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The Role of Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) in Recognition of *Pseudomonas aeruginosa* in Lung Epithelial Cells

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Authors' contributions

This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.

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ABSTRACT

Cystic Fibrosis Transmembrane Regulator (CFTR) plays an important role in chloride and thiocyanate ion homeostasis in human epithelial surfaces. Deletion of phenylalanine at position 508 (Δ508) leads to cystic fibrosis and dysregulation of pro-inflammatory cytokines. *Pseudomonas aeruginosa* gains predominance, contributing over 80% of the lung bacteria in adults with CF and this strongly correlates with the decline of pulmonary function and mortality. The research aimed at understanding the role of CFTR in response to *P. aeruginosa,* (the most common pathogen that colonises the airways of Cystic Fibrosis patients), with the objectives of evaluating the relative expression of epithelial and inflammatory cytokines (IL-17C and IL-6) in five (Calu3, CFBE41o, CFBE41o wt, Calu3 altered and Calu3 knockout) human bronchial epithelial cell lines after two hours of infection with *P. aeruginosa* using Quantitative Polymerase Chain Reaction (qPCR). It was found that Calu3 and Calu3 altered, treated cell lines significantly (p=0.05) increased in the level of IL-17C and IL-6 mRNA in all the experimental repeats compare to untreated.

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The other three (CFBE 41o, CFBE41o wt, and Calu3 knockout) cell lines deficient of CFTR expressed low levels of these cytokines, but the level varied among the experiment in both treated and untreated cells suggesting that CFTR may modulate the level of cytokine production in bronchial epithelial cell lines. CFTR mutations have a direct effect on T cell function; enhance Th17 response which is one of the sources of IL-17. The IL-17C plays a central role in pulmonary host defence by orchestrating the accumulation and associated activity of neutrophils in the bronchoalveolar space. However, the massive neutrophils accumulation in the CF lung does not correlate with bacterial eradication but rather causes extensive tissue damage and inflammation disproportion to infection indicating that the function of neutrophils is dysregulated in CF. Therefore, knocking down IL-17C may minimise inflammation in CF patients.

Keywords: Cystic fibrosis; cytokines; Il-16; Il-17C; inflammatory response; Pseudomonas aeruginosa.

1. INTRODUCTION

Cystic Fibrosis Transmembrane Regulator (CFTR) is a membrane protein and a chloride channel in vertebrates that is coded by CFTR gene [1]. These protein comprises 1480 amino acids with a molecular weight of 1.7kDa and codes for an ABC transporter ion channel. This channel conducts chloride and thiocyanate ion across epithelial surfaces that produce mucus, digestive enzymes, etc. The chloride ion is one of the most important electrolytes in the human blood that balances the level of fluid in and out of the cells. It also maintains proper blood pressure and pH of the body fluid. Thiocyanate plays an integral part in the biosynthesis of hypothiocyanate by a lactoperoxidase. The absence or reduced level of thiocyanate as found in cystic fibrosis patients is damaging to the host defence system [2,3].

Deletion of leucine (CTT) nucleotide base pair or substitution with an amino acid within CFTR gene is the commonest mutation that leads to a pathological condition known as Cystic fibrosis, which occurs as a result of loss of phenylalanine at position 508 (ΔF508) [4]. This mutation affects the proper functioning of chloride ion channels and leads to dysregulation of epithelial fluid transportation in the lung, pancreas and other organs. This leads to complications such as the production of thickened mucus in the lungs (that creates a favourable environment for frequent respiratory infections), and pancreatic insufficiency that is a result of malnutrition, diabetes, accompanied by chronic disability and reduced life expectancy.

In male patients, one of the consequences is a progressive obstruction of the development of vas deferens (spermatic cord) and epididymis from abnormal intraluminal secretions which may lead to male infertility. However, this condition does not cause infertility in women compared to males, but some side effects and complications may affect the female reproductive system and make conception difficult [5].

Cystic Fibrosis is an autosomal inherited disease of the secretory glands that produce mucus and sweat, especially the lungs. The airway surface liquid of a CF patient is defective and characterised by airway obstruction, chronic bacterial infection, salty sweat, and excessive inflammatory responses which are responsible for high morbidity and mortality in CF patients.

In CF patients, chronic endo-bronchial bacterial infections display a limited spectrum of organisms including *H. influenzae*, *S. aureus*, *P. aeruginosa*, *B. cepacia* etc. This pathogen predominates early in life, and 80% of the young adults are chronically infected with *P. aeruginosa.* This organism is highly adaptable, aerobic, Gram-negative bacillus that is motile by means of a single polar flagellum. It is non-pathogenic in normal hosts but becomes a pathogen in individuals (e.g. CF patients) with the weakened immune system. The contributory factor to virulence of *P. aeruginosa* in CF patients is its ability to live in highly thickened mucous that limits the phagocytosis ability of neutrophils, forms biofilms that are resistant to innate immune system, but rather activate Toll-like Receptor 5 (TLR-5) and induce signal through MyD88, IRAK, TRAF6 and MAP kinases that leads to the NF_kB gene transcription pathway as well as induction of inflammatory cytokines such as interleukin 6 and 17 (IL-17C) among others. Interleukin 17 plays a pivotal rule in pulmonary host defence by orchestrating the accumulation of neutrophils (elastase) in the bronchoalveolar space of this individual [6-9].

2. MATERIALS AND METHODS

2.1 Cell Culture

Calu3 cells were grown in 75 cm^2 tissue culture flask containing Dulbecco's Minimum Essential Medium Eagle (DMEM), supplemented with 1% L glutamine, nonessential amino acid (NEA), and 10% foetal bovine serum (FBS) in a humidified incubator at 37ºC and 5% CO_2 [10].

CFBE41o, CFBE 41o (wt CFTR), Calu3 altered and calu3 knockout (KO) cells were grown in Minimum Essential Medium Eagle's (MEM), supplemented with 10% foetal bovine serum (FBS), 4 µg/ml of streptomycin, 2.5 µg/ml amphotericin B and 10 mg/ml puromycin. These cells were isolated from the bronchial epithelial cell of adult cystic fibrosis patients.

The choice of CFTR and non- CFTR cell lines was to examine the influence of CFTR in cytokine induction in response to *P. aeruginosa* in a Calu3 background.

2.1.1 Human bronchial epithelial cell lines used in this study

Cells nomenclature	Gene composition
Calu3	Cells with CFTR
CFBE41o (ΔF508/ΔF508)	Cells with homozygous mutation in CFTR
CFBE 41o (wt CFTR)	Cells transfected with cDNA encoding CFTR(wt CFTR)
Calu3Altered (shCFTR)	5'GCATGCAGAAGTGTAAAGCTACTT CCTGTCATAGCTTTACACTTCTCATG C3'. The 21- mer complementary sequences possess 4 base pair mutations of shCFTR, served as CFTR positive control cells.
Calu-3 knockout	Cells without CFTR

2.1.2 Preparation of *P. aeruginosa* (PA01-L) inoculums

Frozen glycerol of *P. aeruginosa* stocks was streaked on Lennox agar medium (LA) and incubated at 37°C overnight. A single colony was inoculated into 5 ml *X-Vivo* 15 and incubated overnight at 37°C with shaking at 200 rpm. The culture was adjusted to the optical density (OD) 600 nm 0.01 in 25 ml of fresh X-Vivo 15 mediums and incubated at 37°C for three hours. The bacteria cells were collected by centrifugation, washed and re-suspended in 5 ml sterile Hanks' balanced salt solution (HBSS) for cell stimulation.

2.1.3 Cell Lines infected with *P. aeruginosa* (PA01-L)

Undifferentiated cells were seeded at a density of one million (10^6) cells per well in 6 wells plate with appropriate media, and incubated at 37°C 5% CO_2 atmosphere for 3 days prior to the infection. After three days, cells were washed with Phosphate Buffered Saline (PBS) and stimulated with *P. aeruginosa* (PA01-L) at a multiplicity of infection (MOI) of 50 per cell and incubated for two h at 37°C.

2.1.4 RNA isolation

After 2 hrs of incubation, the culture medium was aspirated out of the culture plate, and cells washed with PBS and lysed immediately to extract total RNA using Gen Elute™ Mammalian Total RNA Miniprep Kit (Stragene UK). Briefly, lysed cells were reconstituted in a lysing buffer containing 2- mercaptoethanol and homogenised by repeated pipetting. These were transferred and pre-filtered in a membrane-fitted tube and subsequently centrifuged. Filtrates were mixed with equal volume of 70% ethanol and vortexed briefly. The mixtures were transferred to the RNA binding column and centrifuged. RNA was eluted and re-suspended in DNase free water (Life Technologies UK). The quantity and concentration of isolated RNAs were determined using a Nanodrop 2000 Spectrophotometer (Thermo Scientific UK).

2.1.5 Reverse transcription of RNA to cDNA

The concentration of purified RNA was adjusted to 1 µg in 15 µl of a mixture containing 13.75 µl of nuclease- free water and 1.25 µl random hexamers stock (0.2 µg/µl concentration) before reverse transcription was applied. The first cDNA synthesis was done using random hexamers primers and Moloney Murine Leukemia virus (M-MLV) reverse transcriptase (Life Technologies UK) according to the manufacturer's instructions. In brief, samples were mixed and incubated at 70°C for 5 minutes to denature the secondary template structure. It was then placed on ice for 5 minutes to prevent re-annealing of the template. A 10 µl amount of Master Mix (MM) made up of 5µl of 5× first strand buffer (250 mM Tris-HCl, (pH 8.3), 1.25 µl (10 mM)) dNTP, 1 µl 200 U/µl M-MLV-RT enzyme and 2.75 µl of nuclease- free water was added to template and primer mixture. All were incubated at 37°C for one hr and at 95°C for 10 minutes to inactivate the reverse transcriptase. Non- reverse trans criptase was

prepared to serve as a control. cDNAs were stored at -80°C until qPCR analysis.

2.1.6 Quantitative real- time polymerase chain reaction (RT-PCR)

Another Master Mix (MM) was composed for quantitative real- time polymerase chain reaction and is comprised of 1 µl of 6.25 µM diluted stock (100 µM) to a final concentration of 250 nM of each IL-17C F and R primers. 5 µl of template cDNA, 12.5 µl 2 x SYBR green (Life Technologies, UK), 5.5 µl nuclease free water (total volume prepared = 25 µl). Rhodamine-X (ROX) was used as a reference dye to correct the pipetting error.

The reactions were cycled 40 times as follows: 95°C for 2 seconds (Hot Start); 95°C for 30 seconds (denaturing); 59°C for 30 seconds (annealing) followed by a final extension of 95°C for 30 seconds. Each assay was performed in triplicate including a standard curve of three 10 fold serial dilutions of treated Calu3 cDNA template. Samples were run on an Agilent Mx3005P qPCR system with Hypoxanthine guanine phosphoribosyl transferase 1 (HPRT) serving as normalising agent. The relative expression ratios were calculated by using the cycle threshold (C_T) of the housekeeping gene *HPRT* (*n*-fold expression = $2^{-\Delta CT}$, where ΔC_T represents the difference between the C_T of the cytokines studied).

2.1.7 Primers used in qPCR

Primers	Oligonucleotide sequence (5'3' ⟶
IL 17C_f	GCCGCCACCATGACGCTCCT
IL 17C_r	TTTCACTGAACGGGGCAGCACGC
IL 6_f	TCTAATTCATATCTTCAACCAAGAGG
IL 6_r	TGGTCCTTAGCCACTCCTTC
HPRT_f	GTAATGATCAGTCAACGGGGGAC
HPRT_r	CCAGCAAGCTTGCAACCTTAACCA

3. RESULTS

3.1 IL-17C mRNA Expression in Five Human Bronchial Epithelial Cell Lines in Response to WT (PAO1-L) *Pseudomonas aeruginosa*

The analysis of IL-17C mRNA expression in five human bronchial epithelial cell lines following stimulation with *P. aeruginosa* at an MOI of 50 and subsequent subjection to quantitative polymerase chain reaction (qPCR) revealed a significant (p=0.05) increase in the relative expression of IL-17C mRNA in Calu3 Alt and Calu3 treated cell lines compared to untreated control, low expression in CFBE 41o, CFBE 41o wt, Calu3 ko treated and untreated (Fig. 3.1).

3.2 IL-6 mRNA Expression in Five Bronchial Epithelial Cell lines in Response to WT (PAO1-L) *Pseudomonas aeruginosa*

The same cell lines were infected with *P. aeruginosa* and subjected to qPCR to examine the expression of IL 6 mRNA expression. Significant (p=0.05) expression was also observed in Calu3 Altered (Alt) and Calu3 treated cells compared to untreated. There was an inconsistent low expression in other cells (CFBE41o, CFBE41o wt and calu3 knockout) both treated and untreated cells. It was also observed that CFBE41o untreated expressed more IL-6 mRNA compared to treated cell line (Fig. 3.2).

Expression of mRNA transcripts encoding for IL-17C was increased in Calu-3 altered and Calu3 cell lines after two hours infection with WT PAOI-L compared to uninfected control. CFBE41o, CFBE41o wt, Calu3 knockout infected and uninfected induces a low level of IL-17C (n=2). The QPCR assay was carried out in triplicate. p-values were calculated by One-Way ANOVA.

Expression of mRNA transcripts encoding for IL-6 was increased in Calu3 after 2 hrs of infection with WT PAOI-L compared to untreated control. CFBE41o, CFBE 41o wt, Calu-3 knockout induces a low level of IL-6 but the levels varied among experiments (n=2), except that CFBE41o uninfected induces three times (0.6182) fold change compare to treated (0.3059). Calu3 altered infected induces the highest fold change of IL-6 compared to other cell lines used in this experiment.

4. DISCUSSION

This study was carried out to understanding the role of CFTR in response to *P. aeruginosa,* (the most common pathogen that colonises the airways of Cystic Fibrosis patients), with the objectives of evaluating the relative expression of epithelial and inflammatory cytokines (IL-17C and IL-6) in five different human bronchial epithelial cell lines with and without CFTR. The question is whether the hyperinflammatory condition in the airway epithelium of CF patients is initiated solely by CFTR malfunction. Though previous studies

have shown that defective CFTR protein may alter the consistency of epithelial secretions, this may probably play an important role in hyperactivity and augmentation of inflammatory cytokines that promote neutrophil chemotaxis and enhance inflammation in response to pathogenic stimuli [11-13].

The choice of calu3 cell line was due to the report of Yan et al. [14]. Calu3 cells preserved all the important features of epithelial transport characteristics (expression of high levels of endogenous CFTR protein), hence an excellent model for studying CFTR gene expression.

Findings from this study indicated that differentiated Calu3 Altered and Calu3 infected with Wild- Type (PAO1-L) induced significant (p=0.05) level of IL-17C MRNA compared to untreated control. Upregulation of this cytokine in Calu3 cell line has also been reported by Liu and Yi-Chia [15] and Pfeifer et al. [16], which support our observation that IL-17C is the only member of the IL-17 family that is induced in human respiratory epithelial cells by bacterial stimuli. IL-17 is a pro-inflammatory cytokine that has been implicated in mucosal neutrophil recruitment through regulation of local production of CXCR2 ligands CXCL1, CXCL6 and CXCL8 that are chemoattractants for neutrophils and other immune cells from the airway epithelium of CF. The influx of neutrophils in a normal cell (non CF) is of importance for bacterial clearance during infection, but the massive neutrophil accumulation in the CF lung does not correlate with bacterial eradication but rather correlates with increase interleukin 17C and IL 6 which may be associated with extensive tissue damage and

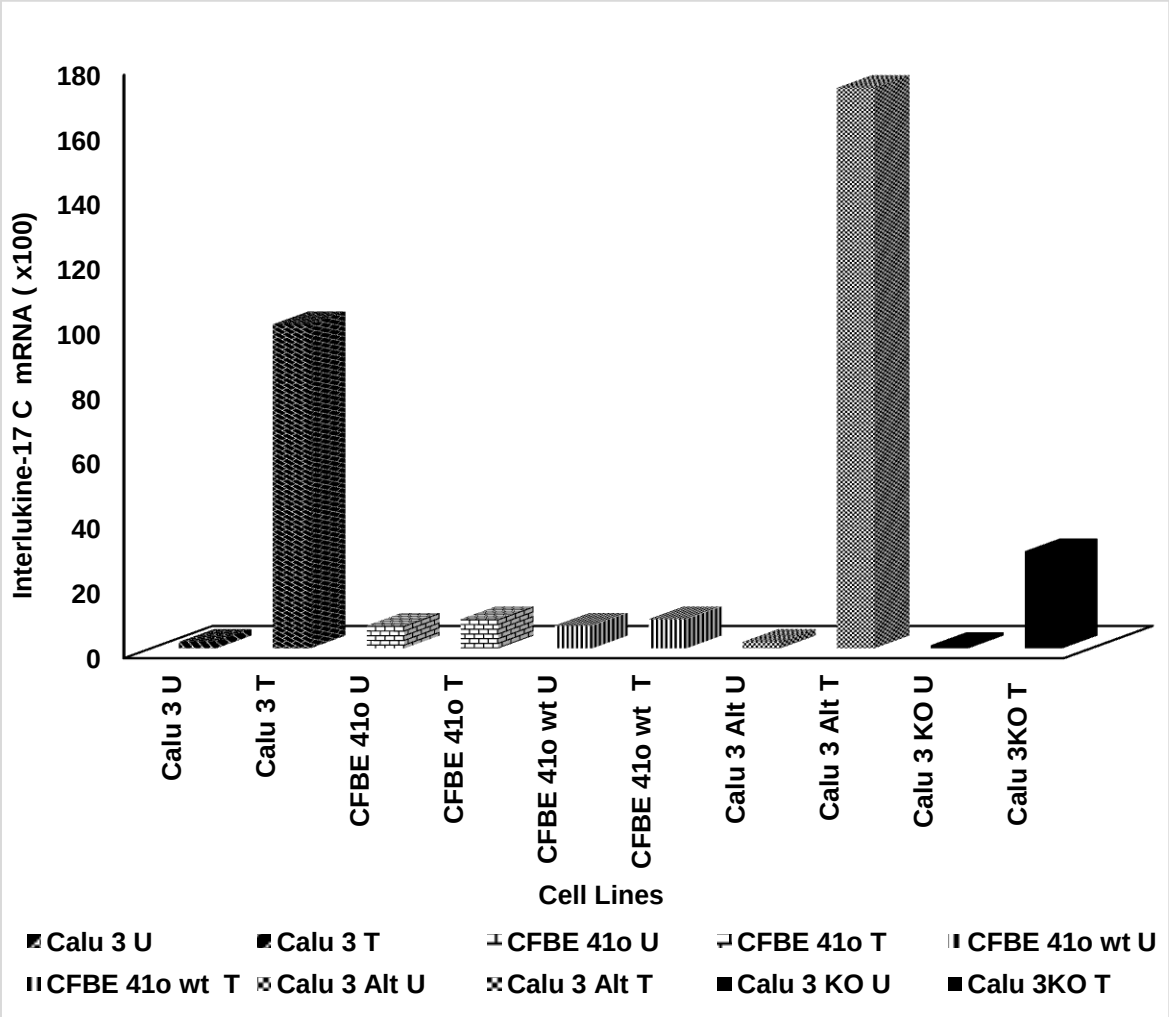

Fig. 3.1. Induction of IL-17C mRNA in five cell lines in response to WT PAOl-L

U= untreated cell lines, T= Treated cell lines, Alt=Altered cell lines, CFBE41o wt = wilt type homozygous CFTR mutated cells.

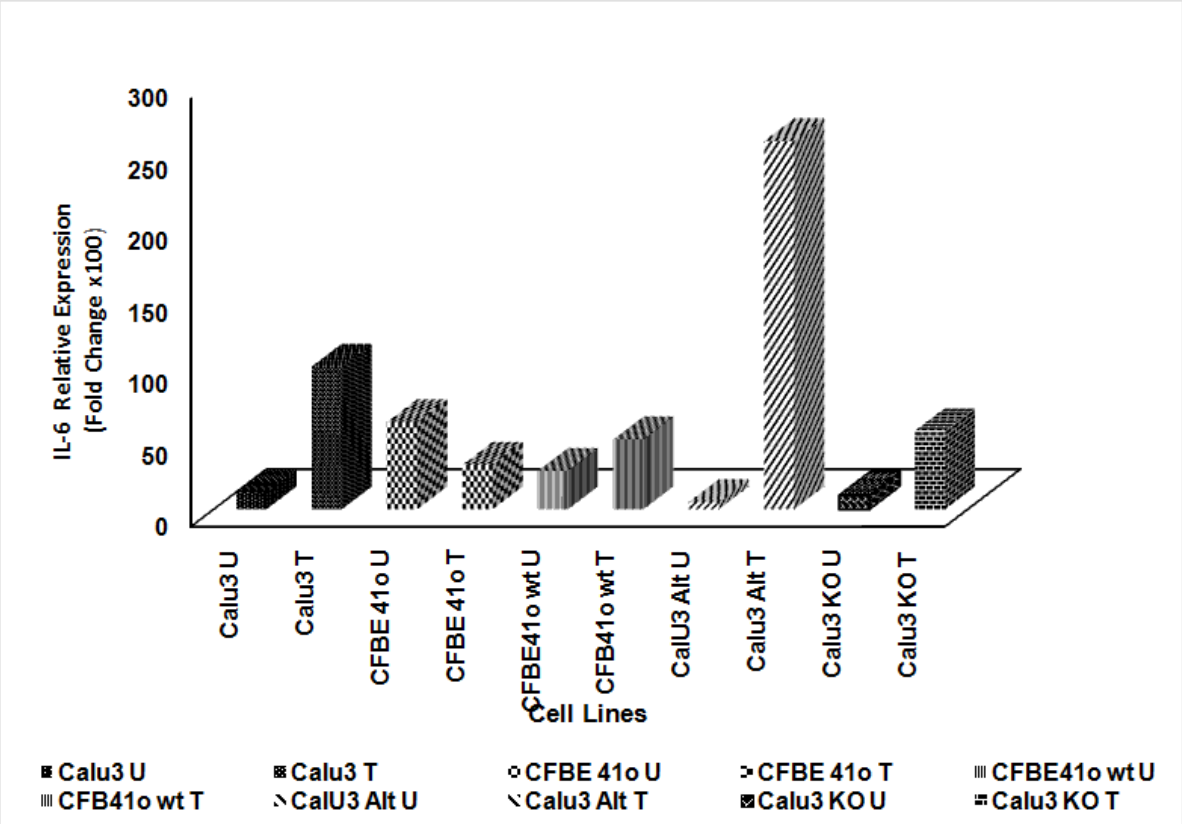

Fig. 3.2. Induction of IL-6 mRNA in Five Cell Lines in Response to WT PAOI-L.

inflammation disproportion to infection indicating that the function of neutrophils is dysregulated in CF [15].

Other cell lines (CFBE 41o, CFBE41o wt, and Calu-3 knockout) express low levels of IL-17C in both treated and untreated cells, but the level varied among the experiments (Fig. 3.1). Maintenance of cellular integrity is essential for epithelium to provide a functional physical barrier against constant foreign invaders, tight junction (TJ) proteins block passage of molecules in extracellular fluid diffusion and prevent the movement of molecules between the cells.

After two hours of cell stimulation with 50 MOI PAOI-L, Calu-3 Altered and Calu-3 cell lines induced significant (p=0.05) increase in the relative expression of IL-6 mRNA compared to other cell lines. The two repeats of this experiment reveal inconsistent induction of the experimented cytokine in (CFBE 41o, CFBE41o wt, and Calu-3 knockout) (Fig. 3.2). A prolonged period of cell passaging may affect cell characteristics. The presence of CFTR and *P. aeruginosa* may influence the induction of proinflammatory cytokines in human bronchial epithelial cell lines.

5. CONCLUSION

CFTR is an crucial membrane protein and a chloride channel in vertebrates. Proper functioning of CFTR is vital for homeostasis of epithelial fluids. Deletion of leucine (CCT) nucleotide or substitution within an amino acid within CFTR gene is the commonest mutation that leads to a pathological condition known as Cystic fibrosis, which occurs as a result of phenylalanine loss at position 508 (ΔF508) [4].

In CF patients, there is a persistence endothelial bacterial infection with a limited spectrum of organisms predominately *P. aeruginosa.* The single polar flagellum in this organism induce signal through MyD88, IRAK, TRAF6 and MAP kinases that lead to NF_kB gene transcription and induction of inflammatory cytokines interleukin 6 and 17 (IL-17C) among others. This experiment analysed the expression of proinflammatory cytokines (IL-17C and IL-6) in five human bronchial epithelial (Clau3, CFBE41o CFBE 41o

wt, Clau3 altered, Clau3 knockout) cell lines with and without CFTR. Clau-3 altered and Clau-3 treated cells induced significant (p=0.05) relative fold change of both cytokines. The level of cytokines expression in other cells (CFBE41o CFBE 41o wt, Clau3 knockout) was low and varied among experiments suggesting that CFTR may contribute to cytokine induction. The physiological change linking the levels of inflammatory cytokines induced by CF patients can be explored further.

6. FURTHER RESEARCH

Knocking down IL-17C may reduce the level of inflammation in CF patients and may also serve some therapeutic purposes. Bacterial infection should be carried out at different time intervals. This will help to establish a relationship between infection time and level of cytokine production.

CONSENT

As per international standard or university standard participants consent has been collected and preserved by the authors.

ETHICAL DISCLAIMER

As per international standard or university standard ethical approval has been collected and preserved by the authors.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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