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IL-17 primes airway epithelial cells lacking functional Cystic Fibrosis Transmembrane conductance Regulator (CFTR) to increase NOD1 responses

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ABSTRACT

In Cystic Fibrosis (CF), the absence of functional Cystic Fibrosis Transmembrane conductance Regulator (CFTR) translates into chronic bacterial infection, excessive inflammation, tissue damage, impaired lung function and eventual death. Understanding the mechanisms underlying this vicious circle of inflammation is key to better therapies for CF. In this manuscript, we have found that the presence of IL-17 in the airways of CF patients not only exacerbates inflammation through the recruitment of neutrophils via secretion of CXCL8, but also by priming airway epithelial cells lacking functional CFTR to increase response to the bacterial sensor NOD1. IL-17 stimulation of airway epithelial cells (AECs) lacking functional CFTR increased the expression of NOD1, NOD2, TLR4 and its own receptors IL-17RA and IL-17RC. Moreover, prior stimulation of AECs expressing the CFTRΔF508 mutant with IL-17 showed much greater CXCL8 secretion in response to a NOD1 agonist and *Pseudomonas aeruginosa* diffusible material. Taken together our results show that IL-17 primes AECs expressing CFTRΔF508 to increase host defence response to bacteria through the up-regulation of PRRs, and in particular of NOD1, and identifies another mechanism of action through which the CFTRΔF508 mutation leads to increase inflammation in response to bacterial ligands. Therefore preventing IL-17 function in CF may prove an important strategy in decreasing lung inflammation due to both direct and indirect effects.

Background

Cystic Fibrosis is one of the most common fatal genetic diseases affecting Caucasians of European decent. It is an autosomal recessive disorder caused by mutations of the CF transmembrane conductance regulator (CFTR). Defective CFTR function in the airway epithelium is responsible for CF lung disease, the most life-threatening complication of CF, characterized by mucus hypersecretion and neutrophil-dominated inflammation. *Pseudomonas aeruginosa* (PA) infections occur in 70% of individuals at an early age and contribute to lung destruction and mortality. Moreover, CF patients suffer from exacerbation episodes, which have a profound effect on the patient's quality of life [1,2]. Therefore in CF, the absence of functional CFTR translates somehow into chronic bacterial infection, excessive inflammation, tissue damage, impaired lung function and eventual death. CF pathogens activate common signaling pathways in airway epithelial cells leading to the production of pro-inflammatory cytokines [3]. Human cells have evolved to recognize pathogens through receptors that bind different molecular patterns like lipids, carbohydrates, peptides and nucleic acids expressed by various microorganisms. Once activated, these patterns-recognition receptors (PRRs) trigger a network of intracellular signaling events leading to the production of inflammatory mediators. The two most studied PRR families are the Toll-like receptors (TLRs) and Nucleotide-binding Oligomerization Domain (NOD)-like receptor (NLR) families. There are currently 12 known mammalian TLRs and more than 20 NLRs [4,5]. Understanding the vicious circle of neutrophil-mediated inflammation may be key to prevent CF lung disease.

Recent studies have highlighted a putative role for IL-17 in CF by driving neutrophilic inflammation [6]. IL-17, CXCL8 and neutrophils have all been found to be elevated in the airways of cystic fibrosis patients [7,8]. The IL-17 family has five members, including IL-17A and IL-17F, which form homodimers and heterodimers considered to be the most potent at recruiting neutrophils in mice [9].

Abbreviations: AECs, airway epithelial cells; CF, cystic fibrosis; CFTR, CF transmembrane conductance regulator; CIKS, Connection to IκB Kinase and Stress-activated protein kinase; CXCL8, Cyst-Xxx-Cys chemokine ligand 8 (also known as IL-8); ERK, extracellular signal-regulated kinase; IL, interleukin; IRAK, IL-1-receptor associated protein kinase; IRF, interferon response factor; LPS, lipopolysaccharides; MAPK, Mitogen-Activated Protein Kinase; MyD88, myeloid differentiating factor 88; NFκB, nuclear factor κB; NLR, NOD-like receptors; NOD, Nucleotide-binding Oligomerization Domain; PAF, Pseudomonas aeruginosa filtrate; PRR, pattern recognition receptor; qPCR, real-time quantitative PCR; RIP, receptor-interacting protein; TAK, TGF-β activated protein kinase; TLRs, Toll-like receptors; TRAF, TNF-receptor associated factor

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IL-17A is synthesized by Th17 lymphocytes subtype, as well as other inflammatory cells including monocytes–macrophages [10]. IL-17A and IL-17F bind to the IL-17RA (IL-17 receptor A) and IL-17RC on target cells [8], which recruit the adaptor protein CIKS (Connection to IκB Kinase and Stress-activated protein kinase). CIKS is a U-box E3 ubiquitin ligase that mediates lysine-63 ubiquitination of TNF-receptor-associated factor 6 (TRAF6) [11], which in turn will recruit the protein kinase TGF-β Activated Kinase (TAK1), that serves as the template for the activation of the transcription factors NFκB and CEBPβ, as well as the Mitogen-Activated Protein Kinase pathways ERK1/ERK2 and p38 MAPK [12]. Human bronchial epithelial cells stimulated with IL-17A and IL-17F show increased secretion of CXCL8 dependent on the MAPK pathways [8].

In this manuscript we have found that innate immune response to pathogens in airway epithelial cells lacking functional CFTR is further enhanced by IL-17 through the up-regulation of PRRs.

Table 1
Primer sequences and amplicon length in qPCR-assays.

Primer name	Sequence (5′–3′)	Length
CXCL8	Forward – GTGCAGTTTTGCCAAGGAGT Reverse – CTCTGCACCCAGTTTTCCTT	177
IL-17RA	Forward – GCCCTGCCACTCTCTCCCGA Reverse – TCATGCACTGGGCCCCTCTG	129
IL-17RC	Forward – ATGCCTGTGCCCTGGTTCTTG Reverse – CTGAACCCTGAGCCTTTCCTCG	150
TLR4	Forward – CAACCAAGAACCTGGACCTG Reverse – GAGAGGTGGCTTAGGCTCTG	151
NOD1	Forward – TCCAAAGCCAAACAGAAACTC Reverse – CAGCATCCAGATGAACGTG	180
NOD2	Forward – CTGTCCAGACCCTGCTCTTC Reverse – CAGAGAAGCCCTTGAGGTTG	141
TLR5	Forward – AGAATGTTGGCGCTGTCC Reverse – CTCCCATGATCCTCGTTGTC	207
GAPDH	Forward – AGCAATGCCTCCTGCACCACC Reverse – CCGGAGGGGCCATCCACAGTC	137

Materials and methods

Materials. PD184352 was bought from USBiological (Swampscott, MA, USA). BIRB07896 was kindly provided by Professor Sir Philip Cohen (MRC PPU, University of Dundee, UK). Recombinant human IL-17A was purchased from Biovision (#4176-25; CA, USA). LPS from *P. aeruginosa* and C12-ie-DAP were bought from Invivogen (CA, USA). The diffusible PA material was prepared as previously reported [13]. Briefly, filtrates of late stationary phase PA (mucoid strain 508) were used. PA 508 is a stable mucoid clinical isolate from the sputum of a patient with CF (Sainte-Justine Hospital, Montréal).

Cell culture. Human airway epithelial cell line NuLi, was derived from the normal lung of a 36-year-old patient by dual retroviral infection to prevent cells undergoing growth arrest in cell culture. NuLi-1 cells (wild type) do not express the ΔF508 mutation. The CuFi airway epithelial cell line was derived from lung of a 14-year-old patient with cystic fibrosis by the same method and is homozygous for the ΔF508 mutation. Cells were obtained from ATCC and maintained at 37 °C, 5% CO_2, 100% humidity, in BGEM (Lonza, MD, USA) with growth factors (SingleQuots, Lonza, except gentamicin), 50 U/ml penicillin G, 50 μg/ml streptomycin, 50 μg/ml geneticin, 2 μg/ml amphotericin B.

RNA extraction and cDNA synthesis. Total RNA was extracted using Trizol (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. The RNA was quantified and 1 μg was treated with DNAse I Amp Grade (Invitrogen, Carlsbad, USA) and reverse-transcribed using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, USA), according to the manufacturer's protocols.

Real-time PCR. Real-time PCR (qPCR) was performed in 96-well plate format using SYBR Green based detection on a Step-One-Plus machine (ABI) with each 20 μl reaction containing approximately 50 ng cDNA, 0.3 μM of sense and antisense primers (see Table 1 for sequences), and 1X Quantitect SYBR Green supermix (Qiagen). The plate was sealed and cycled under the following conditions: 95 °C/10 min, 50 cycles of 95 °C/10 s and 60 °C/45 s. Each reaction was performed in duplicate, mRNA levels of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were used for normalization and fold induction was determined from C_t values using Pfaffl method [14]. PCR efficiency was determined from the slope of a standard curve generated using fivefold dilution series of the DNA template.

ELISA. Human CXCL8/IL-8 (DY208) DuoSet ELISA kits was purchased from R&D Systems (MN, USA). One hundred microliters of supernatant collected after cell stimulation were directly used for CXCL8 quantification.

Immuno-histochemistry. Five micrometer-thick sections from CF alveoli biopsies were prepared from adult subjects undergoing lung transplantation (CF). All patients gave written informed consent, as approved by the hospital Ethics Committee. Slides were deparaffinated in xylene and rehydrated in decreasing concentrations of alcohol. For staining, heat-induced epitope retrieval was performed using citrate buffer, pH 6.0. Sections were then permeabilized with 0.2% Triton, and incubated with 5% hydrogen peroxide. After blocking with universal blocking solution for 30 min (Dakocytomation, Mississauga, ON, Canada), slides were incubated overnight with 10 μg/ml polyclonal IL-17RA antibody (R&D systems #AF177), 10 μg/ml polyclonal IL-17RC antibody (Abcam #69673, Cambridge, MA, USA), NOD1 antibody (Novus biologicals, CO, USA). Isotype controls were prepared by replacing the primary antibody with a non-specific Ig at the same concentration. After rinsing, the slides were incubated with a biotinylated secondary antibodies (1:100), followed by an HRP/AB Complex (both from Dakocytomation). Immunoreactivity was developed with diaminobenzidine (DAB) chromogen (Dakocytomation) and slides were counterstained with hematoxylin and lithium carbonate.

Results and discussion

IL-17 up-regulates NOD1, NOD2, IL-17RA, IL-17RC and TLR4 expression in AECs lacking functional CFTR expression

In order to have a better understanding of the role of IL-17 in cystic fibrosis, we stimulated AECs expressing wild-type (NuLi) and ΔF508 (CuFi) CFTR with IL-17A and measured changes in gene expression. As previously reported [8], IL-17 up-regulated CXCL8 mRNA as determined by real-time PCR in a p38 MAPK and ERK1/ERK2-dependent manner (Fig. 1A). Although the effects of IL-17 on chemokine and cytokine gene expression have been studied, much less is known about its potential effects on the expression of receptors involved in mounting inflammatory responses. In CF, an ongoing problem is the vicious circle of inflammation driven mostly by bacterial infections. Therefore, we decided to check whether IL-17 also played a role in regulating pattern-recognition receptors expression, which could increase the response of AECs to CF pathogens. In AECs expressing wild-type CFTR no major differences were uncovered following IL-17 stimulation for the genes studied (Fig. 1B, white bars). However, unexpectedly, cells expressing the CFTRΔF508, showed upon IL-17 challenge increased levels of TLR4, NOD1, NOD2 and its own receptors IL-17RA and IL-17RC but not TLR5 (Fig. 1B, black bars). These results suggested that increase levels of IL-17 found in the CF airways may contribute to enhance response to bacterial ligands and IL-17 itself, which would promote increased tissue inflammation. We then went onto compare this up-regulation in CFTRΔF508 AECs by IL-17 to other PRR

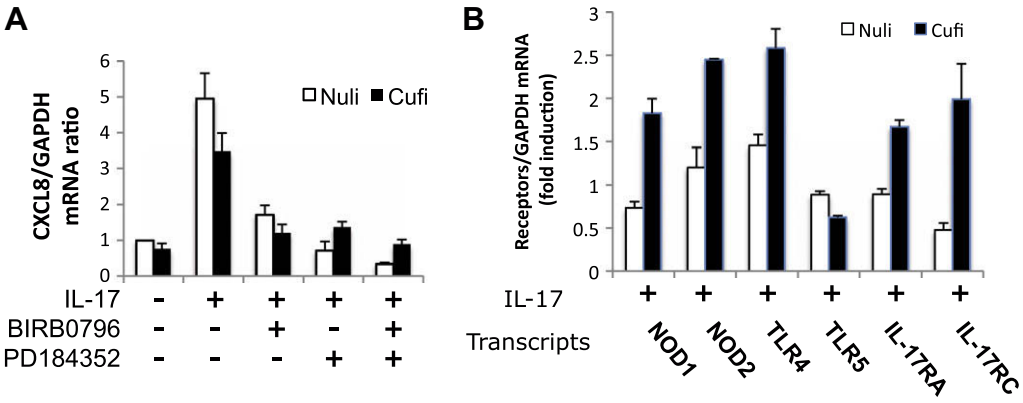

Fig. 1. IL-17 induces expression of NOD1, NOD2, IL-17RA, IL-17RC and TLR4 in AECs expressing CFTRΔF508. AECs expressing wild-type (NuLi) and CFTRΔF508 (CuFi) were pre-incubated (A) or not (B) for 1 h with vehicle (DMSO) (–), 0.1 μM BIRB0796 or 5 μM PD184352 as indicated. The cells were then left untreated (–) or exposed to 20 ng/ml IL-17 (A,B) for 3 h. After stimulation, total RNA was extracted and the amount of CXCL8 (A) or various receptors (B) mRNA were determined by qPCR.

agonists. The cells were exposed to C12-ie-DAP, LPS or *P. aeruginosa* filtrate in addition to IL-17. C12-ie-DAP is an acylated derivative of the dipeptide γ-D-Glu-mDAP, present in the peptidoglycan (PGN) of bacteria to which a lauroyl (C12) group was added to the glutamic residue that binds the intracellular NOD1 receptor and activates downstream signaling via the protein kinase RIP2. This form of PGN derivative is a more potent activator of NOD1 than M-Tri-DAP and ie-DAP according to the manufacturer (Invivogen). LPS isolated from *P. aeruginosa* activates TLR4. We also used *P. aeruginosa* filtrate as a physiological agonist encountered in the CF lungs by AECs. Bacteria present in the lungs of CF patients are found mostly as intra-luminal masses distal from airway epithelial cells [15]. Thus, diffusible *P. aeruginosa* products may represent a more accurate reflection of the pathogenic factors encountered by AECs rather than live organisms. Filtrates of late stationary phase *P. aeruginosa* (mucoid strain 508), a stable mucoid clinical isolate from the sputum of a patient with CF (Hôpital Sainte-Justine, Montréal, Canada), were prepared (see Materials and methods). IL-17 was found to be the most potent agonist tested to up-regulate NOD1, IL-17RA and IL-17RC in CFTRΔF508 cells (Fig. 2B,

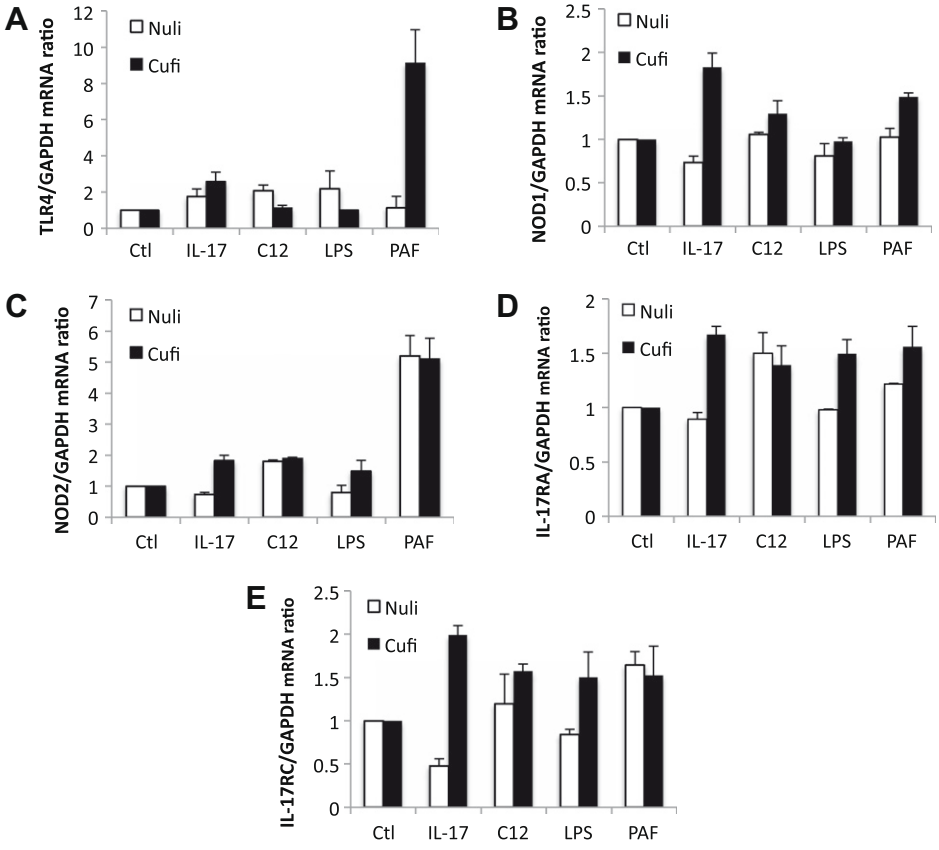

Fig. 2. Differential expression of innate immune and IL-17 receptor subunits in AECs expressing CFTRΔF508 following activation of PRRs. AECs expressing wild-type (NuLi) and CFTRΔF508 (CuFi) were incubated for 3 h with 20 ng/ml IL-17, 1 μg/ml C12-ie-DAP (C12), 100 ng/ml LPS and *Pseudomonas aeruginosa* filtrate (PAF) for 3 h. After stimulation, total RNA was extracted and the amount of different receptors mRNA (TLR4 (A), NOD1 (B), NOD2 (C), IL-17RA (D), IL-17RC (E)) were determined by qPCR.

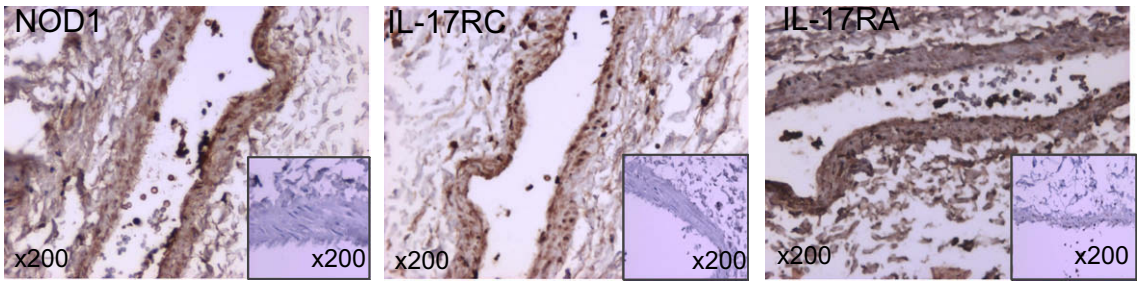

Fig. 3. Expression of IL-17RA, IL-17RC and NOD1 by the airway epithelium of CF patients. The localization of IL-17RA, IL-17RC and NOD1 were determined in tissue sections from a CF lung by immunocytochemistry (see Materials and methods). Isotype controls for each protein are illustrated as inserts.

D and E). *P. aeruginosa filtrate* was a much more potent inducer of TLR4 in CFTRΔF508 cells than IL-17, whereas no increase expression was observed in the wild-type cells (Fig. 2A). Moreover, *P. aeruginosa filtrate* triggered expression of NOD2 in both wild-type and CFTRΔF508 AECs, superior to IL-17 (Fig. 2C). We have previously failed to detect NOD2 activity in AEC stimulated by a panel of NOD agonists [16]. Therefore we concentrated our investigation on the role of IL-17 in up-regulating NOD1 in CFTRΔF508 AECs.

Tissue sections from a CF lung show positive staining of AECS for NOD1, IL-17RA and IL-17RC

Our previous results suggest that IL-17 may increase PRR expression in AECs. We therefore went on check if we could detect *in vivo* expression of the IL-17 receptors and NOD1 in tissue sections from a CF lung. Immuno-histochemistry staining confirmed that AECs lining the airways of a CF lung express IL-17RA, IL-17RC and NOD1 (Fig. 3). This suggests that the action of IL-17 on AECs may potentiate the response of these cells to pathogens encountered in the airways of CF patients.

IL-17 primes airway epithelial cells lacking functional CFTR to increase PRR responses

In order to demonstrate that IL-17 stimulation may increase the response of CFTRΔF508 AECs to pathogen-derived ligands, we exposed both wild-type and CFTRΔF508 AECs to IL-17 for 24 h prior to challenge with various agonists and measured CXCL8 synthesis as a typical response. As expected, C12-ie-DAP, LPS and *P. aeruginosa* filtrate all led to increase CXCL8 synthesis as measured by real-time PCR and ELISA in both wild-type and CFTRΔF508 expressing AECs on their own (Fig. 4A and C). We also found more CXCL8 to be synthesized in the CFTRΔF508 AECs when compared to wild-type cells in response to all PRR agonists. This was especially evident at the level of the secreted protein (Fig. 4C). This is in accordance with previous reports showing that in many, but not all, CF models, AECs respond to *P. aeruginosa* with increased or prolonged CXCL8 synthesis [17,18]. Very interestingly, when AECs were pre-treated with IL-17, cells expressing CFTRΔF508 but not the wild-type channel, showed an increase response to all three agonists (Fig. 4B and D). This is not an additive effect of IL-17 to

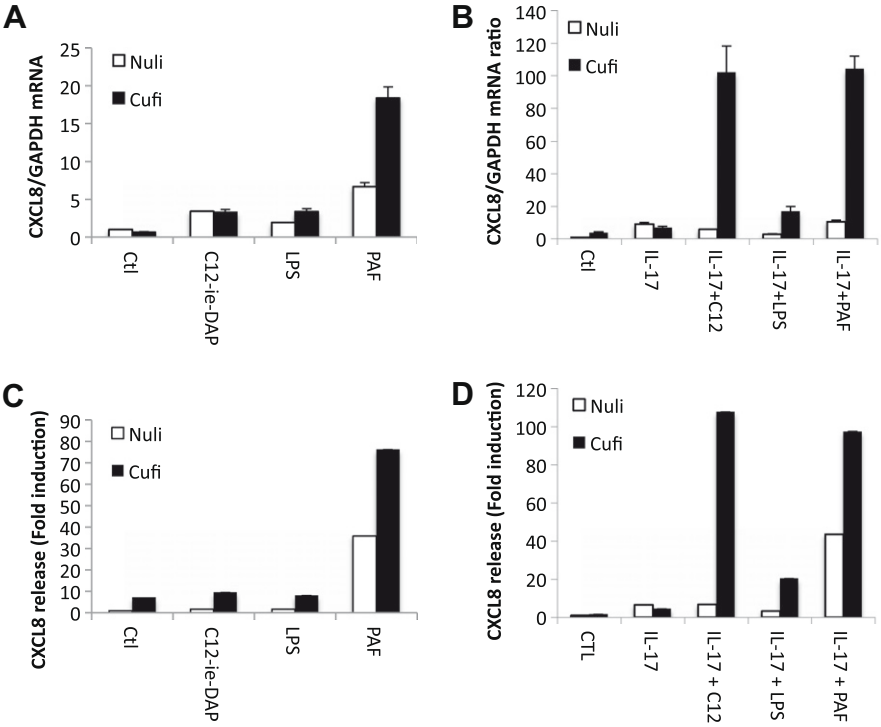

Fig. 4. IL-17 primes AECs expressing CFTRΔF508 to increase CXCL8 synthesis in response to bacterial ligands. AECs expressing wild-type (NuLi) and CFTRΔF508 (CuFi) were pre-incubated avec IL-17 (20 ng/ml, 24 h). The cells were then exposed to 1 μg/ml C12-ie-DAP (C12), 100 ng/ml LPS and *Pseudomonas aeruginosa* filtrate (PAF) for 3 h. After stimulation, the media was collected and total RNA extracted. The amount of CXCL8 mRNA (A) and protein in the media (B) were determined by qPCR and ELISA, respectively.

the other ligands for the production of CXCL8 as (i) the media was changed after the 24 h treatment period and (ii) no effect was seen in the wild-type CFTR AECs (Fig. 4). This was especially striking in response to C12-ie-DAP, the NOD1 agonist, whereas pre-treatment with IL-17 led to 10 times more CXCL8 being synthesized by CFTRΔF508 cells (Fig. 4D). This was also evident with *P. aeruginosa* filtrate, but to a lower extent, most probably due to a lesser contribution of NOD1 relative to other PRR when AECs are stimulated by a mixture of bacterial ligands. These results led us to conclude that IL-17 increase the responses of AECS lacking functional CFTR expression to *P. aeruginosa* via the up-regulation of NOD1. These results show that increase IL-17 in CF lung not only contribute to neutrophilic inflammation through synthesis of CXCL8, but also via the up-regulation of PRR and its own receptor, which prime the AECs to increase responses to pathogens in a feed forward system.

Conclusion

In this manuscript we have discovered an unexpected function of IL-17 in the CF lungs, which is to prime AECs expressing CFTRΔF508 to increase host defence response to bacteria, like the well known CF pathogen *P. aeruginosa*, through the up-regulation of PRR and in particular of NOD1. Therefore preventing IL-17 function in CF may prove an important strategy in decreasing lung inflammation due to both direct and indirect effects. An important question that remains is how the presence of the CFTRΔF508 mutation leads to an increase in the expression of these receptors in response to IL-17? This will require further investigation, but identifies another mechanism of action through which the CFTRΔF508 mutation leads to increase inflammation in response to bacterial ligands.

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