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Infection with *Prevotella nigrescens* induces TLR2 signalling and low levels of p65 mediated inflammation in Cystic Fibrosis bronchial epithelial cells

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Abstract:

Prevotella spp. are frequently identified in Cystic Fibrosis sputum. This study examined whether infection with *Prevotella nigrescens*, a frequently identified member of this species, contributes to inflammation in CF bronchial epithelial cells through activation of TLR- and NF- κ B signalling pathways. CFBE41o- cells were infected with either *P.nigrescens* or *Pseudomonas aeruginosa* and incubated under anaerobic conditions for 4 hours. *P.nigrescens* activated TLR2 signalling but not TLR4 signalling while *P.aeruginosa* activated TLR4 signalling with a lesser effect on TLR2.

P.aeruginosa induced significant I κ B α phosphorylation 10 minutes post infection with a return to control levels by 30 minutes post infection. A significant induction in nuclear p65 DNA binding was observed at 2 hours post infection. In contrast, infection with *P.nigrescens* induced phosphorylation of I κ B α 120 minutes post infection, with significant induction in nuclear p65 DNA binding at 4 hours post infection only. Cytokine gene and protein responses were lower for *P.nigrescens* compared to *P.aeruginosa*.

This study demonstrates the ability of a clinical *P.nigrescens* isolate to provoke a delayed NF- κ B(p65) driven response through induction in TLR2 signalling and activation of sustained levels of IKK α .

Introduction:

Therapeutic advances combined with multi-disciplinary care have dramatically increased life expectancy for people with Cystic Fibrosis (PWCF) and infants born today are predicted to survive for almost 50 years¹. Such therapeutic approaches are helping drive targeted therapies to prevent disease progression^{2,3}. However, although current potentiator/corrector drugs improve the expression and function of the Cystic Fibrosis conductance Transmembrane Regulator (CFTR), they fail to reduce sputum inflammatory markers^{4,5}. Thus, the primary cause of mortality in PWCF is still progressive lung damage, caused by bacterial infection and subsequent inflammation in the lung³. Consequently, in order to prevent excessive infection and lung damage, there is still a need to enhance the understanding of pathogen-host interactions in CF airways.

Bacterial recognition by Toll-Like Receptors (TLRs) results in activation of canonical nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) signalling in mammalian cells, and subsequent release of inflammatory cytokines and chemokines^{6,7}. Induction of these pathways requires the adaptor proteins myeloid differentiation primary response 88 (MYD88), Interleukin-1 Receptor (IL-1R) associated kinases (IRAK), inhibitor of kappa B ($\text{I}\kappa\text{B}\alpha$) and translocation of the NF- κ B subunits p65/p50 into the nucleus. Resolution of the cascade is complex and depends on newly synthesized $\text{I}\kappa\text{B}\alpha$ to localise p65 back to the cytoplasm, the zinc-finger protein A20 to eliminate TNF receptor associated factor 6 (TRAF6) activation, and the induction of $\text{I}\kappa\text{B}$ kinase α (IKK α), which promotes p65 and c-Rel turnover and removal in TLR expressing cells⁸⁻¹⁰.

P.aeruginosa is a major contributor to inflammation in the CF lung, together with other well-studied organisms causative of inflammatory responses in airway cells in PWCF such as *Staphylococcus aureus*, *Haemophilus influenzae* and non-fermenting Gram-negative bacteria such as *Burkholderia cepacia* complex³.

P.aeruginosa infections are dynamic and complex. *P. aeruginosa* can activate TLR4 (through LPS) and TLR5 (through flagellin) and driving a TLR/NF- κ B-dependent inflammatory cascade in bronchial epithelial cells, resulting in secretion of elevated levels of IL-8, further contributing to the variety of inflammatory responses in CF lungs^{11,12}. In mouse lungs, flagellin production gave *P.aeruginosa* a fitness disadvantage, while flagella-negative strains had enhanced fitness¹³. From the host's point, a TLR-based response to a pathogen is the desired event to promote the elimination of the pathogen¹⁴, but in chronic infection of CF airways *in vivo*, in addition to the loss of TLR5-activating flagellin, *P.aeruginosa* modifies its LPS (lipid A) causing less TLR4 activation^{15,16}, and it has been suggested that

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other well-studied *P.aeruginosa* virulence factors (e.g. exopolysaccharide, avoiding adaptive immunity) may also contribute to the progression of CF lung disease ¹⁷.

Molecular techniques based on next generation pyrosequencing have identified a diverse microbiome in the lungs of healthy volunteers and PWCF. Such microbiomes include a number of anaerobic Gram-negative *Prevotella* species; however few studies have explored the biological role of this species in contributing to infection and inflammatory responses in the lungs of PWCF ¹⁸⁻²¹. *P. nigrescens* is an exemplar of this species and has been identified by culture and sequencing in sputum obtained from PWCF sputum ²².

Previous studies utilising heat killed bacteria, bacterial lysates and supernatants have shown that *P. nigrescens* possesses the ability to degrade host anti-proteases and suppress IL-4 responses in mammalian and murine experimental systems, provoking a range of inflammatory responses ²³⁻²⁵.

However, currently no studies have examined the role of live *P.nigrescens* on inflammatory responses in airway cells in PWCF. We hypothesized that the presence of *P.nigrescens* in the lung of PWCF may contribute to the elevated levels of inflammatory responses observed in CF airway epithelial cells and aimed to investigate the role of TLR and nuclear NF-κB signalling pathways involved in this response.

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103 Methods

104 Bacterial cell culture

105 We have used clinical isolates obtained from a patient attending the adult CF clinic at Belfast City
106 Hospital throughout the study. The two isolates studied here were derived from the same patient:
107 *P. nigrescens* isolate B021V1S1 (B = Belfast, 021= patient, V1S1 = first visit stable sample, herein
108 abbreviated B021S) was grown under anaerobic conditions for 72 hours using a Don Whitley anaerobic
109 cabinet as described previously⁹. The *P. nigrescens* strain utilised was *P. nigrescens* B021S identified
110 through the use of 16 S rRNA sequencing²². Further information given in the supplement.
111 *P. aeruginosa* isolate B021 was grown under aerobic conditions (37°C, 5 % CO₂) for 24 hours before
112 infection of cells. This culture was used to inoculate a liquid culture of Lysogeny Broth, which was
113 incubated for 2 hours under aerobic conditions at 200 rpm before being used to infect cells.

114 Cell culture

115 A Cystic Fibrosis bronchial epithelial cell line homozygous for Phe508del (CFBE41o-, a kind gift from D
116 Gruenert, USF) was maintained in the absence of antibiotic supplementation under standard cell
117 culture conditions (37°C, 5 % CO₂, 95 % mixed gas). Cells were passaged as described previously (7).
118 HEK-293-TLR2, HEK-293-TLR4, HEK-293-TLR5 and HEK-293-TLR null cells were maintained as per
119 manufacturer's instructions (InvivoGen). Once infected with the clinical isolates of *P. aeruginosa* or
120 *P. nigrescens*, cells were incubated anaerobically for the duration of the experiments (up to 4h).

121 Inflammatory studies

122 CFBE41o- cells were seeded into 48, 24 and 6 well tissue culture plates (Nunc). At 80% confluency
123 media was removed and the cells were washed with phosphate buffered saline (PBS, Gibco) and pre-
124 warmed fresh media (MEM) was added to each cell population. Cells were infected with an MOI of 100
125 of either *P. nigrescens* or *P. aeruginosa* as defined by plating serial dilutions of each inoculum. Cells
126 were then incubated under anaerobic environments for the duration of the experiments. The MOI
127 used was based on preliminary studies obtaining maximal responses (IL-8, p65) while ensuring cell
128 viability (minimal MTT and LDH responses, no changes in cell morphology) to infections with either
129 *P. nigrescens* and *P. aeruginosa*. Detailed information can be found in supplementary data S1.

130 TLR Reporter assays

131 HEK-293-TLR2, HEK-293-TLR4, HEK-293-TLR5 and HEK-293-TLR null cells were transiently transfected

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with an NF- κ B luciferase containing reporter construct (InvivoGen) and luciferase release from infected and uninfected control cells was assessed 4 hours post infection as described previously ²⁶.

Cytoplasmic and nuclear fraction extraction for DNA binding ELISA

CFBE410- cells were infected as described, and nuclear and cytoplasmic cell fractions were extracted from infected and uninfected control cells for assessment of IKK α , I κ B α , p65 and p50 by sandwich ELISA (TransAM™ Activ Motif I κ B α function ELISA, Cell Signalling Technology PathScan® Phospho-IKK α (Ser 176/180), TransAM™ Activ Motif NF- κ B family function ELISA).

Gene expression analysis

Innate immune response profilers were purchased from SA Biosciences. Total RNA was extracted from infected and uninfected cells and genomic DNA was eliminated from each sample as per manufacturer's instructions (SA Biosciences).

RT-PCR for TLR and Cytokine gene expression

Total RNA was extracted from infected cell and uninfected control cell populations using a combination of Trizol reagent and column extraction (Thermo Fisher Scientific). qPCR analysis was carried out on an MX300-P (Agilent technologies) using SYBR green primers (QuantiTect, Qiagen) and the SYBR Green PCR master mix kit (Thermo Fisher Scientific).

siRNA knockdown

Reverse transfection was used in all siRNA assays. siRNA (Thermo Fisher Scientific) was diluted to a working concentration of 20 pmol/mL and reverse transfection was carried out as per manufacturer's instructions (Thermo Fisher Scientific). Target gene and protein expression were assessed at 24-48 hours post transfection by qPCR to confirm successful knockdown. Average knockdown was found to be >70%. Further details can be found in the supplementary data S2.

Statistical analysis

All experiments were performed in duplicates and data of three independent replicates are expressed as mean +/- standard deviation. Parametric tests including t-test to assess the null hypothesis, or 1-Way ANOVA with Bonferroni post-test correction were used to assess any significant differences in the mean values in the following experiments. Two-way ANOVA was used to assess the variation between matched samples over time with Bonferroni correction.

Results:

NF- κ B signalling in CFBE410- cells infected with *P.nigrescens* or *P.aeruginosa*

NF- κ B activation and cytokine gene expression were determined 2 and 4 hours after infection. In response to *P.aeruginosa* CFBE410- cells exhibited a significant induction in phosphorylated I κ B α protein at 10 minutes (67% increase) and a significant increase in nuclear p65 DNA binding at both time points analysed (2 and 4 h, 2.8- and 2-fold increase, respectively), (Figure 1A-1B). In keeping with the significant p65-DNA binding in response to *P.aeruginosa*, a 5-fold increase in IL-6 gene expression and an 11-fold increase in IL-8 gene expression were observed 4 hours post infection (Figures 1C-1D). When infected with *P.nigrescens* CFBE410- cells displayed 70% increase in phosphorylated I κ B α protein 2 hours post infection leading to a 2-fold increase in nuclear p65 observed at 4 hours post infection (Figures 1A, 1B). Significantly lower levels of IL-6 and IL-8 gene expression were observed in *P.nigrescens* infected cells compared to *P.aeruginosa* infected cells at 4 hours, however the 2 hour levels of IL-6 mRNA observed from cells infected with *P.nigrescens* were significantly higher when compared to baseline controls.

Figure 1: Inflammatory responses from CFBE410- cells infected with *P.nigrescens* or *P.aeruginosa* (MOI 100) and uninfected controls. **1A)** Time course of I κ B α phosphorylation; **1B)** p65 DNA binding at 2 and 4 hours; **1C)** IL-6 gene expression at 2- and 4-hour post infection and **1D)** IL-8 gene expression at 2- and 4-hours post infection. All n=3, *p<0.05, **p<0.01, ***p<0.001, 2-Way ANOVA with Bonferroni post-test, overall ANOVA given within the graph.

TLR signalling in response to infection with *P.nigrescens* in HEK-293 TLR cells and CFBE41o- cells

HEK 293 reporter cell lines were infected with *P.aeruginosa* or *P.nigrescens* in order to determine if infection with either isolate would activate TLR signalling.

A 20-fold induction in TLR2 signalling was observed at 4 hours post infection with *P.aeruginosa* while a 30- and 40-fold induction was observed at 2 and 4 hours post infection with *P.nigrescens* in HEK-293-TLR2 cells. A 16-fold up regulation in TLR4 signalling was observed in HEK-293-TLR4 cells at 1 hour post infection with *P.aeruginosa* and a 20-fold induction was observed at both 2 and 4 hours post infection with *P.aeruginosa* in HEK-293-TLR4 cells (Figures 2A, 2B). An 11-fold induction in TLR5 signalling was observed in HEK-293-TLR5 cells in response to infection with *P.aeruginosa* at 2 hours post infection and a 19-fold induction was observed at 4 hours post infection (Figure 2C).

Figure 2: TLR signalling using HEK-TLR reporter cell lines: **2A)** HEK-293-TLR2; **2B)** HEK-293-TLR4 and **2C)** HEK-293-TLR5 signalling in response to infection with either *P.nigrescens* or *P.aeruginosa* (MOI 100). Infected cell populations were compared to non-infected cell populations. All n=3, *p<0.05, **p<0.01, ***p<0.001, 2-Way ANOVA with Bonferroni post-test, overall ANOVA given within the graph.

TLR signalling activates p65 NF-κB signalling in CFBE41o- cells infected with *P.nigrescens*.

siRNA mediated knockdown of TLR2 and TLR5 in CFBE41o- cells confirmed that *P.nigrescens* infection activated TLR2 signalling in CFBE41o- cells and not TLR5 signalling, as measured by a 39 % reduction in p65 DNA binding observed at 4 hours post infection in the TLR2 knockdown experiments (Figure 3B).

IL-6 gene expression was reduced in TLR2 knockdown cells infected with *P.nigrescens* when compared to wild-type cells infected with *P.nigrescens* however this reduction was not statistically significant (Figure 3D). No significant decreases in IL-6 gene expression or the DNA binding abilities of nuclear p65 were observed in cells transfected with an siRNA targeted against TLR5 at any time point (Figures 3A, 3C).

Figure 3: Effect of TLR2 and TLR5 knockdown on NF- κ B induced inflammation in CFBE41o- cells. CFBE41o- cells were infected with *P.nigrescens* or *P.aeruginosa* (MOI 100, 2 or 4 hours) and compared to non-infected and to wild-type *P.nigrescens* infected CFBE41o- cells. Scrambled and sham transfections were compared to non-stimulated controls. **3A)** IL-6 gene expression in response to TLR2 knockdown (MOI 100, 2 hours). **3B)** p65 phosphorylation with TLR2 siRNA knockdown (MOI 100, 4 hours). **3C)** IL-6 gene expression in response to TLR5 knockdown (MOI 100, 2 hours). **3D)** p65 DNA binding in CFBE41o- cells with TLR5 siRNA knockdown (MOI 100, 4 hours). All n=3, *p<0.05, **p<0.01, ***p<0.001, 1-Way ANOVA with Bonferroni post-test, overall ANOVA given within the graph.

The contribution of IKK α to inflammation induced by infection by *P.nigrescens* in CF bronchial epithelial cells.

CFBE41o- cells displayed sustained 3-fold induction in IKK α phosphorylation in response to infection with *P.nigrescens* as measured at t=0-60 minutes (Figure 4A). *P.aeruginosa* infection, in contrast, only resulted in a 2-fold increase in IKK α phosphorylation 60 minutes post infection (Figure 4A). siRNA mediated knockdown of IKK α resulted in the restoration of nuclear p65 DNA binding at 2 hours in cells infected with *P.nigrescens*. A 2.7-fold induction in nuclear p65 DNA binding was observed in CF cells transfected with siRNA targeted against IKK α and infected with *P.nigrescens* suggesting a role for IKK α in repressing inflammation in CF bronchial epithelial cells in response to infection with *P.nigrescens* (Figure 4 B).

Figure 4: CFBE410- cells infected *P.aeruginosa* or *P.nigrescens* (MOI of 100) incubated under anaerobic conditions. **4A)** Time course IKK α phosphorylation in CFBE410- for up to 1 hour (n=3, ***p<0.001, 2-way ANOVA with Bonferroni post-test). **4B)** p65 DNA binding in CFBE410- cells transiently transfected with siRNA targeted against IKK α and infected with *P.nigrescens* (MOI 100) for 2 hours. Infected cell populations were compared to non-infected cell populations; siRNA knockdown populations were compared to *P.nigrescens* infected cells and scrambled and sham transfections were compared to non-stimulated controls (n=3-6, **p<0.01, 1-way ANOVA with Bonferroni post-test).

Infection by *P.nigrescens* activates MAPK/p38 pathway in CF bronchial epithelial cells.

Finally, as our data strongly suggest that *P.nigrescens* B021S signals through TLR2 without the involvement of IKK α , we investigated the MAPK/p38 pathway. Our gene expression data (mRNA) shows a significant induction of IL-12 after 4h of stimulation with *P.nigrescens* B021S (Figure 5).

Figure 5: Gene expression (mRNA) of selected genes of the TLR and MAPK/p38 pathway 4h after infection with *P.aeruginosa* B021 and *P.nigrescens* B021S (n=3-5, ***p<0.001, 2-way ANOVA with Bonferroni post-test, overall ANOVA given within the graph).

Discussion:

Prevotella spp. are prevalent commensal colonizers at mucosal sites; both in the respiratory system²⁷ as well as airway secretions and saliva²⁸. *Prevotella* spp. have also been identified in high numbers in CF airway secretions and sputum^{20, 29}, although their contribution to lung disease in PWCF through changing quantity and diversity is still debated^{29, 30}. The presented study is the first to examine the contribution of a clinical *P. nigrescens* isolate to inflammatory responses in CF epithelial cells. Our clinical isolate *P. nigrescens* (B021S) activates TLR2 signalling resulting in a moderate induction of NF- κ B mediated inflammation compared to the inflammatory response from one *P. aeruginosa* strain (B021) isolated from the same patient (Figure 3A).

In PWCF, infection with *P. aeruginosa* results in NF- κ B-driven IL-8 release from bronchial epithelial cells³¹. Infection with *P. nigrescens* B021S also induced an increase in IL-8 gene expression in CFBE410- cells, however, at a much slower rate than in *P. aeruginosa* B021.

P. aeruginosa B021 induces NF- κ B-driven inflammatory responses through a robust induction of TLR4 signalling in CFBE410- cells, activating transcription of inflammatory cytokines¹¹. Infection with *P. nigrescens* B021S significantly induced early TLR2 signalling in CFBE410- (starting 2 hours post infection), and TLR5 signalling (4 hours post infection). However, our *P. nigrescens* strain did not utilise TLR4 signalling. Furthermore, we observed a delayed and attenuated inflammatory response from CFBE410- cells (Figure 1C, 1D) compared to *P. aeruginosa* B021 infection, which could possibly be attributed to the increased activation of IKK α in response to infection (Figure 4A).

Surprisingly, CFBE410- cells infected with *P. nigrescens* B021S demonstrated significantly elevated levels of IL-6 gene expression at 2 hours, in contrast to the levels observed for the *P. aeruginosa* infection (Figure 1C), indicating a different inflammatory pathway was activated in response to infection with this isolate.

Some species of *Prevotella* may engage TLR2 signalling rather than TLR4 signalling and it has been hypothesised that this may result in a less damaging inflammatory response and a switch to adaptive T_H2 responses *in vivo*³². In order to confirm if infection with our *P. nigrescens* strain resulted in the activation of TLR2 signalling rather than TLR4 signalling, HEK-293-TLR2 and HEK-293-TLR4 cells were transfected with an NF- κ B reporter plasmid and luciferase release from both cell types in response to infection was measured over time. In this model, infection with *P. nigrescens* B021S induced TLR2 signalling rather than TLR4 signalling (Figure 2A, 2B), confirming our earlier results. In contrast to *P. nigrescens*, infection with *P. aeruginosa* B021 induced the expected predominantly TLR4 signalling

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response, as measured by siRNA knockdown in CFBE410- cells, in part explaining the differences in responses from the cells in response to infection with the two different bacteria.

We also included TLR5 (HEK-293-TLR5) into our investigations. TLR5 signalling of *in vitro* grown flagellated *P.aeruginosa* (LPS smooth) is well-established^{33,34}, but the actual pathogens in CF lung disease causing chronic infections are mucoid isolates that to some degree, may have shut off flagella production to avoid immune detection by the host^{34,35}. However, up to 75% of mucoid clinical isolates retain their TLR5 activating capacity during chronic CF lung infection³⁶. The clinical isolate of *P.aeruginosa* we studied here may still express flagella, as HEK-293-TLR5 show significant TLR5 signalling after infection (Figure 2C). *Prevotella spp.* may lack flagellin as none or only one flagella synthesis pathway gene has been identified and therefore may use other structures for attachment and motility³⁷. Although we show TLR5 activation 4h after infection with *P.nigrescens* B021S, we did not further investigate this TLR5 activation in our experimental cell model as our TLR-siRNA experiments showed that NF-kB activation (p65-DNA binding) in B021S infected CFBE410- cells was due to TLR2 and not TLR5 signalling (Figure 3B, 3D). To date, flagellin is the only ligand identified for TLR5³⁸, but TLR5 activation may also occur through non-flagellin agonists³⁹. Therefore, the increase in TLR5 activation seen here could be due to non-specific (non-flagellin) activation, for instance, through residual broth in the bacterial preparations.

Despite a significant decrease in NF-kB activation (Figure 3B), IL-6 gene expression was not significantly reduced in *P.nigrescens* B021S infected CFBE410- cells with TLR2 knock down (Figure 3A), suggesting that activation of other signalling pathways may contribute IL-6 expression in response to this isolate.

IL-6 induction is generally regarded as NF-kB and also MAPK/p38 driven, but using LPS stimulated epithelial cells of the bladder, Song *et al.* showed that in addition to the classical NF-kB-associated pathway, TLR4 triggers a distinct and more rapid signalling response involving secondary messengers (Ca²⁺, adenylyl cyclase 3-generated cAMP, and a transcriptional factor, cAMP response element-binding protein). Although they did not investigate if this pathway is also activated after sole TLR2 engagement⁴⁰, such non-NF-kB induction of IL-6 could provide a possible explanation of our finding.

In order to ascertain why the inflammatory response was delayed and less robust in CFBE410- cells infected with *P.nigrescens* B021S when compared to the responses observed for *P.aeruginosa* B021 infection we studied phosphorylation of IKK α in response to infection. Studies in neuronal cells have shown that IKK α can reduce production of inflammatory cytokines through repression of IKK β while *ikk α* ^{AA/AA} macrophages (bone marrow derived) are resistant to apoptosis and display elevated levels of p65 signalling, demonstrating that IKK α can also have a regulatory effect on TLR expressing cells⁹.

Phosphorylation of IKK α by NF- κ B-inducing kinase (NIK) contributes to resolution of the inflammatory response in macrophages⁹. We therefore measured phosphorylated IKK α in response to infection with and without siRNA knockdown of IKK α . CF bronchial epithelial cells transfected with siRNA against IKK α display significantly elevated levels of p65 DNA binding at 2 hours post infection (fold change) as compared to WT controls, suggesting that sustained phosphorylation of IKK α in CF bronchial epithelial cells contributes to resolving inflammation in response to infection.

Finally, as our data strongly suggest that *P. nigrescens* B021S signals through TLR2 activating NF- κ B/p65 without the involvement of IKK α (canonical and non-canonical NF- κ B pathway), we investigated the MAPK/p38 pathway. MAPK/p38 activation drives IL-12-induced IFN- γ expression in activated T cells⁴¹ and infected macrophages⁴². Our mRNA analyses shows a significant induction of IL-12 after 4h of stimulation with *P. nigrescens* B021S compared to infection with *P. aeruginosa* B021. Furthermore, IRF-7 (IFN Regulatory Factor-7) expression is also significantly increased 4h after infection with *P. nigrescens* B021S. In humans dendritic cells MAPK/p38 enhances IRF-7 nuclear translocation⁴³, therefore, IRF-7 expression may also be interpreted as a MAPK/p38 target. Further to this we also observed elevated levels of TNF α mRNA as well as up-regulation of the TNF receptor TNFRSF1A (TNFR1). TNF α activates and regulates MAPK pathways, causing both activation of MAPK3-MKKK1/2-ERK1/2, and regulation of a secondary, downstream cytokine response in response to an initial stimulation with LPS. This may in part explain our IL-6 data, which was not NF- κ B dependent.⁵⁵ Our data on IL-6 mRNA (Fig 1C) and TLR2 activation (Fig 2A) suggest that *P. nigrescens* B021S causes an earlier response (2h) than *P. aeruginosa*. We only investigated the response of CFBE41o- cells to *P. nigrescens* B021S 4h after the infection, therefore, we might have missed the early upregulation of upstream TAK1 and MAPK genes AP1 and MAPK14. Taken together, these data tie the early TLR2 activation by *P. nigrescens* B021S to the MAPK pathway (Figure 5 and 6).

Microbiome studies have identified the presence of *Prevotella* spp. in CF sputum and some studies suggest that *Prevotella* spp. may provoke a consistent low grade inflammatory response in the lung which is profoundly less damaging to the host than the exuberant responses observed during infection with other Gram-negative bacteria^{12,44}. Further studies have correlated declining lung function and elevated levels of CRP with a lower microbial diversity in the lung and reduced levels of *Prevotella* spp.^{45,46}. Larsen *et al.* proposed that the activation of TLR2 by Gram-negative anaerobic bacteria and the subsequent weak inflammatory response from the host cell ensures that colonisation of the respiratory system by these bacteria is tolerable for the host³². Our study supports these observations as *P. nigrescens* B021S infection resulted in a low grade TLR2 signalling response in CF bronchial epithelial cells, which is possibly less destructive and more tolerable to the host than that induced by

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Gram-negative *P.aeruginosa* B021. TLR2 signalling could also contribute to the establishment of host-microbial symbiosis ⁴⁷, but the beneficial effect on lung immune response to infection in CF needs further investigation.

While it appears that infection with *P.nigrescens* B021S may induce an inflammatory response in CFBE41o- cells which is less 'aggressive' than that induced by *P.aeruginosa* B021, we are aware that the inability of the cell lines to remain under anaerobic conditions for more than 4 hours has resulted in a significantly shorter experimental time-frame than other studies which assess inflammatory responses to infection for up to 24 hours post infection ⁴⁸. Further, the reduced oxygen levels in the media may have hampered the cells' ability to respond to infection, however, even under the applied anaerobic conditions, the cells displayed a robust inflammatory response to infection with *P.aeruginosa* indicating that the environment did not hinder the ability of the CFBE41o- cells to respond to infection.

The main limitation of our study is the use of only one *Prevotella* strain and one *P.aeruginosa* strain. While only one *P.aeruginosa* isolate was used throughout this study we also screened a range of clinical *P.aeruginosa* isolates and compared them to the inflammatory responses obtained from CFBE41o- cells infected with PAO1. This was to ensure that our *P.aeruginosa* induced inflammatory responses were representative of those published in previous studies.

P.nigrescens was chosen as a representative strain of many *Prevotella* species found in PWCF. This strain was from a clinical sputum sample from a person with CF. Previous studies have investigated other clinical isolates of *P.aeruginosa* and *P.nigrescens* ^{22, 49, 50}, and we aimed to compare a representative strain of *P.aeruginosa* to a representative strain of a common *Prevotella* species, in this case *P.nigrescens*.

Figure 6: Schematic representation of the potential signal transduction pathways utilised by the clinical isolate *P.nigrescens* B021S in comparison to the pathways used by *P.aeruginosa* B021 in CF airway epithelial cells.

In conclusion, there is evidence that the clinical isolate *P.nigrescens* B021S is associated with a lower inflammatory response in CF epithelial cells than the clinical isolate *P.aeruginosa* B021. Several studies have shown that *Prevotella intermedia* activates immune responses through the MAPK/AP1 pathway^{51,52} and our study suggests that *P.aeruginosa* B021 may also employ the MAPK/AP1 pathway. Furthermore, our data would suggest that TLR2 signalling by *P.nigrescens* B021S may not only activate MAPK, but also p65, even when IKKα is knocked down. MAPK-p38 activation lead to the activation of AP1, may also activate p65/p50 via an NF-κB translocation-independent mechanism⁵³. While we did not explore MAPK-38 driven pathways in CFBE41o- cells in response to infection with our clinical *P.nigrescens* isolate (B021S), our gene expression data outlined here indicate that this signalling

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pathway may also be activated in our cells in response to infection with *P.nigrescens* B021S. This may in part also explain the differences observed in NF-κB driven inflammatory responses in this study between the *P.nigrescens* B021S and *P.aeruginosa* B021 isolates. Figure 6 summarises the potential signalling pathway of *P.nigrescens* B021S in comparison to the pathways used by *P.aeruginosa* B021. However, to confirm these findings across *Prevotella* spp., additional *P.nicrescens* strains need to be investigated. Furthermore, to fully understand the complex inflammatory environment present in the CF lung future work will also investigate mixed infections.

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Conflict of interest statement:

The authors have no conflict of interest, financially or otherwise.

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