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The Role of Short-Chain Fatty Acids, Produced by Anaerobic Bacteria, in the Cystic Fibrosis Airway.

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Citation
Short-chain fatty acids cause an IL-8 response in cystic fibrosis airways via increased GPR41

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AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

A large number of culture-dependent and -independent studies have revealed a significant anaerobic bioburden in the cystic fibrosis (CF) airways; however, the role of anaerobic
bacteria in the pathogenesis of infection and inflammation in the CF airways remains largely underexplored.

What This Study Adds to the Field

In the current study we demonstrate a high prevalence of anaerobes in the airways of people with CF that increases with age. Short-chain fatty acids (SCFAs) were secreted by anaerobic bacteria in vitro and were also detected in vivo in CF bronchoalveolar lavage. SCFAs induced a significant IL-8 response in bronchial epithelial cells that was more pronounced in CF than normal bronchial epithelium. Receptors for SCFAs, GPR41 and GPR43, were both expressed in bronchial epithelial cells. However, only GPR41 was upregulated in CF compared to normal airway epithelium in vitro and in vivo. GPR41 upregulation was intrinsically driven by the lack of CFTR activity and endoplasmic reticulum stress and further corroborated by inflammatory stimuli. This study provides novel insight into the pathophysiological role of anaerobic bacteria in the CF airways.

This article has an online supplement, which is accessible from this issue’s table of content online at www.atsjournals.org.
Abstract

Rationale: Anaerobic bacteria are present in large numbers in the airways of people with cystic fibrosis (PWCF). In the gut, anaerobes produce short-chain fatty acids (SCFAs) that modulate immune/inflammatory processes.

Objectives: To investigate the capacity of anaerobes to contribute to CF airway pathogenesis via SCFAs.

Methods: Samples from 109 PWCF were processed using anaerobic microbiological culture with bacteria present identified by 16S RNA sequencing. SCFAs levels in anaerobe supernatants and bronchoalveolar lavage (BAL) were determined by gas chromatography. The mRNA and/or protein expression of SCFAs receptors, GPR41 and GPR43, in CF and non-CF bronchial brushings, and 16HBE14o- and CFBE41o- cells were evaluated using RT-PCR, western blot, laser scanning cytometry and confocal microscopy. SCFAs-induced IL-8 secretion was monitored by ELISA.

Measurements and Main Results: Fifty seven of 109 (52.3%) PWCF were anaerobe-positive. Prevalence increased with age, from 33.3% to 57.7% in PWCF under (n=24) and over 6 years (n=85). All evaluated anaerobes produced millimolar concentrations of SCFAs, including acetic, propionic and butyric acid. SCFAs levels were higher in BAL samples from adults than children. GPR41 levels were elevated in; CFBE41o- versus 16HBE14o- cells; CF versus non-CF bronchial brushings; 16HBE14o- cells after treatment with CFTR inhibitor CFTR(inh)-172, CF BAL, or inducers of endoplasmic reticulum stress. SCFAs induced a dose-dependent and pertussis toxin-sensitive IL-8 response in bronchial epithelial cells with a higher production of IL-8 in CFBE41o- than 16HBE14o- cells.

Conclusions: This study illustrates that SCFAs contribute to excessive production of IL-8 in CF airways colonized with anaerobes via upregulated GPR41.
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Keywords: cystic fibrosis; anaerobic bacteria; short-chain fatty acids; inflammation.
**Introduction**

The main cause of morbidity and mortality in cystic fibrosis (CF) is respiratory failure due to persistent microbial infection and neutrophil-dominated inflammation (1, 2). CF airway infection is polymicrobial and bacteria such as *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Haemophilus influenzae*, and *Burkholderia cepacia* complex have been recognized as key pathogens in CF airway infection (3). The detection of bacterial pathogens in CF has widely depended on routine aerobic culture techniques. Consequently, the prevalence of anaerobic and other fastidious bacteria has remained largely understudied. With the implementation of enhanced culture and culture-independent molecular-based techniques, interest in the prevalence and potential pathogenic role of anaerobes has been sparked (4–6).

Anaerobic bacteria are present in the airways of 66-91% people with CF (PWCF) (7, 8) in numbers equal to those of *P. aeruginosa*, a well-established pathogen in the CF airways (3, 7). Studies exploring the role of anaerobes have failed to show a direct impact on airway function in CF (7, 8). However, more recent studies have associated decreasing CF airway microbial diversity, to which anaerobic bacteria contribute, with poorer lung function (9–11).

Contrary to this, *Prevotella intermedia* was shown to induce a humoral immune response in PWCF and mediate an influx of neutrophils and macrophages thereby increasing CF airway inflammation (12). Furthermore, anaerobes belonging to the oropharyngeal flora have been shown to enhance the virulence of *P. aeruginosa* in a number of infection models (13–17).

Anaerobic bacteria comprise an integral part of the normal gut microflora, where they produce copious amounts of short-chain fatty acids (SCFAs), including acetic, propionic and butyric acid (18). In addition to their role as fuel for intestinal epithelial cells, SCFAs modulate various processes, including cell proliferation and differentiation, hormone secretion, metabolic homeostasis and modulation of immune/inflammatory responses (18–23).
by binding to the recently deorphanized G protein-coupled receptors, GPR41 (FFAR3) and
GPR43 (FFAR2) and by the inhibition of histone deacetylase (HDAC) (24–26).

In the current study, we investigated the capacity of anaerobic bacteria, found in the CF
airways, to induce an inflammatory response by the production of SCFAs. The results reveal
that these anaerobes produce copious amounts of SCFAs that were also present in the
bronchoalveolar lavage (BAL) of PWCF. SCFAs induced a dose-dependent and pertussis
toxin (PTX)-sensitive IL-8 response in bronchial epithelium that was higher in CF when
compared to normal bronchial epithelial cells. This effect coincided with increased GPR41
expression in CF when compared to normal bronchial epithelial cells that was intrinsically
driven by lack of CFTR activity and endoplasmic reticulum (ER) stress, in addition to the
proinflammatory milieu of the CF airways.
Methods

Comprehensive detail on all methods, including Quantification of SCFA, Quantification of SCFAs Receptors, CF and non-CF Bronchial Epithelial Cell Culture and Treatments and Measurement of IL-8 is provided in the online supplement.

Study population. A total of 109 PWCF (range 0-61, mean age 20.7 ± 12.1 years; 65 male, 44 female) were recruited to this study. Seventy-three PWCF were attending Beaumont Hospital, Dublin (range 18-61, mean age 27.4 ± 8.2; 44 male, 29 female), 24 were attending Our Lady’s Children’s Hospital, Crumlin (range 0-6, mean age 3.5 ± 1.6; 11 male, 13 female) and 12 were attending Children’s University Hospital Temple Street, Dublin (range 7-17, mean age 14.6 ± 2.4; 10 male, 2 female). CF was confirmed by sweat testing and/or genotyping. Only clinically stable PWCF were included in this study. Clinical stability was defined as no change in symptoms, forced expiratory volume in the first second (FEV₁) within 10% of best value in the previous 6 months, and no new antibiotics started. Full informed consent was obtained from all participants or their parents prior to the collection of samples and ethical approval was obtained from Beaumont Hospital, Our Lady’s Children’s Hospital or Children’s University Hospital Institutional Review Board.

Collection and processing of sputum and BAL. Spontaneously expectorated sputum was collected in a sterile container that was placed into an Anaerogen™ Compact anaerobic pouch (Thermo Fisher Scientific, Waltham, MA, USA) and immediately transported to an anaerobic cabinet for further processing. BAL samples were obtained from individuals undergoing diagnostic or therapeutic fiber-optic bronchoscopy as part of routine care. In adults, 60 ml of sterile 0.9% NaCl was instilled into the right or left sub-segmental bronchi. In children, flexible fiber-optic bronchoscopy was performed via a laryngeal mask airway. To
prevent upper airway contamination suction was not performed until the tip of the bronchoscope was past the carina. During the procedure, 1 ml/kg of sterile 0.9% NaCl was instilled twice in the right middle lobe and twice in the lingula and all four samples were pooled. BAL intended for anaerobic bacterial isolation and identification was handled as described for sputum above and BAL samples intended for SCFAs determination were filtered through gauze and centrifuged at 500g for 10 min at 4°C. The microbiological culture and identification of bacteria in sputum and BAL were carried out as described previously (7).

**Bronchial brushing sample collection.** All patients (control subjects and PWCF) were undergoing diagnostic or therapeutic fiber-optic bronchoscopy as part of routine care. Bronchial brushings were recovered as previously described (27).

**Statistical Analysis.** Data were analyzed with GraphPad Prism 4.0 software (GraphPad Software, San Diego, CA). Unless stated otherwise, all data are presented as mean ± SEM and are representative of at least three independent experiments. Normal data were compared using the Student’s t-test, non-normal data were compared by Mann-Whitney U test and where appropriate ANOVA was performed. Differences were considered significant at $P < 0.05$. 

6
Results

Anaerobic bacteria are present in a high percentage of PWCF. To determine the prevalence of anaerobic bacteria in the CF airways, 80 sputum and 29 BAL samples from 109 PWCF were collected and analyzed (one sample per patient). The presence of ≥1 obligate anaerobe was found in 57 of 109 analyzed samples (52.3 %). The most frequently detected obligate anaerobes belonged to the genera *Prevotella* (35.8 %), *Actinomyces* (16.5 %), *Veillonella* (8.3 %), and *Fusobacterium* (5.5 %) (Table E1) and the most commonly occurring aerobes (facultative anaerobes) belonged to the genus *Pseudomonas* (44.0 %), *Staphylococcus* (41.3 %) and *Streptococcus* (40.4 %), including *S. anginosus*, *S. constellatus*, *S. intermedius*, *S. parasanguinis* and *S. sanguinis*. The prevalence of obligate anaerobes was greater than that of *P. aeruginosa* (43.2%). Higher prevalence of anaerobes was observed in PWCF over 6 years of age (57.7 %, n = 85, mean age 25.6 ± 8.9 years, 54 males and 31 females) compared to PWCF under 6 years of age (33.3 %, n = 24, mean age 3.5 ± 1.6 years, 11 males and 13 females; Figure 1A), demonstrating that the anaerobic prevalence increases with age. Based on these data, four representative obligate anaerobes and one facultative anaerobe were selected for further work, namely *Prevotella melaninogenica*, *Actinomyces odontolyticus*, *Veillonella parvula*, *Fusobacterium nucleatum*, and *Streptococcus sanguinis*.

Selected anaerobic strains secrete copious amounts of SCFAs in vitro. To investigate whether anaerobic bacteria found in the CF airway secrete SCFAs, bacterial supernatants from five representative species grown under anaerobic conditions were collected and analyzed using gas chromatography. ATCC and patient-isolated strains of all selected species secreted a variety of SCFAs in millimolar concentrations (Figure 1B). Each individual species had its own secretion profile which was identical for the ATCC and patient isolate.
strain. For example, while *A. odontolyticus* and *S. sanguinis* secreted acetic acid, *V. parvula* and *F. nucleatum* displayed more complex SCFAs secretomes, consisting of acetic and propionic acid and acetic, propionic and butyric acid, respectively. *P. melaninogenica*, produced the highest number of SCFAs, including acetic, i-butyric, 2-methylbutyric and i-valeric acid, albeit in lower concentrations. Of note, *P. aeruginosa* grown under anaerobic or aerobic conditions did not secrete any SCFAs (data not shown). These data suggest that anaerobic bacteria found in the CF airway produce copious amounts of SCFAs.

**SCFAs are present in the airways of PWCF and are elevated in adults when compared to children with CF.** To investigate whether anaerobes produce SCFAs in the CF airway in vivo, BAL samples from adults (*n* = 11; mean age 29.2 ± 9.3 years; 3 males and 8 females) and children with CF (*n* = 7; mean age 4.0 ± 2.2 years; 2 males and 5 females) were collected and analyzed using gas chromatography. Six SCFAs detected in bacterial supernatants were also observed in μM concentration range in BAL samples from adult PWCF (Figure 1C). A significant increase in acetic (*P* < 0.001) was observed in BAL samples from adult PWCF when compared to children with CF. These results illustrate that SCFAs are present in the airways of PWCF and are elevated in adult PWCF compared to children with CF.

**IL-8 is elevated in BAL from adult PWCF when compared to children with CF and correlates with acetic acid.** IL-8 is an inflammatory marker in the airways of PWCF that mediates neutrophil trafficking to the bronchial lumen (28). As evaluated with an IL-8 ELISA, IL-8 was markedly increased in BAL samples from adult PWCF (*n* =10; mean age 30.1 ± 9.7 years; 3 males and 7 females) compared to children with CF (*n* = 6; mean age 4.5 ± 1.9 years; 2 males and 4 females; Figure 1D). A correlation analysis was performed to investigate whether this phenomenon was associated with SCFAs levels in CF BAL. IL-8
levels displayed a strong correlation with acetic acid in BAL samples from children and adults with CF (n = 16, $r^2 = 0.6409$, $P = 0.002$; Figure 1E). Similarly, a significant correlation was also observed between IL-8 and acetic acid in BAL samples from adult PWCF (n = 10, $r^2 = 0.4200$, $P = 0.0427$). No significant correlation between IL-8 and other SCFAs in CF BAL was observed (data not shown). Collectively, these results showcase that elevated IL-8 levels in CF BAL are associated with high levels of acetic acid in BAL.

**Anaerobe supernatants induce an inflammatory response in CF epithelial cells.**

CFBE41o- cells were treated with supernatants from representative species and monitored for IL-8 secretion using ELISA. All five strains induced a dose- and PTX-dependent IL-8 response in CFBE41o- cells (Figure 2, Figure E1 and E2). The highest IL-8 response was observed with *V. parvula* and *F. nucleatum* supernatants which were shown to produce high amounts of acetic, propionic and butyric acid. Interestingly, the IL-8 response induced by *V. parvula* and *F. nucleatum* was equal or greater than that induced by major CF pathogens, *S. aureus* and *P. aeruginosa*. When CFBE41o- cells were treated with *S. sanguinis* or *A. odontolyticus* which secreted acetic acid only, a less pronounced IL-8 response was observed and higher concentrations of bacterial supernatant (5 and 10 %) were needed to achieve significant cytokine production (Figure E1). *P. melaninogenica* displayed an intermediate but significant impact on IL-8 production. Upon stimulation of 16HBE14o- cells with anaerobe supernatants, only *F. nucleatum* induced a significant IL-8 response over baseline levels (Figure 2). Furthermore, anaerobe-induced IL-8 responses in 16HBE14o- cells were strikingly lower when compared to CFBE41o- cells, suggesting that the CF epithelium is more susceptible to stimulation by anaerobe supernatants.
SCFAs generate a notable IL-8 response in CF epithelial cells. To elucidate whether SCFAs in anaerobe supernatants contribute to IL-8 secretion, bronchial epithelial cells were treated with SCFAs and analyzed for IL-8 secretion. The influence of SCFAs on cell viability was evaluated to exclude any SCFAs-induced cytotoxicity (Figure E3) and in the following experiments the cells were treated with non-toxic concentrations of SCFAs. All SCFAs, apart from 2-methylbutyric acid, induced a significant and a dose-dependent IL-8 response from CFBE41o- cells (Figure 3) with the potency rank order: butyric ($EC_{50} = 0.170$ mM, $R^2 = 0.8816$) > $i$-valeric ($EC_{50} = 1.0$ mM, $R^2 = 0.7711$) > propionic ($EC_{50} = 4.6$ mM, $R^2 = 0.9107$) > acetic ($EC_{50} = 15.5$ mM, $R^2 = 0.8443$) > $i$-butyric acid ($EC_{50} = 64$ mM, $R^2 = 0.8304$). At the same time, no basal or SCFA-stimulated IL-1$\beta$ levels were detected in CFBE41o- supernatants (data not shown). Additionally, a small but insignificant increase in SCFA-induced IL-6 over baseline was observed in CFBE41o- supernatants (Figure E4). This response was markedly lower when compared to IL-8, confirming that IL-8 is the predominant pro-inflammatory cytokine induced by SCFAs in CF bronchial epithelial cells. When CFBE41o- cells were treated with a combination of SCFAs mimicking the complex SCFAs secretomes of *P. melaninogenica*, *F. nucleatum* and *V. parvula*, no synergistic effect on IL-8 secretion was observed (Figure E5). SCFAs also induced a dose-dependent IL-8 response in 16HBE14o- cells (Figure 3); however, IL-8 secretion was significantly less pronounced in 16HBE14o- compared to CFBE41o- cells corroborating our earlier observations with anaerobe supernatants. These results confirm that SCFAs in anaerobe supernatants are at least in part responsible for the inflammatory response in bronchial epithelial cells *in vitro*. Of note, upon incubation of representative SCFAs (acetic, propionic and butyric acid) with human serum albumin or alpha-1 antitrypsin no changes in intrinsic fluorescence of either proteins were observed (Figure E6), suggesting that SCFAs do not bind
human serum albumin or alpha-1 antitrypsin. Moreover, the latter also failed to attenuate the SCFAs-induced IL-8 response in CFBE41o- cells (Figure E7).

SCFAs-mediated release of IL-8 by CFBE41o- cells is GPR41-dependent. Previous studies have shown that GPR41 couples exclusively through the PTX-sensitive Gαi/o family, whereas GPR43 displays dual coupling through Gαi/o and PTX-insensitive Gαq protein families (24, 25). To explore whether SCFAs-mediated IL-8 release was indeed mediated by SCFAs receptors, CFBE41o- cells were treated with PTX prior to SCFAs. PTX (250 ng/ml) significantly attenuated IL-8 secretion in CFBE41o- cells induced by all evaluated SCFAs (Figure 4A). Higher PTX concentrations (500 and 1000 ng/ml) did not fully restore the IL-8 baseline levels (Figure E8). These data suggest the involvement of PTX-sensitive receptors GPR41 and GPR43 in SCFAs-mediated cytokine response in CFBE41o- cells.

To further confirm the involvement of GPR41 in SCFA-mediated IL-8 response, a small interfering RNA (siRNA) knockdown approach was employed. siRNA (80 nM) -mediated knockdown of GPR41 by the was confirmed using RT-PCR and western blot analysis 48 h after transfection (Figure 4B and 4C). Even a partial reduction of GPR41 protein expression (~50 %) was sufficient to significantly inhibit IL-8 production induced by acetic (10 mM), propionic (1 mM) and butyric acid (1 mM; Figure 4D). Collectively, these data corroborate the involvement of GPR41 in SCFA-mediated IL-8 release by CF bronchial epithelial cells.

GPR41 expression is increased in CF when compared to normal bronchial epithelial cells. To confirm the expression of SCFAs receptors in human bronchial epithelium, mRNA and protein levels of GPR41 and GPR43 were evaluated. Western blot analysis revealed that the GPR41 receptor was expressed by both 16HBE14o- and CFBE41o- cells, with significantly higher protein expression in CFBE41o- than in 16HBE14o- cells (P < 0.001;
Figure 5A). GPR43 was expressed in 16HBE14o- and CFBE41o- cells at equivalent levels (Figure 5A). The cell surface expression of SCFAs receptors was further examined with laser scanning cytometry and confocal microscopy. Staining for the GPR41 receptor was significantly increased on the surface of CFBE41o- compared to 16HBE14o- cells as assessed with laser scanning cytometry (55.5 ± 19.5%, P < 0.001; Figure 5B). Although both cell lines displayed intracellular staining for GPR41 receptor as visualized with confocal microscopy (Figure 5C), a significant increase in co-localization of the GPR41 receptor with the plasma membrane marker, Na/K-ATPase, was observed in CFBE41o- (11.9 ± 2.0%) compared to 16HBE14o- (6.9 ± 1.1%, P = 0.0434) cells. No differences in GPR43 cell surface expression were detected in 16HBE14o- and CFBE41o- cells when analyzed with laser scanning cytometry or confocal microscopy (Figure E11). These results suggest that while GPR41 and GPR43 are both present in bronchial epithelial cells, only GPR41 is upregulated at the surface of CF epithelial cells when compared to normal bronchial epithelial cells.

To investigate whether GPR41 mRNA was also increased in CFBE41o- cells, qRT-PCR was performed. GPR41 mRNA levels were significantly increased in CFBE41o- compared to 16HBE14o- cells (P < 0.001; Figure 5D). The in vitro trend was also observed in vivo as an increase in GPR41 mRNA levels in bronchial brushings from PWCF compared to non-CF controls (P = 0.0064; Figure 5E).

**Endoplasmic reticulum stress upregulates GPR41 expression in CF bronchial epithelial cells in vitro.** Previous studies have associated dysfunctional cystic fibrosis transmembrane conductance regulator (CFTR) with endoplasmic reticulum (ER) stress and activation of the unfolded protein response (UPR) (29). To elucidate whether the latter could have an impact on GPR41 expression, 16HBE14o- cells were treated with tunicamycin (2 µg/ml),
thapsigargin (2 µM) or dithiothreitol (DTT, 1 mM), well established inducers of ER stress and UPR (30). ER stress and UPR induction by these compounds was confirmed by monitoring the expression of glucose-regulated protein (GRP) 78 and GRP 94 (Figure E10). Tunicamycin and thapsigargin significantly increased GPR41 protein levels in 16HBE14o-cells (P = 0.0297 and P = 0.0198, respectively; Figure 6A) These data suggest that ER stress and UPR may have a role in the GPR41 upregulation in CF bronchial epithelial cells.

Dysfunctional CFTR and the inflammatory CF environment promote GPR41 upregulation in CF bronchial epithelial cells in vitro. To explore the possibility that GPR41 upregulation in the CF airway epithelium could also be associated with reduced CFTR activity, 16HBE14o-cells were treated with a CFTR inhibitor, CFTR(inh)-172 (10 µM), and subsequently evaluated for GPR41 mRNA expression. CFTR inhibition significantly increased GPR41 mRNA levels in 16HBE14o-cells (P = 0.0166; Figure 6B) matching them to levels found in CFBE41o-cells (Figure 5). Similarly, 10% CF BAL caused a significant upregulation of GPR41 mRNA in 16HBE14o-cells (P < 0.001; Figure 6B). These findings were translated to the protein level, as both CFTR(inh)-172 and 10% CF BAL significantly elevated GPR41 protein levels in 16HBE14o-cells (P = 0.0233 and P = 0.0098, respectively; Figure 6C). Similarly, GlyH-101, another inhibitor of CFTR also increased GPR41 protein levels (data not shown). These results imply that reduced CFTR activity may be associated with GPR41 upregulation in CF bronchial epithelial cells in vitro, an effect that is also driven by the inflammatory CF airway milieu.
**Discussion**

Recent advances in culture-independent molecular techniques for bacterial identification have sparked a renewed interest in the role of anaerobic bacteria in the CF airways. Therefore, we aimed to explore the contribution of anaerobes via SCFAs to the pathogenesis of CF airway disease. In the current study, we show that anaerobic bacteria commonly found in the CF airways secrete SCFAs *in vitro* and *in vivo*, and by acting through GPR41 and GPR43, mediate IL-8 release promoting an inflammatory response in the CF airway.

Similar to previous studies (7, 8, 16) our cohort of PWCF displayed a high prevalence of anaerobic bacteria (52.3 %). This number was greater than that of *P. aeruginosa* (43.2 %), an established CF airway pathogen, confirming that anaerobes are prevalent in the CF airways, and may play a role during the course of the disease. Indeed, the percentage of PWCF positive for anaerobes increased with age. Five representative species, that were selected based on their prevalence, secreted high quantities of SCFAs, with each displaying a distinct SCFA secretome (Figure 1B). SCFAs were also detected in CF BAL and were elevated in BAL samples from adult PWCF compared to children with CF (Figure 1C). This suggests that alongside anaerobes, SCFAs also increase with age. Although SCFAs were previously studied in the context of allergic airway inflammation (31), to our knowledge, this is the first report proving the presence of SCFAs in the lower airways.

CF airway disease is characterized by an exaggerated neutrophil influx that plays a key role in tissue damage and disease progression (32). We have previously shown that IL-8, a potent neutrophil chemotactic agent, is readily secreted from CF airway epithelium by various stimuli (33–35) and is found in high concentrations in CF BAL (28). Anaerobe supernatants induced a potent and dose-dependent IL-8 response that was more pronounced in CF than in normal bronchial epithelium (Figure 2 and Figure E11). Similarly, SCFAs generated a dose-dependent IL-8 response in bronchial epithelial cells that was higher in CF than in normal
bronchial epithelial cells (Figure 3), suggesting that SCFAs in anaerobe supernatants contribute to the IL-8 response and may have a detrimental role in the CF airways by promoting neutrophil mobilization.

In addition to the activation of airway epithelial cells to produce cytokines, SCFAs are known to display a plethora of other effects. SCFAs were recently shown to act as chemotactic agents for neutrophils via GPR43 (36). At the same time, butyric acid was also reported to impair neutrophil reactive oxygen species production, phagocytosis and microbial killing (37). This suggests that in addition to IL-8-mediated recruitment of neutrophils, SCFAs may display a direct effect on neutrophil migration to the CF airways and their function.

Both SCFAs receptors, GPR41 and GPR43, couple to the PTX-sensitive Gα\textsubscript{i/o} family of proteins, but only GPR43 also couples to the PTX-insensitive Gα\textsubscript{q} family of proteins (24, 25). PTX blocks the signaling mediated by Gα\textsubscript{i/o} proteins (38) and PTX treatment significantly reduced the SCFAs-mediated IL-8 response (Figure 4) confirming that SCFAs mediate their effects via SCFAs receptors GPR41 and GPR43. However, increasing the concentration of PTX did not return the basal levels of IL-8 production after stimulation by propionic and butyric (Figure E8). Propionic and butyric are well established inhibitors of HDAC (38) and previous studies have shown that butyric acid induces an IL-8 response in human intestinal epithelial cells by inhibiting HDAC activity and promoting histone acetylation (26).

Therefore, SCFA-induced IL-8 response in bronchial epithelial cells may also be mediated by HDAC inhibition in addition to SCFAs receptors.

GPR41 expression is increased in CF bronchial epithelium in vitro and in vivo (Figure 5) and the mechanism of upregulation was subsequently investigated. F508del CFTR is the most common cause of CF. It causes misfolding of CFTR and its subsequent degradation after synthesis leading to a lack of the functional CFTR on the cell surface (39). Treatment with CFTR(inh)-172, a CFTR inhibitor, increased mRNA and protein levels of GPR41
16HBE14o- cells (Figure 6B and 6C), suggesting that the lack of CFTR activity could be associated with GPR41 upregulation in CF bronchial epithelial cells and that the mechanism could be related to defective chloride ion and/or bicarbonate conductance. Previously, we have demonstrated altered expression of microRNAs (miRNAs) in CF (27). miR-182, miR-23b and miR-544 were shown to be decreased in CF bronchial brushings (27), and were identified to target GPR41 mRNA using various miRNA target prediction databases (40, 41). However, overexpression of these miRNAs failed to reduce GPR41 mRNA and protein expression in CFBE41o- cells (data not shown) excluding the possibility of regulation of GPR41 expression by these miRNAs. Accumulation of misfolded F508del CFTR in the ER causes ER stress that activates UPR, a signal transduction pathway to alleviate this stress and restore ER homeostasis (29, 42, 43). Exposure of 16HBE14o- cells to tunicamycin, thapsigargin or DTT, well-known inducers of ER stress and UPR, increased protein expression of GPR41 (Figure 6A), implying that the ER stress and UPR may also, independent of CFTR activity, contribute to GPR41 upregulation. Lastly, the CF airway harbors high concentrations of proinflammatory markers, including proteases and chemokines (28). Treatment of 16HBE14o- cells with CF BAL increased GPR41 mRNA and protein levels (Figure 6B and 6C) suggesting that in addition to intrinsic upregulation of GPR41 in CF, the latter could further be corroborated by the inflammatory milieu of the CF airways.

In conclusion, we propose a novel mechanism by which anaerobic bacteria, through the production of SCFAs, contribute to the inflammatory environment in the CF airways in this way, potentially amplifying mobilization of neutrophils, tissue destruction and disease progression. This data suggests that eradication of anaerobes in PWCF may be beneficial in order to resolve the excessive inflammation brought on by these microorganisms.
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33. Cosgrove S, Chotirmall SH, Greene CM, McElvaney NG. Pulmonary proteases in the cystic fibrosis lung induce interleukin 8 expression from bronchial epithelial cells via a


Figure legends

Figure 1. Anaerobic species are found in high numbers in the CF airways and secrete copious amounts of SCFAs in vitro and in vivo. (A) The percentage of anaerobe-positive cultures in children with CF up to 6 years of age (n = 24; mean age 3.5 ± 1.6 years; 11 males and 13 females) and people with CF over the age of 7 (n = 85; mean age 25.6 ± 8.9 years; 54 males and 31 females). Sputum or BAL samples from 109 PWCF were collected and screened for the presence of anaerobic bacteria as stated in materials and methods. Only four genera of the most frequent obligate anaerobes (Prevotella, Actinomyces, Veillonella, and Fusobacterium) and the most commonly occurring aerobes (facultative anaerobes) belonging to the genera Pseudomonas, Staphylococcus and Streptococcus are shown for clarity reasons. (B) SCFAs levels in bacterial supernatants from ATCC and patient isolate strains of P. melaninogenica, A. odontolyticus, V. parvula, F. nucleatum and S. sanguinis as determined by gas chromatography. Data are presented as means ± SEM (n ≥ 3). (C) SCFAs levels in BAL samples from children with CF (n = 7; mean age 4.0 ± 2.2 years; 2 males and 5 females) and adults with CF (n = 11; mean age 29.2 ± 9.3 years; 3 males and 8 females). (D) IL-8 levels in BAL samples from children with CF (n = 6; mean age 4.5 ± 1.9 years; 2 males and 4 females) and adults with CF (n = 10; mean age 30.1 ± 9.7 years; 3 males and 7 females) as evaluated with an IL-8 ELISA. (E) Correlation between IL-8 and acetic acid in BAL samples from people with CF (n = 16, $r^2 = 0.6409$, $P = 0.002$). ** $P \leq 0.01$, *** $P \leq 0.001$.

Figure 2. Supernatants from anaerobic bacteria promote a pro-inflammatory response in CF airway epithelial cells. 16HBE14o- and CFBE41o- cells were treated with 5% bacterial supernatants or growth medium as control for 24 h and IL-8 secretion was assessed
with ELISA. All data are presented as means ± SEM of at least three independent experiments. ** $P \leq 0.01$, *** $P \leq 0.001$.

Figure 3. Short-chain fatty acids induce a dose-dependent IL-8 response in bronchial epithelial cells. 16HBE14o- and CFBE41o- cells were treated with increasing concentrations of SCFAs for 24 h and monitored for IL-8 production using ELISA. All data are presented as means ± SEM of at least three independent experiments. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

Figure 4. Pertussis toxin and GPR41 siRNA attenuate SCFAs-mediated IL-8 secretion in CF bronchial epithelial cells. (A) CFBE41o- cells were treated with pertussis toxin (PTX; 250 ng/ml) and increasing concentrations of SCFAs for 24 h. Afterwards, cell supernatants were monitored for IL-8 secretion using ELISA. CFBE41o- cells were transfected with GPR41 or control siRNA and were evaluated for GPR41 mRNA and protein levels 48 h after transfection using qRT-PCR and western blot analysis, respectively. (B) mRNA expression of GPR41 relative to GAPDH was determined using the $2^{(-\Delta\Delta Ct)}$ method and presented as fold differences. (C) Whole cell lysates were separated by 12.5 % SDS-PAGE, transferred to a PVDF membrane and detected with anti-GPR41 antibody. Signal intensities of all bands were quantified by densitometry and normalized against GAPDH that served as loading control. Bar graph depicts densitometric analysis of GPR41 expression. All data are presented as means ± SEM (n ≥ 3). (D) CFBE41o- cells were transfected with GPR41 or control siRNA (80 nM) 24 h before treatment with acetic (10 mM), propionic (1 mM) and butyric acid (1 mM) for additional 24 h. Cell supernatants were monitored for IL-8 secretion using ELISA. The results shown are representative of three independent experiments. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. 
Figure 5. Short-chain fatty acid receptor GPR41 is upregulated at the surface of CF bronchial epithelial cells. (A) A representative western blot depicting GPR41 and GPR43 protein expression in 16HBE14o- and CFBE41o- cells. Whole cell lysates were separated by 12.5 % SDS-PAGE, transferred to a PVDF membrane and detected with anti-GPR41 or GPR43 antibodies. Signal intensities of all bands were quantified by densitometry and normalized against GAPDH that served as loading control. Bar graph depicts densitometric analysis of GPR41 expression in 16HBE14o- and CFBE41o- cells. Data are shown as means ± SEM (n ≥ 3). (B) GPR41 cell surface staining was analyzed in CFBE41o- and 16HBE14o- cells using LSC as stated in methods. Histograms, representative of three independent experiments are shown. Inlet values represent a fold increase in mean fluorescence intensity between cells stained with primary anti-GPR41 and secondary antibody (black line) and control cells stained only with the secondary antibody (grey line). (C) 16HBE14o- or CFBE41o- cells were grown on coverslips and probed with anti-GPR41 and anti-Na/K-ATPase antibody followed by FITC-labeled and Alexa Fluor 568 secondary antibody, respectively. Controls were labeled with secondary antibodies only and are shown in Figure E11. Cell nuclei were visualized using DAPI (blue), GPR41 and Na/K-ATPase were visualized as green and red fluorescence, and colocalization is shown in white. Images shown are representative of three independent experiments. Scale bar, 5 μm. (D) qRT-PCR was performed in vitro using CFBE41o- and 16HBE14o- epithelial cells and (E) in vivo using bronchial brushings from PWCF (n = 3, mean age 23.0 ± 4.0 years, 2 males and 1 female) and non-CF controls (n = 3, mean age 56.3 ± 9.5 years, 3 females). Expression of GPR41 relative to GAPDH was determined using the $2^{(-\Delta\Delta Ct)}$ method and presented as fold differences. Data are presented as means ± SEM (n ≥ 3). ** $P \leq 0.01$, *** $P \leq 0.001$. 
Figure 6. The mechanism of GPR41 upregulation in CF bronchial epithelial cells. (A) 16HBE14o- cells were treated with thapsigargin (2 µM), tunicamycin (2 µg/ml) or DTT (1 mM) for 24 h and the GPR41 protein levels were evaluated by western blot analysis. Whole cell lysates were separated by 12.5 % SDS-PAGE, transferred to a PVDF membrane and detected with anti-GPR41 antibody. Signal intensities of all bands were quantified by densitometry and normalized against GAPDH that served as loading control. Bar graph depicts densitometric analysis of the GPR41 expression. (B) 16HBE14o- cells were treated with CFTR(inh)-172 (10 µM) or 10% CF BAL for 24 and 6 h, respectively and the GPR41 mRNA levels were evaluated using qRT-PCR as described in materials and methods. Expression of GPR41 relative to GAPDH was determined using the $2^{(-\Delta\Delta C_t)}$ method and presented as fold differences. (C) 16HBE14o- cells were treated with CFTR(inh)-172 (20 µM) for 48 h or 10% CF BAL for 24 h and GPR41 protein levels were evaluated with western blot analysis as above. Data are presented as means ± SEM of at least three independent experiments. * $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$. 
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