of functions. The analysis was run under default options, and the resulting output was filtered for association of genes with GOTERM_BP_FAT terms at a raw p-value < 0.01. DAVID assigned 131 genes to 130 functional terms under these conditions. In order to organize and visualize clusters of related genes and functions, a binary matrix encoding the association of each gene with each GO FAT term was subjected to a two-way hierarchical clustering within JMP-software; this process simultaneously groups genes according to similarity of functional profiles and groups functions by similarity of gene membership (Figure 5).

Data analysis (other than microarray studies)

Data are expressed as mean +/− SEM. Significance was assessed using Student's t-test when independent variables were studied; by ANOVA and *post-hoc* Dunnett's test when results of trends were compared. Significant changes were marked as * when $p < 0.05$, ** when $p < 0.01$ and *** when $p < 0.001$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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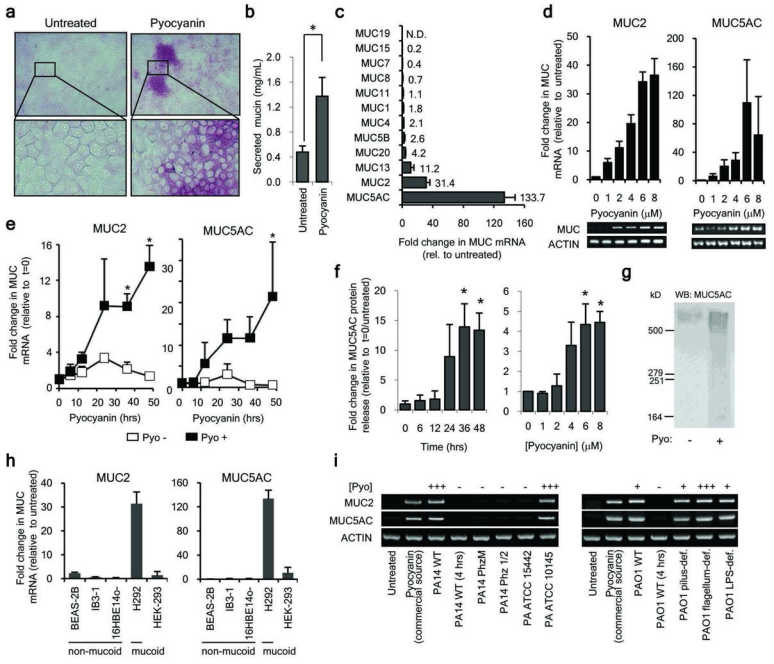

Figure 1. Pyocyanin induces mucin synthesis in bronchiolar epithelial cells

(**a**) Pyocyanin (10 μM, 2 days) induces mucin production in bronchiolar epithelial cells as detected by Periodic acid-Schiff staining (one representative result shown, n=3). (**b**) Quantitation of released mucins by dot-blot assay reveals significant increase in mucin production in cells exposed to pyocyanin (mean+/−S.E.M., n=3). (**c**) A survey of mucin transcripts expressed in the human airway epithelium identified MUC5AC and MUC2 as the two major mucins induced by pyocyanin (mean+/− S.E.M., n=4, qPCR). Pyocyanin-triggered induction of MUC5AC and MUC2 gene expression is dose- (**d**) and time-dependent (**e**) (mean+/− S.E.M. of at least three independent experiments). Primers used: graphs (qPCR): MUC2-2, MUC5AC-2; gels (RT-PCR): MUC2-1, MUC5AC-1 (Table S5). (**f**) Dose- and time-dependent release of MUC5AC protein in bronchiolar epithelial cells treated with pyocyanin (ELISA, mean +/−S.E.M., n=3; dose-dependence at 2 days; time-dependence with 10 μM Pyo). (**g**) Western blotting detects increased MUC5AC protein in supernatant of pyocyanin-exposed cells (another experiment gave similar results). (**h**) Only mucoid airway epithelial cells respond to pyocyanin by MUC5AC and MUC2 induction (qPCR, mean +/− S.E.M., n=4, primers: MUC2-2, MUC5AC-2). (**i**) MUC2 and MUC5AC induction in cells treated with pyocyanin (8 μM, 2d) extracted from 24 hr-old *Pseudomonas aeruginosa* culture supernatants of different wild-type strains (PA14 WT, PA ATCC10145, PAO1 WT). Pyocyanin prepared from pilus-, flagellum- and LPS-deficient strains of PAO1 also triggered MUC5AC and MUC2 induction. Equivalent extracts from pyocyanin-free supernatants of either short-term cultures of wild-type strains (PAO1 WT (4hrs), PA14 WT (4hrs)) or 24 hr-old cultures of pyocyanin-deficient strains (PA14 PhzM, PA14 Phz1/2, PAATCC 15442) failed to induce mucin expression. The +/− signs denote pyocyanin concentrations detected in each culture supernatant (+++ = 20–100 μM; + = 5–20 μM; − = none), (WT = wild type). Primers used: MUC2-1, MUC5AC-1 (Table S5).

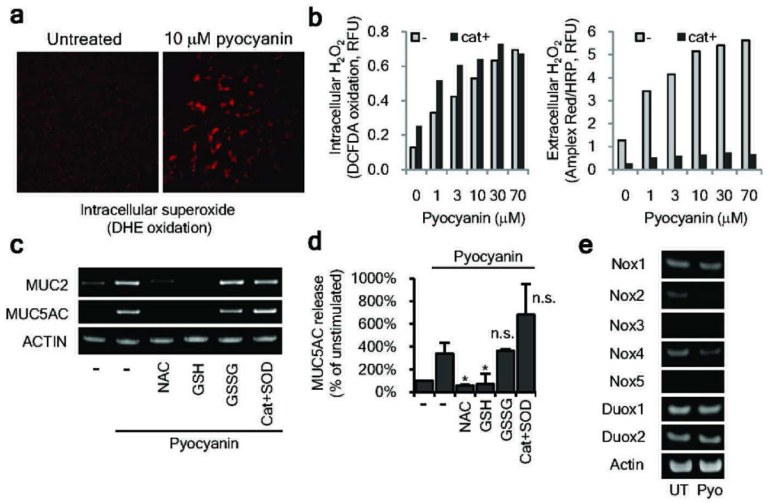

Figure 2. Reactive oxygen species mediate pyocyanin-triggered MUC2 and MUC5AC induction

(**a**) Intracellular superoxide detection by DHE oxidation in pyocyanin-treated bronchial epithelial cells (10 μM, 3 hrs; one representative experiment, n=3). (**b**) Intracellular hydrogen peroxide detected by DCF-DA oxidation in cells exposed to pyocyanin (3 hrs) is insensitive to extracellular catalase, whereas extracellular hydrogen peroxide release detected by Amplex Red/HRP is sensitive to exogenous catalase (mean, one representative experiment, n=3). Induction of MUC2 and MUC5AC gene expression (**c**) and MUC5AC release (**d**) in pyocyanin-treated cells (10 μM, 2 days) is prevented by intracellular antioxidants (10 mM NAC, GSH). Oxidized glutathione (GSSG, 10 mM) and extracellular ROS scavangers (1500 U/mL catalase + 12.5 μg/ml SOD) have no effect (RT-PCR: one representative experiment, n=3; MUC5AC release: mean+/−S.E.M., n=4). (**e**) Pyocyanin does not increase mRNA transcripts of ROS-producing NADPH oxidases (10 μM Pyo, 2 days, one representative, n=4).

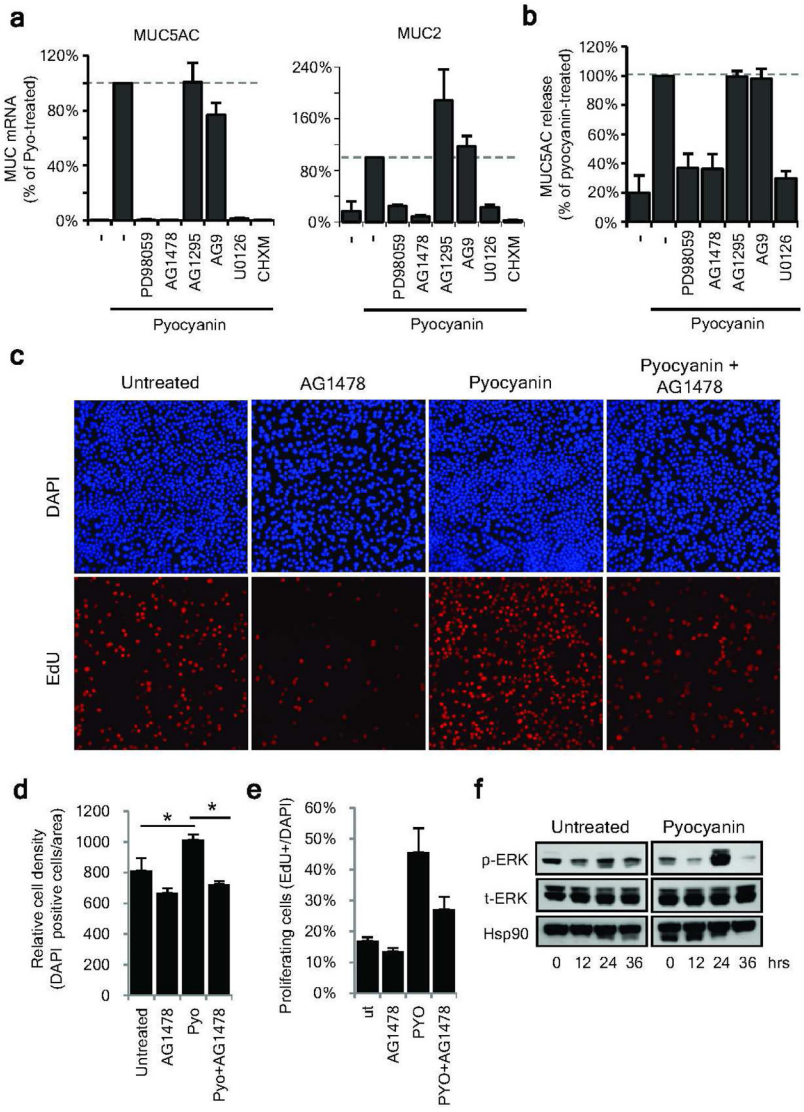

Figure 3. Pyocyanin activates epidermal growth factor receptor pathway and promotes proliferation in airway epithelial cells

Inhibitors of MEK1/2 (PD98059, U0126), EGFR (AG1478) and protein synthesis (cycloheximide, CHXM) blocked pyocyanin-mediated induction of MUC2 and MUC5AC gene expression (**a**) and MUC5AC release (**b**) in bronchiolar epithelial cells. The inhibitor of the platelet-derived growth factor receptor (AG1295) or the control analogue (AG9) had no effect. (Mean+/− S.E.M., n=3). (**c**) Pyocyanin-induced proliferation of bronchiolar epithelial cells is inhibited by AG1478 (detected by EdU-staining of cells treated with 8 μM pyocyanin for 36 hrs). (**d**) Quantitation of changes in epithelial cell density upon pyocyanin-treatment (DAPI-stained nuclei/unit surface, mean+/−S.E.M, n=3). (**e**) Pyocyanin exposure results in increase in proportion of actively dividing epithelial cells (EdU-positive cells/all (DAPI); mean+/− S.E.M., n=3). (**f**) Pyocyanin greatly enhanced phosphorylation of ERK at 24 hrs post-exposure in bronchiolar epithelial cells (another experiment gave similar results, t=total, p=phospho).

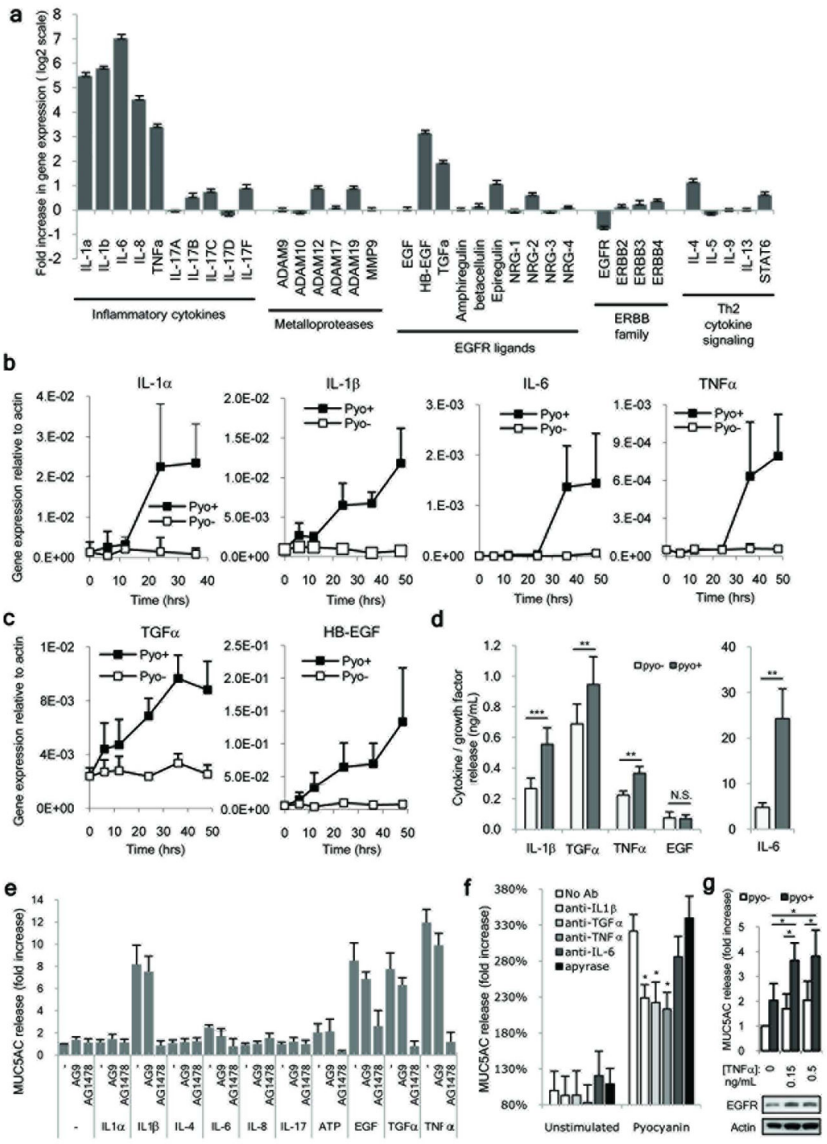

Figure 4. Pyocyanin-induced transcription of inflammatory cytokines and EGFR ligands mediate MUC5AC secretion

(**a**) Pyocyanin-induced transcriptional changes for genes known as inducers of mucin synthesis or involved in their signaling in bronchiolar epithelial cells assayed by microarray. Transcription of interleukin-1α, IL-1β, IL-6, IL-8, TNFα, HB-EGF and TGFα were strongly induced by pyocyanin. (**b**) Pyocyanin-induced transcriptional changes of the inflammatory cytokines, IL-1α, IL-1β, IL-6, TNFα were followed over time by real-time RT-PCR (mean +/−S.E.M., n=3–5). (**c**) Transcriptional changes of the EGFR ligands, TGFα and HB-EGF were confirmed by real-time RT-PCR (mean +/−S.E.M., n=3–5). (**d**) IL-1β, TGFα, TNFα, EGF and IL-6 protein levels were determined in supernatants of pyocyanin-treated cells (mean+/−S.E.M., n=4). (**e**) MUC5AC release from cells exposed to a variety of stimuli was measured in the presence of the EGFR inhibitor (AG1478) or its control compound (AG9) (mean+/− S.E.M., n=4). Concentration of cytokines/growth factors: 10 ng/mL, [ATP]= 100 μM. (**f**) Neutralizing antibodies against IL-1β, IL-6, TNFα and TGFα inhibit MUC5AC

release from pyocyanin-treated bronchiolar epithelial cells (mean +/−S.E.M., n=7–12). (**g**) Pretreatment of H292 cells with TNFα (2 days) increases EGFR levels and further potentiates pyocyanin-stimulated MUC5AC release (mean +/− S.E.M., n=4. [pyocyanin]= 4μM, 2 days).

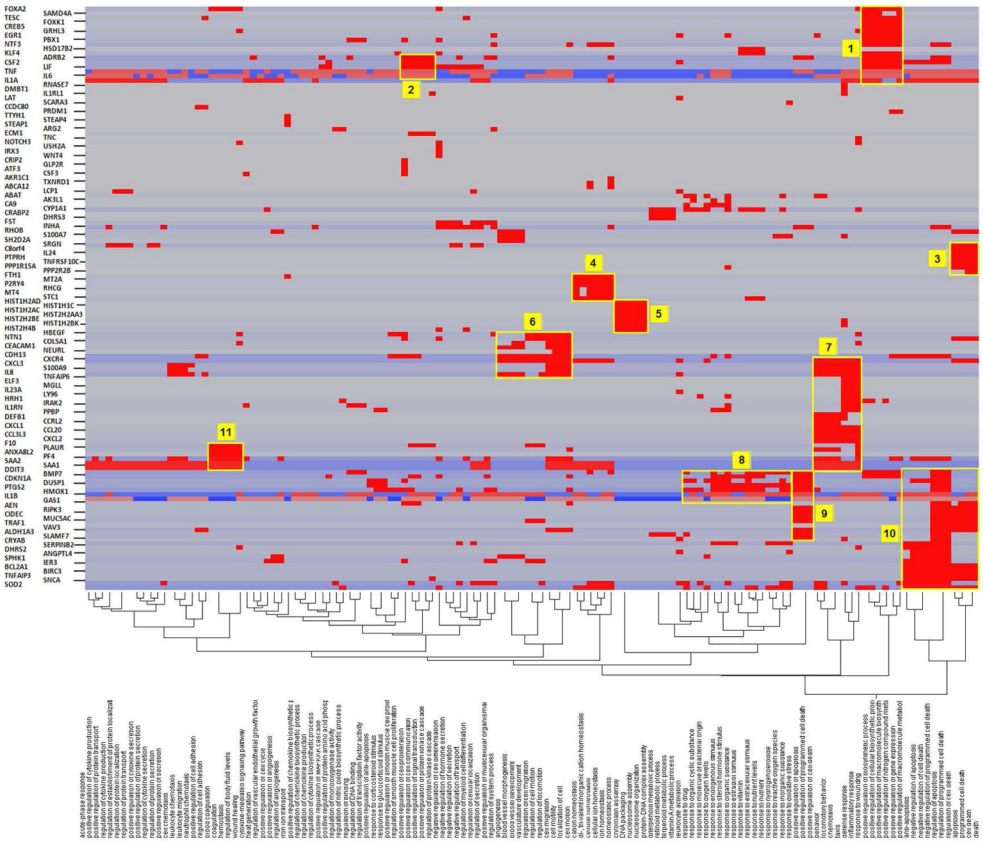

Figure 5. Functional clustering of pyocyanin-responsive genes in airway epithelial cells

The heat map shows a two-way clustering of genes (vertical axis) and the associated GO FAT functions (horizontal axis). Red cells indicate assignment of an altered gene to a function. (Due to data standardization, genes that participate in many functions, such as TNF, IL1A, IL1B and IL6, produce pinkish cells.) Clusters encompassing at least 5 genes were selected visually and numbered arbitrarily from 1–11. The clustered genes and corresponding functions are listed in Table 1 and Table S3. The data are obtained from H292 cells exposed to 8 μM pyocyanin for 48 hrs.

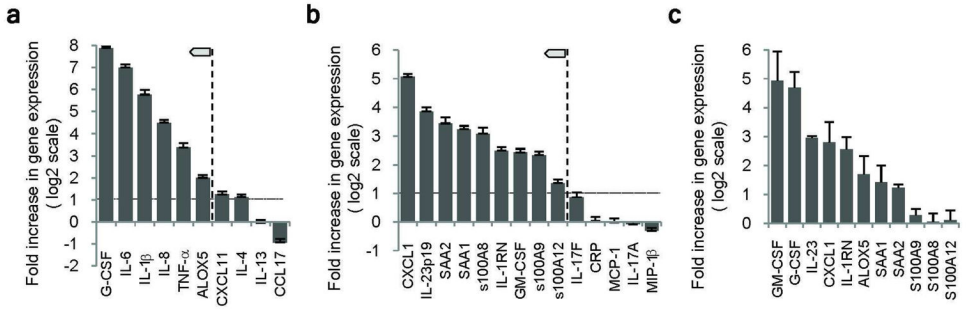

Figure 6. Pyocyanin induces epithelial transcription of several inflammatory mediators reported as elevated in CF patients

Gene expression changes in pyocyanin-treated epithelial cells (48 hrs, 8 μM) are shown for 24 inflammatory mediators (cytokines, chemokines or acute phase proteins) previously reported as displaying increased levels in CF patients (Table S4). (**a**) Six of ten genes specifically associated with *Pseudomonas* infection in CF are induced at least 2 fold by pyocyanin in bronchiolar epithelial cells (microarray data). (**b**) Nine of fourteen additional CF-associated genes (not studied in relation to *Pseudomonas*) were also induced by pyocyanin in bronchiolar epithelial cells (microarray data). (**c**) Real-time PCR confirmation of gene expression changes of CF-associated genes up-regulated by pyocyanin. qPCR results were not only obtained from samples used for microarray but from additional, independent experiments as well (n= 4–9).

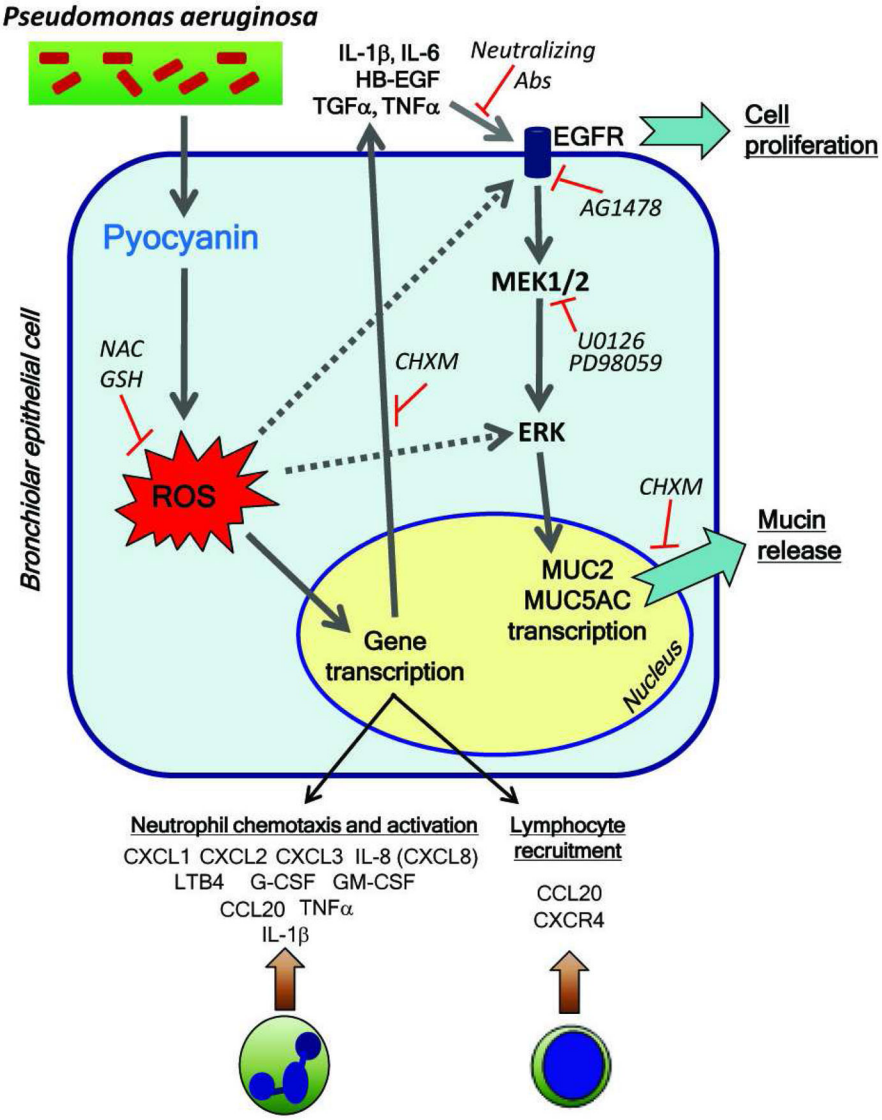

Figure 7. Proposed model of pyocyanin's inflammatory action in airway epithelial cells

The scheme shows proposed mechanisms for pyocyanin-induced mucin release and transcriptional changes. Reactive oxygen species produced in the host cell by pyocyanin result in transcriptional changes of several genes involved in the host's response to oxidative stress and in the recruitment of white blood cells to the infected mucosa. Inductions of TNFα, IL-1β, TGFα, IL-6 and HB-EGF collectively lead to activation of EGFR, followed by activation of MEK1/2, ERK, MUC2/MUC5AC transcription and cell proliferation. Red lines indicate points of experimental intervention with inhibitors, ROS scavengers and neutralizing antibodies.

Table 1

Summary of gene-function clusters.

Cluster	Functions	Genes (Log ratio)
C3 (3.34)	Apoptosis	C8orf4 (3.50), IL24 (6.87), PPP1R15A (3.34), *PPP2R2B (−2.78)*, PTPRH (4.25), SRGN (2.53), TNFRSF10C (2.01)
C7 (3.33)	Inflammatory/wound response, chemotaxis	*ANXA8L2 (−2.14)*, CCL20 (5.88), CCL3L3 (2.08), CCRL2 (2.00), CXCL1 (5.07), CXCL2 (4.56), CXCL3 (5.04), CXCR4 (4.37), *DEFB1 (−3.33)*, ELF3 (3.44), F10 (3.66), HRH1 (2.03), IL1RN (2.49), IL23A (3.85), IL8 (4.51), IRAK2 (2.43), LY96 (2.55), MGLL (2.17), PF4 (3.34), PLAUR (2.46), PPBP (3.48), S100A9 (2.34), SAA1 (3.24), SAA2 (3.45), TNFAIP6 (2.58)
C11 (3.29)	Hemostatis, wound healing	*ANXA8L2 (−2.14)*, F10 (3.66), PF4 (3.34), PLAUR (2.46), SAA1 (3.24), SAA2 (3.45)
C2 (2.94)	Positive regulation of cell proliferation	ADRB2 (2.21), CSF2 (2.44), IL6 (6.82), LIF (2.94), TNF (3.38)
C8 (2.86)	Response to stimuli	*BMP7 (−2.00)*, CDKN1A (3.41), DDIT3 (2.24), DUSP1 (2.73), HMOX1 (2.86), IL1B (5.71), PTGS2 (3.93)
C6 (2.76)	Angiogenesis, cell migration	CDH13 (2.55), CEACAM1 (2.87), *COL5A1 (−2.65)*, CXCL3 (5.04), CXCR4 (4.37), HBEGF (3.00), IL8 (4.51), NEURL (2.34), NTN1 (2.30), S100A9 (2.34)
C10 (2.73)	Anti-apoptosis, apoptosis	AEN (2.03), ALDH1A3 (2.34), ANGPTL4 (2.69), BCL2A1 (4.90), BIRC3 (3.41), *BMP7 (−2.00)*, CDKN1A (3.41), CIDEC (2.11), *CRYAB (−3.63)*, DDIT3 (2.24), DHRS2 (2.16), DUSP1 (2.73), *GAS1 (−4.10)*, HMOX1 (2.86), IER3 (2.86), IL1B (5.71), MUC5AC (4.11), PTGS2 (3.93), RIPK3 (2.73), SERPINB2 (3.72), SLAMF7 (2.19), *SNCA (−2.06)*, SOD2 (2.88), SPHK1 (2.02), TNFAIP3 (2.99), TRAF1 (2.53), *VAV3 (−2.73)*
C9 (2.73)	Positive regulation of apoptosis	AEN (2.03), ALDH1A3 (2.34), *BMP7 (−2.00)*, CDKN1A (3.41), CIDEC (2.11), DDIT3 (2.24), DUSP1 (2.73), HMOX1 (2.86), IL1B (5.71), MUC5AC (4.11), PTGS2(3.93), RIPK3 (2.73), SLAMF7 (2.19), *VAV3 (−2.73)*
C5 (2.61)	Chromatin assembly	HIST1H1C (2.92), HIST1H2AC (2.75), HIST1H2AD (2.12), HIST1H2BK (3.16), HIST2H2AA3 (2.61), HIST2H2BE (2.52), HIST2H4B (2.18)
C1 (2.49)	Positive regulation of gene expression/ macromolecule biosynthesis	ADRB2 (2.21), CREB5 (5.20), CSF2 (2.44), *EGR1 (−2.04)*, FOXA2 (2.59), FOXK1 (2.09), GRHL3 (2.22), IL1A (5.32), IL6 (6.82), KLF4 (2.13), LIF (2.94), NTF3 (2.27),*PBX1 (−2.54)*, SAMD4A (2.89), TESC (2.10), TNF (3.38)
C4 (2.22)	Cation homeostasis	FTH1 (2.13), MT2A (2.05), MT4 (2.73), P2RY4 (3.27), RHCG (2.27), STC1 (2.17)

Clusters are numbered as in Figure 5 and ordered by the median absolute value of the log ratios for the cluster members (in parentheses). Log_2 ratios for individual genes are shown as up (black) or down (red) for pyocyanin treated vs. untreated. The individual GO FAT functions constituting each cluster are given in Table S3. Data are obtained from cells exposed to 8 μM pyocyanin

Table 2

Transcriptional changes of novel pyocyanin-responsive genes involved in airway inflammation and responses to oxidative stress in airway epithelial cells.

Gene symbol	Gene name	Microarray	qPCR
	Interleukins, interleukin receptors		
IL11	Interleukin-11	2.7	23.0
IL19	Interleukin-19, transcript variant 1	3.1	4.3
IL20	Interlekuin-20	3.6	21.0
IL24	Interleukin-24, transcript variant 2	128.6	1601.0
IL1R2	Interleukin 1 receptor, type II	13.1	24.3
	Chemokines, chemokine receptors		
CXCL2	Chemokine (C-X-C motif) ligand 2	34.5	40.1
CXCL3	Chemokine (C-X-C motif) ligand 3	50.8	38.9
CCL20	Chemokine (C-C motif) ligand 20	58.7	53.4
CXCR4	Chemokine (C-X-C motif) receptor 4	22.0	115.2
	Oxidativee stress-responsive genes		
CRYAB	Crystallin, alpha B	0.081	0.060
SCARA3	Scavenger receptor class A, member 3, transcript variant 1	0.239	0.071
SNCA	Synuclein, alpha (non A4 component of amyloid precursor)	0.267	0.076
DHRS2	Dehydrogenase/reductase (SDR family) member 2	4.5	3.0
DDIT3	DNA-damage-inducible transcript 3	4.7	9.1
DUSP1	Dual specificity phosphatase 1	6.6	5.0
HMOX1	Heme oxygenase (decycling) 1	7.3	26.1
SOD2	Superoxide dismutase 2, mitochondrial	7.4	8.5
S100A7	S100 calcium binding protein A7	10.8	4.2
PTGS2	Prostaglandin-endoperoxide synthase 2	16.0	4.4

Microarray: fold changes, mean, n=4; qPCR: fold changes, mean, n=3–5.

qPCR results were not only obtained from samples used for microarray but from additional, independent experiments as well.

Bronchiolar epithelial cells were exposed to 8 μM pyocyanin for 48 hrs.