Original Article

P. aeruginosa drives CXCL8 synthesis via redundant toll-like receptors and NADPH oxidase in CFTRΔF508 airway epithelial cells

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Abstract

Background: Understanding the mechanisms underlying bacterial-driven inflammation and neutrophil recruitment is important to design better therapies for CF. CXCL8 is an important chemokine found elevated in the airways of CF patients that recruits neutrophil to sites of the inflammation.

Methods: Airway epithelial cells (AECs) expressing wild-type CFTR or CFTRΔF508 were challenged with *Pseudomonas aeruginosa* diffusible material (PsaDM) and the synthesis of CXCL8 was measured by quantitative real-time PCR and ELISA in absence or presence of MAPK inhibitors, TLR antagonists, glutathione and a NADPH oxidase inhibitor.

Results: CFTRΔF508 AECs secrete more CXCL8 in response to PsaDM than their wild type counterpart, which can be reversed by addition of extracellular glutathione or incubating AECs at 27 °C to favour folding and expression of CFTR at the cell membrane. Moreover, in CFTRΔF508 AECs, TLR2, TLR4 and TLR5 act redundantly to drive CXCL8 synthesis via the activation of NADPH oxidase.

Discussions: These results demonstrate that NADPH oxidase is necessary for CXCL8 synthesis in response to TLRs activation by *P. aeruginosa*.
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Keywords: Cytokines; Reactive oxygen species; MAPK; Cystic fibrosis; Gene expression

1. Introduction

Defective CF transmembrane conductance regulator (CFTR) function is responsible for CF lung disease, the most life-threatening complication of CF, characterized by mucus hyper secretion and neutrophil-dominated inflammation. Intensity of airway neutrophilia was shown to be associated with decreasing lung functions as measured by the decline in FEV_1 [1]. Unfortunately, diseases associated with neutrophilic inflammation are generally found to be poorly responsive to glucocorticoids while patients are still susceptible to their side effects, highlighting the need for alternative treatments [2]. Neutrophils can be attracted to sites of inflammation from the circulation via the action of chemo-attractants of the Cys-Xaa-Cys chemokine family, such as

CXCL8 (also known as IL-8) [3], which has been found elevated in the airways of CF patients [4].

Secretion of CXCL8 by airway epithelial cells can occur upon infection to initiate the innate immune response. *Pseudomonas aeruginosa* (Psa) is the most commonly found pathogen in the airways of chronically infected CF patients. Moreover, Psa is also the predominant pathogen encountered during exacerbation episodes, which have a profound effect on the patient's quality of life [5]. Human cells detect pathogens in part through Pathogen-Recognition Receptors (PRRs) such as the Toll-Like Receptors (TLRs) and Nucleotide-binding Oligomerization Domain (NOD)-like receptor (NLR) families. Once activated, these receptors will engage various intracellular signalling pathways responsible for host responses to infection (reviewed in [6]). Among the major signalling pathways activated in response to motile gram negative bacteria like Psa are the NFκB and MAPK pathways.

We have recently shown that in airway epithelial cells expressing the most common mutation in CF (CFTRΔF508)

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[7], the extracellular glutathione levels are decreased, leading to a greater sensitivity to ROS, hyper-activation of the p38 and ERK MAPKs and increased IL-6 synthesis [8]. In this manuscript we have investigated the impact of the CFTRΔF508 mutation on the synthesis of the neutrophil chemo-attractant CXCL8 in response to *P. aeruginosa* diffusible material.

2. Materials and methods

2.1. Materials

The CFTR inhibitor, GlyH-101 was purchased from EMD Biosciences (NJ, USA). DPI (diphenyliodonium chloride) was bought from Sigma-Aldrich (Ontario, Canada). All other materials were purchased from sources previously reported [8]. The *P. aeruginosa* diffusible material (PsaDM) was prepared from late stationary phase Psa (mucoid strain 508) isolated from the sputum of a patient with CF (Hôpital Sainte-Justine, Montréal) as previously reported [8].

2.2. Neutralizing antibodies

Neutralizing antibodies against TLR2, TLR4 and TLR5 were used at 5 µg/ml and purchased from Invivogen (CA, USA). CXCL8 neutralizing antibody was used at 50 µg/ml and purchased from R&D systems (Minneapolis, USA).

2.3. Cell culture

Human airway epithelial cell line NuLi derived from a normal lung of a 36-year-old male patient and CuFi derived from the lung of a 14-year-old female patient with cystic fibrosis homozygous for the CFTRΔF508 mutation were generously provided by Dr Emmanuelle Brochiero (Centre de Recherche, Hôtel-Dieu du CHUM, Université de Montréal, Canada) and were cultured as described previously [8]. For CFTR temperature rescue experiments, NuLi and CuFi were seeded at 2.5×10^5 cells/cm^2, grown until near confluence and then incubated at 27 °C for 30 h. The cells were incubated for 1 h at 37 °C prior to stimulation with PsaDM.

2.4. Neutrophil chemotaxis assay

Neutrophil migration assays were performed as described previously [8].

2.5. ELISA

Human CXCL8 DuoSet ELISA kit (DY208) was purchased from R&D Systems (MN, USA). A 10 times supernatant dilution collected after cell stimulation was used for CXCL8 quantification according to manufacturer's protocol.

2.6. RNA extraction and cDNA synthesis, quantitative real-time PCR and mRNA stability assays

These methods were all performed as described previously [8] using the following sense and antisense primers: CXCL8 (5′-GTGCAGTTTTGCCAAGGAGT-3′; 5′-CTCTGCACC-CAGTTTTCCTT-3′), and GAPDH (5′-AGCAATGCCTCCTG-CACCACC-3′; 5′-CCGGAGGGGCCATCCACAGTC-3′).

2.7. Statistical analysis

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

3.1. PsaDM-driven CXCL8 levels are higher in AECS expressing the CFTRΔF508 mutation

P. aeruginosa is mostly found in the lungs of CF patients as intra-luminal masses distal from airway epithelial cells [9]. Therefore, Psa diffusible material (PsaDM) represents a model of the pathogenic factors encountered by AECs. Upon exposure to PsaDM, AECs expressing the most common mutation encountered in CF, CFTRΔF508, secrete more CXCL8 than cells expressing wild-type CFTR (Fig. 1A). This increased secretion of CXCL8 by CFTRΔF508 AECs is the result of both increased transcription (Fig. 1B) and enhanced mRNA stability (Fig. 1C). Moreover, PsaDM-driven CXCL8 synthesis is dependent on the ERK1/ERK2 and p38 MAPKs in both non-CF and CF AECs (Fig. 1A and B), as demonstrated by pre-incubating cells with the p38 MAPK inhibitor BIRB0796 [10] or the MKK1/MKK2 inhibitor PD184352 [11]. MKK1/MKK2 are direct upstream activators of ERK1/ERK2 [12]. This is in accordance with previous results obtained for CXCL8 in airway epithelial cells stimulated by TLR agonists [13] or IL-6 synthesis in response to PsaDM [8].

In the initial secreted cytokine array performed to identify p38 MAPK-dependent cytokines secreted upon PsaDM challenge in AECs, CXCL8 enhanced secretion had not been clearly detected and became apparent only with a more detailed and robust analysis combining quantitative real-time PCR and ELISA. This prompted us to investigate the other p38 MAPK-dependent cytokines up regulated by PsaDM in AECs that were initially found in the screen: GM-CSF, RANTES and LTα [8]. For these three cytokines, no significant up-regulation was detected in both non-CF and CF AECs upon PsaDM challenge (Supplementary Fig. 1). Therefore in the AEC model investigated, IL-6 and CXCL8 are the major cytokines up regulated in a p38 MAPK-dependent fashion upon PsaDM challenge.

As CXCL8 is a powerful chemo-attractant for neutrophils, we looked at its contribution to the chemotactic potential of PsaDM-stimulated AECs medium. Neutralizing CXCL8 action with an antibody at concentrations shown to be effective previously [14], led to a clear reduction of neutrophils
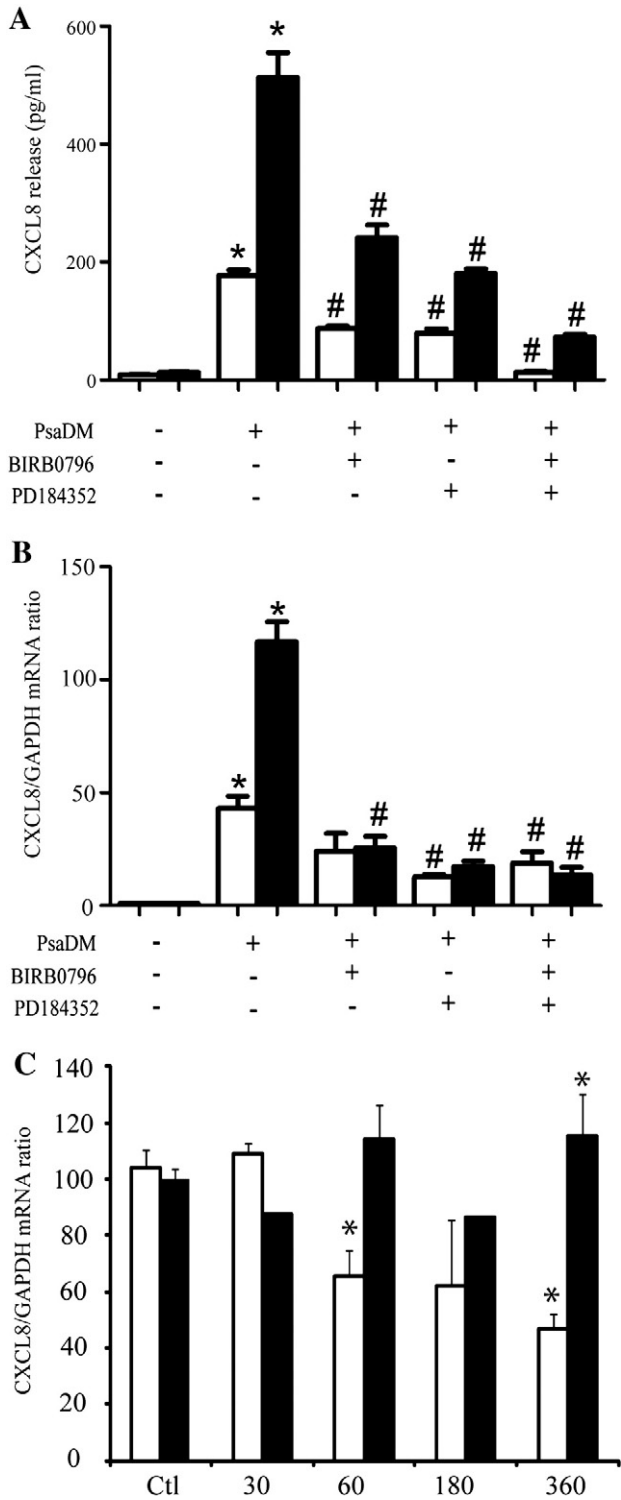
Fig. 1. MAPK-dependent CXCL8 synthesis is higher in CFTRΔF508 compared to their wild type counterpart in response to PsaDM. Non-CF (white bars) and CFTRΔF508 (black bars) AECs were left untreated or pre-treated for 1 h with 0.1 μM BIRB0796 (A, B) or 2 μM PD184352 (A, B) and exposed to 3 h PsaDM. (C) Alternatively, following a 3 h PsaDM stimulation, 5 mg/mL Actinomycin D was added to block transcription and the RNA extracted at various intervals thereafter from 30 min to 360 min. The amount of CXCL8 mRNA (B, C) was determined by quantitative real-time PCR and the presence of CXCL 8 in the supernatant (A) quantified by ELISA. $*=p<0.05$, compared to respective control; $\#=p<0.05$, Compared to PsaDM stimulation.

recruitment by conditioned medium from both non-CF and CF AECs (Fig. 2).

3.2. Increased CXCL8 secretion in response to PsaDM is dependent on the loss of functional CFTR in AECs

As the two immortalized cell lines used in this study come from different genetic backgrounds, it was important to assess whether the differences were due to the loss of functional CFTR or the contribution of other genetic factors. It has been previously reported that incubating AECs carrying the CFTRΔF508 mutation at lower temperatures favours correct folding of the protein and promotes its functional expression at the cell membrane [15]. Incubating both AECs lines at 27 °C for 30 h prior to restoring the temperature to 37 °C and exposure to PsaDM abolished the difference in CXCL8 synthesis observed in the CFTRΔF508 AECs (Fig. 3A). Conversely, incubating AECs expressing wild-type CFTR in the presence of GlyH-101, a CFTR inhibitor, for 1 or 3 days prior to exposure to PsaDM led to a significant increase in CXCL8 synthesis (Fig. 3B). Taken together these experiments link the absence of functional CFTR to increase CXCL8 synthesis in response to PsaDM.

3.3. Multiple TLRs act redundantly in driving CXCL8 synthesis by CFTRΔF508 AECs exposed to PsaDM

PsaDM may contain one or more agonists contributing to CXCL8 synthesis. Among the various bacteria derived products commercially available, a triacylated synthetic lipoprotein (Pam3CSK4) that activates TLR1/TLR2, LPS from *P. aeruginosa* that activates TLR4, flagellin from *Salmonella typhimurium* that activates TLR5 or C12-ie-DAP an acylated derivative of the dipeptide γ-D-Glu-mDAP, present in the peptidoglycan (PGN) of bacteria that activates the intracellular receptor
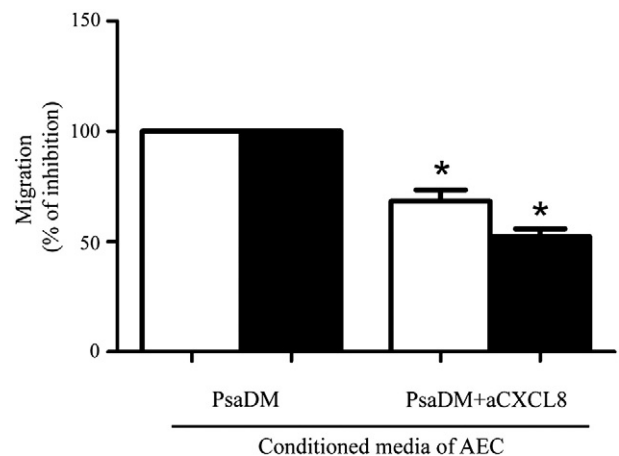

Fig. 2. CXCL8 is an important but not sole driver of neutrophil chemotaxis of AECs stimulated by PsaDM. Neutrophils chemotaxis was assayed in presence of PsaDM (3 h) treated Non-CF (white bars) and CFTRΔF508 (black bars) AECs conditioned medium (PsaDM). The contribution of CXCL8 was determined by the addition of a neutralizing antibody (aCXCL8, 50 ug/ml). $*=p<0.05$, compared to respective PsaDM stimulation.
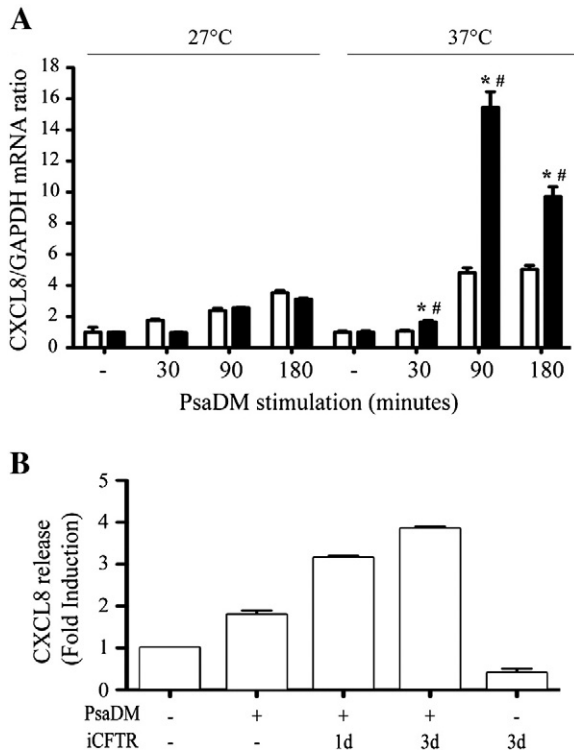
Fig. 3. Enhanced CXCL8 synthesis in response to PsaDM is linked with loss of functional CFTR. (A) Non-CF (white bars) and CFTRΔF508 (black bars) AECs were incubated at 27 °C or 37 °C for 30 h before they were left to recuperate for 1 h at 37 °C. AECs were then left untreated (-) or exposed for increasing length of times to PsaDM as indicated. The amount of CXCL8 mRNA was determined by quantitative real-time PCR. Results from three independent experiments are shown *$=p<0.05$, CFTRΔF508 compared to wild-type CFTR; #$=p<0.05$, 37 °C compared to 27 °C. (B) Non-CF AECs were left untreated or incubated in the presence of 20 μM GlyH-101 for 1 (1d) or 3 (3d) days prior to exposure to PsaDM (+) for 3 h. The presence of CXCL8 in the supernatant was quantified by ELISA.

NOD1, were all found to increase CXCL8 synthesis more potently in the CFTRΔF508 AECs than in the non-CF counterpart (Fig. 4A). The cells were also stimulated with IL-17A, a cytokine driving neutrophil recruitment to inflamed areas via the synthesis of CXCL8 [16], but in this case, non-CF AECs were more responsive, indicating that enhanced cytokine secretion in CFTRΔF508 AECs appears specific to bacterial-derived products. The more potent agonists were those activating TLR2 and TLR5.

In order to check whether these TLRs contributed to the synthesis of CXCL8 by PsaDM, we prevented TLR2, TLR4 and TLR5 activation with neutralizing antibodies. Neutralizing TLR2 or TLR4 had a weak effect on CXCL8 secretion, whereas neutralizing TLR5 was more potent in non-CF AECs (Fig. 4B). Combining all three neutralizing antibodies showed no additive effect compared to blocking TLR5 alone (Fig. 4B). In contrast, in CFTRΔF508 cells, although each neutralizing antibody had a partial effect on CXCL8 synthesis upon exposure to PsaDM (Fig. 4C), combining all three antibodies brought the CXCL8 levels back to those observed in the non-CF cells (Fig. 4B and C).
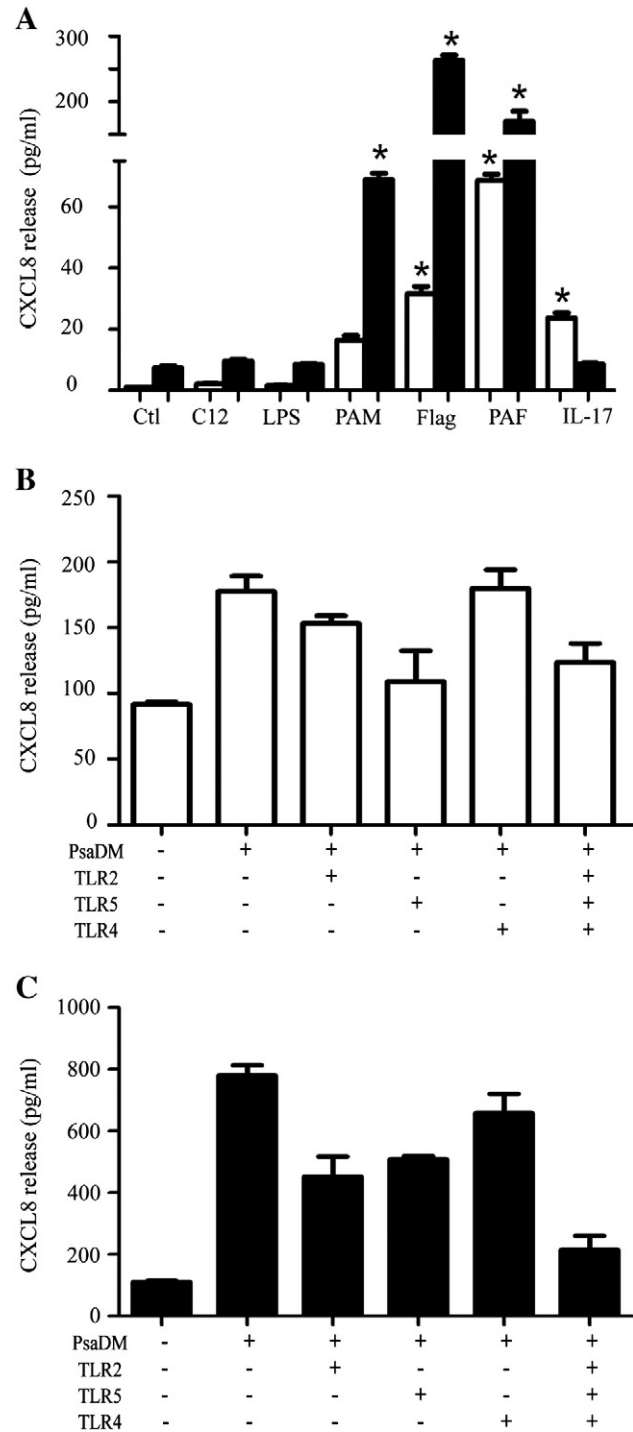

Fig. 4. CFTRΔF508 induced CXCL8 in response to PsaDM through the action of multiple redundant TLRs. Non-CF (white bars) and CFTRΔF508 (black bars) AECs were incubated with 20 ng/ml IL-17A, 1 μg/ml C12-ie-DAP (C12), 100 ng/ml LPS and 1 μg/ml Pam3CSK4 (PAM), 10 ng/ml flagellin (Flag) or PsaDM all for 3 h (A). Non-CF (B) and CFTRΔF508 (C) AECs were pre-treated for 30 min with 50 μg/ml neutralizing antibodies against TLR2, TLR5 and/or TLR4 and exposed for 3 h to PsaDM (B, C). The presence of CXCL8 in the supernatant was quantified by ELISA. Results from two independent experiments are shown. *$=p<0.05$, compared to respective control.

Therefore the up-regulation of CXCL8 synthesis in CFTRΔF508 AECs is occurring through the action of multiple TLRs acting redundantly.

3.4. NADPH oxidase is essential for cytokine synthesis by AECs in response to PsaDM

In CFTRΔF508 AECs, lower concentrations of the anti-oxidant glutathione in the extracellular media, lead to hyper activation of the ERK1/ERK2 and p38 MAPK pathways and enhanced IL-6 synthesis [8]. We found that similarly, adding glutathione to the CFTRΔF508 AECs medium greatly diminished the synthesis of CXCL8 (Fig. 5A). Additionally, in the case of IL-6, intracellular ROS generation was found to be essential for its synthesis in response to PsaDM by an uncharacterized mechanism [8]. We looked at NADPH oxidase as a plausible enzyme generating ROS following TLR activation [17]. Non-phagocytic cells have NADPH oxidases that are sensitive to the action of the pharmacological inhibitor diphenyliodonium chloride (DPI) [18]. Therefore, we checked whether NADPH oxidase played a role in response to PsaDM. Pre-treating CFTRΔF508 AECs with increasing concentration of DPI, greatly reduced CXCL8 transcription with doses as low as 3 μM (Fig. 5B). These results suggest that NADPH oxidase is an important component of the innate immune response of AECs to *P. aeruginosa*.
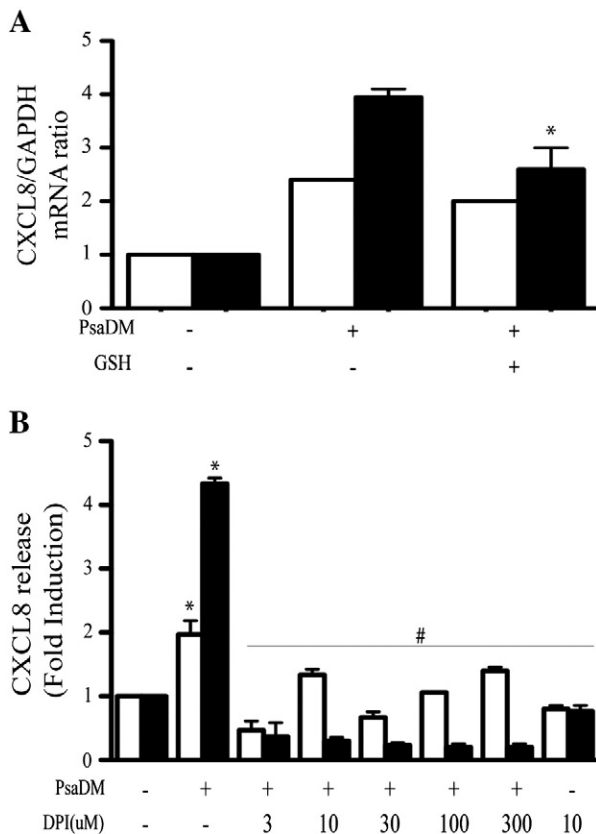

Fig. 5. NADPH oxidase is essential for CXCL8 synthesis by CFTRΔF508 AECs in response to PsaDM. Non-CF (white bars) and CFTRΔF508 (black bars) AECs were left untreated or pre-treated for 15 min with 10 mM extracellular glutathione (GSH) (A), or 30 min with increasing concentrations of DPI (B) and exposed 3 h to PsaDM. (A) After stimulation, the amount of CXCL8 mRNA was determined by quantitative real-time PCR $* = p < 0.05$, GSH compared to PsaDM. B. The presence of CXCL8 in the supernatant was quantified by ELISA. $* = p < 0.05$, compared to control; $\# = p < 0.05$, compared to PsaDM stimulation.

4. Discussion

In this manuscript we have shown that loss of functional CFTR leads to enhanced CXCL8 synthesis upon exposure to PsaDM in a TLRs and NADPH oxidase-dependent fashion. In many, but not all CF models, AECs respond to *P. aeruginosa* with increased or prolonged IL-6 and CXCL8 synthesis [19]. Moreover, freshly harvested cells from inflamed CF lungs showed evidence of enhanced cytokine production [20]. This matches the clinical observations that pro-inflammatory cytokines, TNFα, IL-1β, IL-6 and CXCL8 are all found elevated in the airways of CF patients accompanied by increased neutrophilia [4], although an intrinsic increased inflammatory response of AECs expressing the CFTRΔF508 mutation is still much debated. We have recently showed that increased IL-6 synthesis in CFTRΔF508 AECs is the result of enhanced MAPK activation leading to increased mRNA transcription and stability. In this manuscript we have found that similar to IL-6, but to a lesser extent, CXCL8 is also increased at both the transcriptional and post-transcriptional level in a MAPK-dependent fashion. Moreover, we have linked the functional absence of CFTR to increased CXCL8 synthesis using a reconstitution model in CFTRΔF508 AECs and a CFTR inhibitor in wild-type CFTR AECs. This means that the major changes observed in our cell culture model were not due to differences in the genetic background of the two cell lines. However, other genes as well as epigenetic factors were shown to have an important impact on CF [21], which may be lost in *ex vivo* and *in vitro* assays.

Although many similarities were found between CXCL8 and IL-6 synthesis in response to PsaDM in both CFTRΔF508 and non-CF AECs, including their dependence on MAPK pathways for transcriptional activation, a number of interesting differences were also revealed. First, whereas neutralizing IL-6 in the conditioned medium of CFTRΔF508 cells brought the chemotaxis of neutrophil back to the non-CF levels, CXCL8 was found to potently inhibit neutrophil recruitment in both CFTRΔF508 and non-CF AECs. Moreover, CXCL8 synthesis by CFTRΔF508 AECs is downstream of multiple redundant TLRs (TLR2, TLR4 and TLR5), whereas IL-6 synthesis is dependent on one or more additional pathways [8]. These results would have a clear impact on therapeutic strategies aimed at targeting only one or both of these important inflammatory cytokines. They also suggest that attempts at preventing inflammation by targeting a single TLR may not be as efficient as predicted. It is therefore essential to identify common signalling events leading to inflammatory mediator production to find more efficient ways to interfere with inflammation. When three different pattern-recognition receptors (TLR2, TLR3 and NOD1) acting through distinct adaptor molecules are activated by their respective ligands in AECs they convey signals towards common signalling modules that regulate the synthesis of CXCL8 [13]. This convergence mechanism to signalling module from multiple cellular receptors was also found in a systems biology study of RAW macrophages [22].

A common mechanism may be the dependence of the response to the generation of ROS. Therefore we have investigated the potential role of a well-known enzymatic

system involved in ROS production, NADPH oxidase, which can be activated following TLR stimulation [17]. Once activated, NADPH oxidase catalyses the transfer of electrons from NADPH to molecular oxygen, forming O_{2-} [23]. Here we report that DPI, a relatively specific inhibitor of NADPH oxidase, blocked CXCL8 transcription in response to PsaDM. Although we cannot discard that other proteins modified by DPI mediate this effect, it highlights a common pathway downstream of TLR receptors leading to CXCL8 synthesis. Moreover, this may provide an explanation for the difference we observed between TLR and IL-17 initiated responses; TLR-signalling may be more sensitive than the IL-17Rs to the pro-oxidative shift observed in CFTRΔF508 AECs.

Taken together our results suggest that in order to block CXCL8 synthesis, individual TLR may not be ideal targets, but targeting ROS or NAPDH oxidase may be a more attractive strategy. Moreover, these findings support the notion that ROS are very important intermediates of the lung inflammatory response to multiple insults [24], including CF [25]. Therapies aimed at increasing the anti-oxidant potential of AECs or neutralizing the action of ROS may be beneficial to decrease inflammation in chronic lung diseases. However a careful monitoring of the adverse impact on the innate immune system capacity to deal with invading pathogens will be needed.

Supplementary materials related to this article can be found online at doi:10.1016/j.jcf.2010.11.005.

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