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Mucoid *Pseudomonas aeruginosa* caused by *mucA* mutations result in activation of TLR2 in addition to TLR5 in airway epithelial cells

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ABSTRACT

The presence of the mucoid phenotype of *Pseudomonas aeruginosa* is a marker of poor survival in cystic fibrosis. As CF lung disease results from chronic infection leading to airway inflammation, we determined whether the switch to a mucoid phenotype by *P. aeruginosa* has an impact on the inflammatory response of airway epithelial cells. Exposure of airway epithelial cells to non-mucoid and mucoid *P. aeruginosa*-derived material leads to p38α MAPK activation, a key protein kinase involved in transmitting inflammatory signals. However, while the non-mucoid strain PAO1 activates p38α MAPK pathway solely via TLR5, the mucoid strain PACF508 activates p38α MAPK via both TLR5 and TLR2. Inactivation of *mucA* (the gene responsible for the mucoid phenotype) in PAO1 leads to p38α MAPK activation by both TLR2 and TLR5, as observed in the clinical mucoid isolate PACF508. Therefore, the switch to mucoid phenotype may contribute to more inflammation via TLR2 activation in addition to TLR5. Our findings highlight an important and under recognized role for TLR2 in the response of airway epithelial cells to infection.

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1. Introduction

Cystic fibrosis (CF) is characterized by mucus hyper-secretion, chronic infection and inflammation associated with decreasing lung function [1,2]. Therefore signals that increase inflammation in chronically infected CF patients may worsen decline in lung function. Markers of inflammation are increased at the onset of pulmonary exacerbations [3] and following these episodes, a net decline in lung function has been documented from pre-exacerbation state [21]. Unfortunately, very little is known about pulmonary exacerbations. They are probably related to a complex relationship between host defense and airway microbiology. One model proposes that exacerbations are caused by the release and proliferation of planktonic bacteria from biofilm aggregates [4].

Pseudomonas aeruginosa is the most significant pathogen in CF with up to 80% of patients eventually chronically infected with *P. aeruginosa* [5]. Moreover, clinically the presence of the mucoid phenotype of *P. aeruginosa* is a marker of poor survival in CF [6,7]. The mucoid phenotype is typically attributed to mutations in the *mucA* gene, a negative regulator of the stress sigma factor AlgU [8].

Activation of innate immunity in response to pathogens is mediated via pattern-recognition receptors (PRRs) expressed by host cells. The p38α mitogen-activated protein kinase (MAPK) is an important mediator of inflammatory signaling that plays a role in host defenses against *P. aeruginosa* in vertebrates [9]. Activation of p38α MAPK in response to *P. aeruginosa* has been linked to the flagellin receptor Toll-like receptor 5 (TLR5) expressed at the surface of airway epithelial cells [10].

In this report, we investigated if the switch to a mucoid phenotype by *P. aeruginosa* has an impact on the activation of p38α MAPK in airway epithelial cells.

2. Materials and methods

2.1. Materials

All chemicals were bought from Fisher Scientific (Fair Lawn, NJ, USA). Zeocin, Hygromycin, Blasticidin, Normocin, FSL-1, LPS from *P. aeruginosa* and *S. typhimurium* flagellin were bought from InvivoGen (San Diego, CA, USA).

2.2. P. aeruginosa strains

Two strains of *P. aeruginosa* were investigated: the common laboratory strain PAO1, and PACF508, a stable mucoid clinical isolate from the sputum of a patient with CF (CFTRΔF508 homozygous;

Abbreviations: AEC, airway epithelial cells; CF, cystic fibrosis; CFTR, CF transmembrane regulator; MAPK, mitogen activated protein kinase; PRR, pattern recognition receptor; PsaDM, *P. aeruginosa* diffusible material; SCFM, synthetic CF media; TLR, Toll-like receptors.

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Hôpital Sainte-Justine, Montréal) [11]. The *flgK* and *mucA* mutants are transposon mutants obtained from the PAO1 transposon library [12].

2.3. P. aeruginosa diffusible material preparation

P. aeruginosa diffusible material from planktonic bacteria (PsaDM) was obtained from bacteria grown in peptone (Fisher Scientific) or synthetic CF medium SCFM [13] as previously described [14,15]. Prior to use, bacterial filtrates were heat inactivated at 95 °C for 10 min (to inactivate proteases) and allowed to cool to room temperature.

2.4. Antibodies

Neutralizing antibodies against TLR2, TLR4 and TLR5 were used at 5 μg/mL and purchased from InvivoGen (CA, USA). Anti-GAPDH (used at a concentration of 1/4000) and anti-phospho p38 MAPK (Thr180/Thr182; used at 1/1000 dilution) were purchased from Millipore (Temecula, CA). Anti-p38 MAPK (used at a dilution of 1/1000) was purchased from Cell signaling (Boston, Ma). Goat anti-rabbit IgG DyLight™800 (35,571; 1:15,000) and Goat anti-mouse IgG DyLight™680 (35,518; 1:15,000) were bought from Thermo Scientific (Rockford, IL, USA).

2.5. Cell culture

BEAS-2B AECs were cultured as previously described [11]. HEK-Blue TLR5 cells and HEK-Blue Null1 cells were purchased from InvivoGen (San Diego, CA). HEK-Blue TLR5 were grown and maintained in DMEM supplemented with 10% FBS with 100 U/mL penicillin G, 100 μg/mL of streptomycin, 100 μg/mL normacin, 100 μg/mL Zeocin and 30 μg/mL of blasticidin. 24 h prior to stimulation, cells were starved in DMEM without antibiotics. HEK-Blue Null1 were grown and maintained in DMEM supplemented with 10% FBS with 100 U/mL penicillin G, 100 μg/mL of streptomycin, 100 μg/mL normacin and 100 μg/mL zeocin. 24 h prior to stimulation, cells were starved in DMEM without antibiotics. Human airway epithelial cell line NuLi was derived from a normal lung of a 36-year-old male patient and CuFi airway epithelial cell line derived from lung of a 14-year-old female patient with cystic fibrosis homozygous for the CFTRΔF508 mutation were cultures as previously described [15].

2.6. Cell lysis and immunoblotting

Following stimulation, cells were lysed in ice-cold buffer (50 mM Tris–Cl pH 7.5, 1 mM EGTA, 1 mM EDTA, 1% (v/v) Triton X-100, 1 mM sodium orthovanadate, 5 mM sodium pyrophosphate, 0.27 M sucrose, complete mini protease inhibitor cocktail and 2 mM DTT). Proteins were quantified using the Bradford

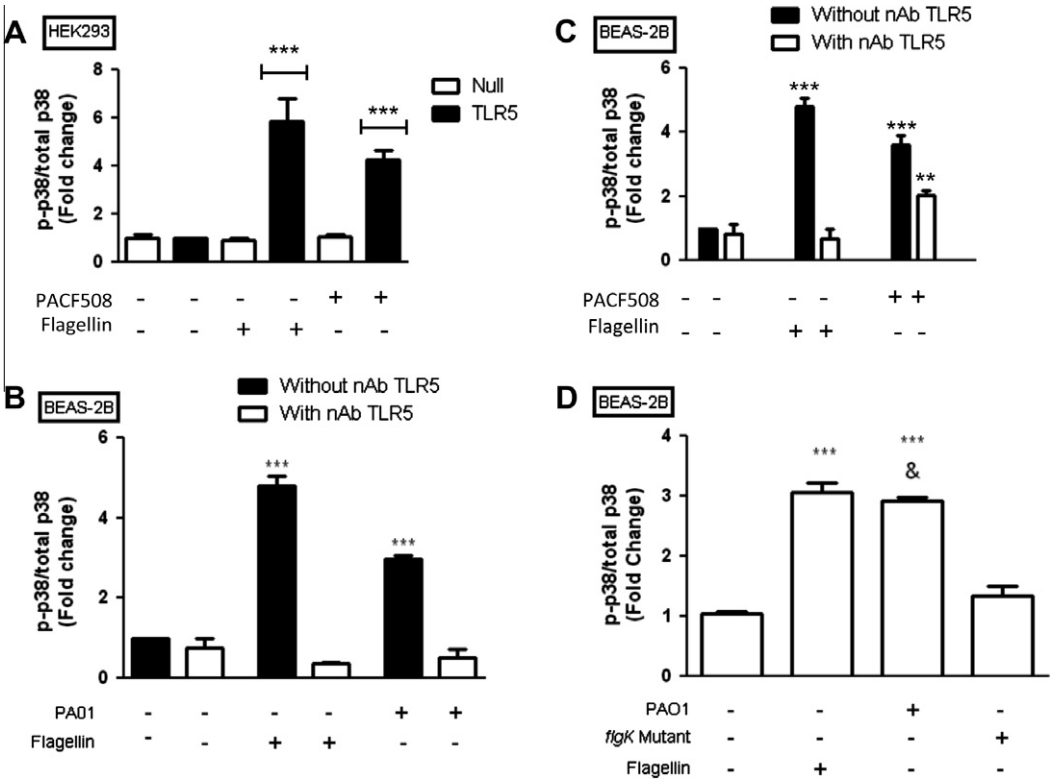

Fig. 1. TLR5 leads to p38 MAPK activation in AECs exposed to Planktonic PsaDM. (A) Human embryonic kidney (HEK) cells lacking (null, white bars) or expressing TLR5 (TLR5, black bars) were left untreated (−) or treated (+) with 0.4 μg/mL flagellin or 1 μg/mL of planktonic PACF508 PsaDM grown in SCFM for 30 min. Following stimulation, cells were lysed and 20 μg of Triton-soluble materials was subjected to SDS–PAGE. After transfer to nitrocellulose, the membranes were probed with antibodies recognizing only the phosphorylated forms of p38α MAPK or antibodies that recognize all forms of p38α MAPK. Quantitative analysis of the signals from each antibody was performed using Li-Cor infrared Odyssey imaging system. (B and C) BEAS-2B AECs were left untreated (−) or pretreated for 30 min without (black bars) or with 5 μg/mL neutralizing TLR5 antibody (white bars) followed by exposure (+) for 30 min to 0.4 μg/mL of flagellin or 1 μg/mL of planktonic PACF508 PsaDM. p38α MAPK activation was determined as in Fig. 1A. (D) BEAS-2B AECs were left untreated (−) or stimulated (+) for 30 min with 1 μg/mL planktonic PsaDM from wildtype PAO1 or a *flgK* mutant. p38α MAPK activation was determined as in Fig. 1A. TLR5 nAb: TLR5 neutralizing antibody. The mean of three experiments is shown +/− SEM. Statistics were performed using one-way ANOVA with Bonferroni post-test analysis. $^{***,**}p \leqslant 0.001$ or 0.01, respectively compared to unstimulated; $^{\#}p \leqslant 0.05$ in absence of TLR5 nab compared to its presence. $^{\&}p \leqslant 0.05$ wildtype PAO1 compared to PAO1*flgK*.

method, 30 μg of lysates were submitted to SDS–PAGE, transferred to nitrocellulose and immunoblotted with specified antibodies. The signal was detected and quantified using a Licor Odyssey imaging system.

2.7. Statistical analysis

Analyses of variance (ANOVA) followed by a multiple comparison test (Bonferroni) were used to test differences in mean between groups. p values <0.05 were considered significant.

3. Results and discussion

3.1. TLR5 leads to p38α MAPK activation in AECs exposed to planktonic

To compare the impact of the switch to a mucoid phenotype by *P. aeruginosa* on activation of host inflammatory pathway activation, two *P. aeruginosa* strains were studied: the non-mucoid common laboratory strain PAO1 and a mucoid clinical isolate of PA (PACF508). Moreover, to further simulate growth conditions relevant to pulmonary exacerbations in CF airways, the two strains were prepared as planktonic cultures in synthetic CF sputum medium (SCFM) that mimics the nutritional composition of CF sputum [13]. Furthermore, since bacteria are found distal from airway surface, AECs likely interact and respond to diffusible bacterial products [16]. Therefore, AECs were exposed to planktonic *P. aeruginosa* diffusible material (PsaDM) form the two strains and the activation of p38α MAPK measured in view of the critical role of this protein kinase in transmitting inflammatory signals.

Given that flagellin is recognized by TLR5 at the surface of mammalian cells and is a potent activator of p38α MAPK [10], we first checked the contribution of TLR5 to host activation. To test this, HEK293 cells with or without TLR5 expressed at their surface were challenged with PACF508 PsaDM. p38α MAPK activation was only detected in TLR5 expressing HEK293 cells exposed to planktonic PsaDM (Fig. 1A). This shows that like PAO1, PACF508 also activates TLR5.

We next tested whether neutralizing TLR5 on AECs was sufficient to suppress p38α MAPK activation in response to planktonic PsaDM. As expected, neutralizing TLR5 prevented p38α MAPK activation in BEAS-2B AECs stimulated with flagellin (Fig. 1B) and abolished p38α MAPK activation in response to planktonic PsaDM from strain PAO1 (Fig. 1C). This was further supported by our finding that a PAO1 mutant lacking a flagella due to a *flgK* gene mutation [17] failed to activate p38α MAPK in BEAS-2B AECs (Fig. 1D). Intriguingly, blocking TLR5 activation only reduced p38α MAPK activation by 46% in response to the planktonic PACF508 PsaDM (Fig. 1B). Therefore, in contrast to the common laboratory strain used in many studies investigating activation of host defense

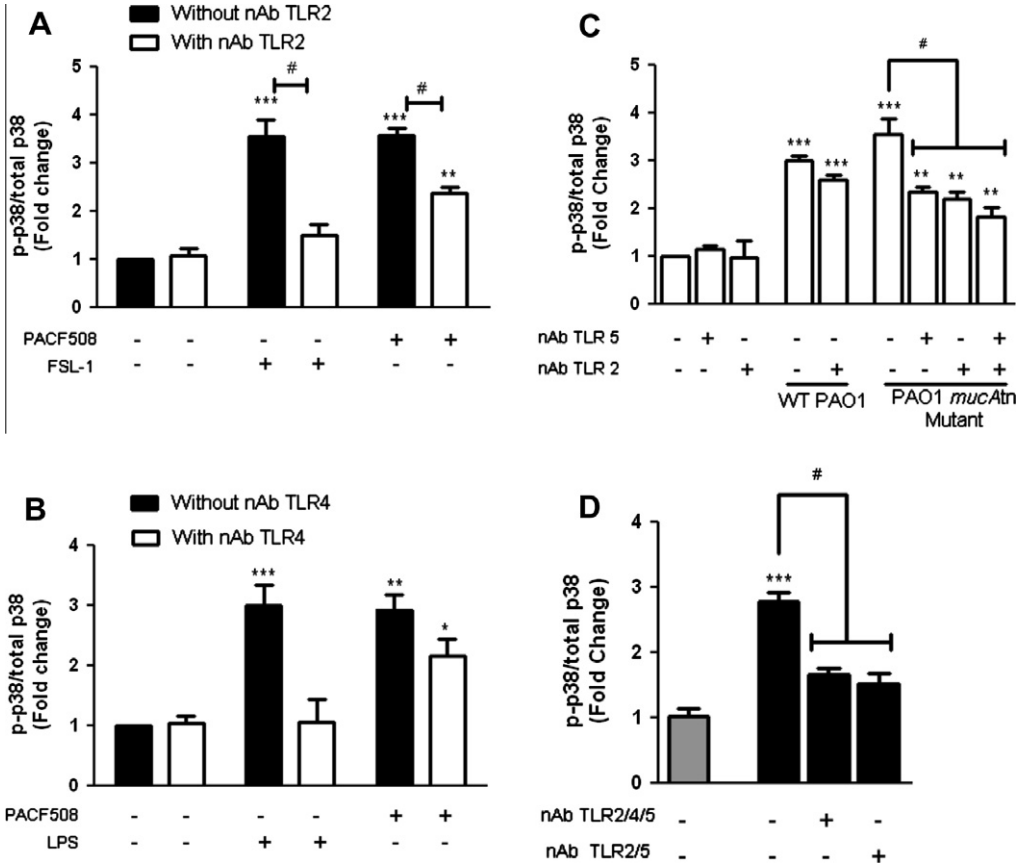

Fig. 2. The mucoid phenotype of *P. aeruginosa* leads to activation of TLR2. (A and B) BEAS-2B AECs were left untreated (−) or pretreated for 30 min without (black bars) or with (white bars) 5 μg/mL neutralizing TLR2 (A), TLR4 (B) neutralizing antibodies followed by exposure (+) for 30 min to 1 μg/mL of planktonic PsaDM, or 0.4 μg/mL of FSL-1 (A) or 0.5 μg/mL LPS (B). p38α MAPK activation was determined as in Fig. 1A. (C) BEAS-2B AECs were left untreated (−) or pretreated with (+) 5 μg/mL neutralizing TLR2 and/or 5 μg/mL neutralizing TLR5 for 30 min followed by exposure to wild type planktonic PAO1 PsaDM (PAO1 wt) or a PAO1 *mucA* mutant (PAO1 mucAtn). p38α MAPK activation was determined as in Fig. 1A. (D) BEAS-2B were left untreated (−) or pretreated with a combination of 5 μg/mL TLR2 and TLR5 μg/mL TLR 5 neutralizing antibodies (+) or a combination of 5 μg/mL TLR 2, 5 μg/mL TLR4 and 5 μg/mL TLR5 (+) neutralizing antibodies for 30 min prior to stimulation with 1 μg/mL of planktonic PACF508 PsaDM. TLR2 nAb: TLR2 neutralizing antibody; TLR4 nAb: TLR4 neutralizing antibody; TLR2/5 nAb: combination of TLR2 and TLR5 neutralizing antibodies; TLR2/4/5 nAb: Combination of TLR2, TLR4 and TLR5 neutralizing antibodies. The mean of three experiments is shown +/− SEM. Statistics were performed using one-way ANOVA with Bonferroni post-test analysis. $^{***,**,*}p \leqslant 0.001$, 0.01 or 0.05 respectively compared to control; $^{\#}p \leqslant 0.05$ in the absence compared to the presence of neutralizing antibodies.

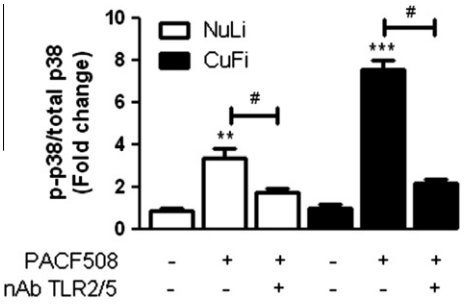

Fig. 3. TLR2 and TLR5 lead to p38α MAPK activation in airway epithelial cells expressing wildtype CFTR and CFTRΔF508. NuLi (non-CF, white bars) or CuFi (CF, black bars) AECs were left untreated (−) or pretreated with a combination of 5 μg/mL TLR2 and 5 μg/mL TLR5 (+) before stimulation for 30 min with 1 μg/mL of planktonic PACF508 PsaDM grown in SCFM. p38α MAPK activation was determined as in Fig. 1A. Statistics were performed using one-way ANOVA with Bonferroni post-test analysis. $^{***,**}p \leqslant 0.001$ or 0.01 respectively compared to unstimulated; $^{\#}p \leqslant 0.05$ in absence of neutralizing antibodies compared to their presence.

mechanism, a mucoid clinical isolate presents a more complex profile of activation; while flagellin-mediated TLR5-dependent mechanisms are dominant in PAO1, additional pathways contribute to p38α MAPK activation in the PACF508 clinical isolate.

3.2. The mucoid phenotype of P. aeruginosa is linked to TLR2 activation

In addition to TLR5, TLR2 and TLR4 bind bacterial membrane constituents and activate the p38α MAPK pathways [18]. As expected, both TLR2 and TLR4 neutralizing antibodies prevented p38α MAPK activation by their respective ligands, FSL-1 (a synthetic TLR2/TLR6 agonist) and lipopolysaccharides (LPS) (Fig. 2A and B). When stimulated with PACF508 PsaDM, inhibition of TLR2 reduced the p38α MAPK activation by 34% (Fig. 2A), while TLR4 inhibition had a small reduction in p38α MAPK activation that was not found to be statistically significant (Fig. 2B).

The PACF508 strain is mucoid, a phenotype attributed to mutations in the *mucA* gene [8]. While such mutation leads to over-production of alginate, it is also associated with increased expression of bacterial lipoproteins, which can be potent TLR2 agonists [19]. We therefore hypothesized that *mucA* mutations are responsible for the greater TLR2-dependent activation of p38α MAPK in mucoid strains. To test this hypothesis, we used a PAO1-*mucA*-deficient strain and found that, in contrast to planktonic PsaDM from wildtype PAO1 where p38α MAPK activation was completely dependent on TLR5 and not TLR2 (Figs. 1C and 2C), the *mucA*-deficient bacteria activated p38α MAPK in both a TLR5 and TLR2-dependent manner (Fig. 2C). Inhibition of TLR4 in addition to TLR2 and TLR5 did not further decrease p38α MAPK activation by planktonic PACF508 PsaDM (Fig. 2D). These results demonstrate that in addition to activating TLR5, mucoid strains activate TLR2, which contributes to p38α MAPK activation.

These results were confirmed in two other airway epithelial cell lines, one expressing wildtype CFTR (NuLi) and one expressing the most common mutation leading to CF, CFTRΔF508 (CuFi). Neutralizing TLR2 and TLR5 greatly reduced p38α MAPK activation by planktonic PACF508 PsaDM in both NuLi and CuFi AECs (Fig. 3). It is worth noting that consistent with previous observations, p38α MAPK activation is greater in the CFTRΔF508 expressing cells [15,20].

Overall, these results indicate that planktonic-derived material is not uniform in its capacity to stimulate host responses and that changes of bacterial gene expression modulate this response. These changes may occur at different moments in the evolution of the disease, for example during episodes of pulmonary exacerbations, where CF patients experience worsening of their respiratory symptoms [21]. The underlying mechanisms of these pulmonary exacerbations is unknown, but it has been proposed that they can be caused by the release of planktonic bacteria from the biofilm aggregates [4]. In the planktonic form, these bacteria express flagellin, which binds and activates TLR5. Interestingly, TLR5 has been identified as a modifier gene in CF and proposed as an anti-inflammatory target [22,23]. Moreover, if these bacteria have mutations in their *mucA* gene, linked to a mucoid phenotype, they will also activate innate immune responses through TLR2. Since the presence of the mucoid phenotype of *P. aeruginosa* is a marker of poor survival in CF [6,7], our findings highlight an important and under recognized for TLR2 in the response of airway epithelial cells to infection. These findings also raise the question whether mucoid *P. aeruginosa* can induce stronger pulmonary exacerbations than non-mucoid clinical isolates due to the engagement of both TLR2 and TLR5. It would also be interesting to test whether TLR2 is another CF modifier gene by itself or in combination with TLR5. Finally, in the optic of designing better treatments against mucoid infections, which are found to be deadlier in children suffering from CF, our report adds a novel avenue of decreasing lung inflammation.

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