UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS

Functional characterization of complexes regulating chloride and mucus transport and their significance in disease

" Documento Definitivo"

Doutoramento em Biologia
Especialidade de Biologia de Sistemas

Filipa Bica Simões

Tese orientada por:
Professor Doutor Karl Kunzelmann e Professora Doutora Margarida Amaral

Documento especialmente elaborado para a obtenção do grau de doutor

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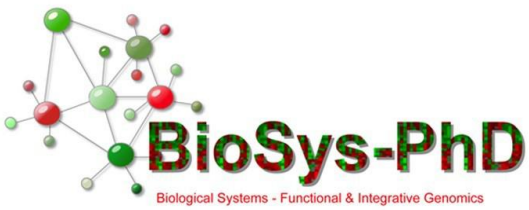

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No cumprimento do disposto da referida deliberação, o autor esclarece serem da sua responsabilidade, exceto quando referido o contrário, a execução das experiências que permitiram a elaboração dos resultados apresentados, assim como da interpretação e discussão dos mesmos. Os resultados obtidos por outros autores foram incluídos com a autorização dos mesmos para facilitar a compreensão dos trabalhos e estão assinalados nas respetivas figuras e metodologias.

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Summary

Mucus hyperproduction is a feature of several pulmonary diseases, such as cystic fibrosis (CF). CF is caused by mutations in the Cl^-/HCO_3^- channel CFTR, which result in airway mucus obstruction, bacterial infections, chronic inflammation, and epithelial remodelling. A possible therapy is the activation of alternative Cl^- channels, such as TMEM16A, that can compensate for the absence of functional CFTR. However, some studies describe a role of Cl^- channels in mucus hyperproduction, inflammation and differentiation, although the mechanisms behind these processes are still unclear.

Thus, the aim of this work was to understand the importance of Cl^- channels for mucus production, epithelial differentiation and airway inflammation.

In the **first chapter**, we showed that, although essential for airway hydration, TMEM16A is not required for mucus production. Particularly, our results show that TMEM16A is upregulated by interleukin-4 (IL-4) due to an increase in cell proliferation that is independent of mucus hyperproduction. Hence, we conclude that TMEM16A is a promising drug target for CF.

In the **second chapter**, we generated a novel CF airway basal cell line and observed that dysfunctional CFTR is sufficient to impair epithelial differentiation, even in the absence of bacterial infection or inflammation. This cell line is the first *in vitro* model allowing the study of the CFTR-dependent differentiation, thus contributing to the understanding of CF pathogenesis.

In the **third chapter**, we studied the interaction between inflammatory mediators and the airway epithelium. Our results describe differential responses according to the inflammatory stimuli in terms of mucus production, expression of Cl^- channels and epithelial differentiation. These findings may help to design novel cytokine-specific therapies to solubilise mucus secretions for the treatment of pulmonary diseases.

Altogether, these results contribute to the understanding of how mucus hyperproduction, ion transport and epithelial differentiation are regulated, particularly upon airway inflammation.

Key words: Cystic Fibrosis, Mucus, Chloride channels, Epithelial differentiation, Inflammation

Resumo

O muco constitui a primeira linha de defesa da imunidade inata do pulmão, protegendo o epitélio respiratório de microrganismos, alergénios e poluentes inalados. Em indivíduos saudáveis, o muco é um gel formado por 98% de água e 2% de sólidos, que incluem maioritariamente mucinas (MUC), uma família de glicoproteínas de elevada massa molecular (2 – 0.2 MDa) cuja expressão varia de tecido para tecido. Em particular, o muco respiratório é essencialmente constituído pelas mucinas 5AC e 5B (MUC5AC, MUC5B), as quais são secretadas por células caliciformes (em inglês: goblet cells).

Após secreção, as mucinas hidratam e expandem para formar um gel com as propriedades viscoelásticas necessárias para a limpeza mucociliar dos agentes inalados, através do batimento coordenado de cílios das células ciliadas. Para isto, é essencial manter a hidratação das vias respiratórias, as quais são revestidas por um líquido (ASL – do inglês: "Airway Surface Liquid"), cuja composição é regulada por canais iónicos que maioritariamente secretam cloreto (Cl^-) ou absorvem sódio (Na^+), criando um gradiente osmótico para o transporte de água.

Apesar do muco ser essencial para a limpeza mucociliar, a sua hiperprodução é prejudicial, ocorrendo em várias doenças pulmonares, tais como a asma, a Fibrose Quística (FQ) e a Doença Pulmonar Obstrutiva Crónica (DPOC). Em particular, na FQ a obstrução das vias respiratórias com um muco densamente viscoso e desidratado promove o aparecimento de infeções bacterianas recorrentes e inflamação crónica, levando à destruição progressiva da função pulmonar. Assim, os doentes com FQ estão constantemente expostos a agentes infeciosos e inflamatórios que danificam as vias respiratórias. Nestas condições, a proliferação, migração e diferenciação epitelial são essenciais para a regeneração do tecido. No entanto, estes processos também estão comprometidos na FQ, o que deteriora ainda mais a função pulmonar.

A FQ é causada por mutações no gene CFTR (do inglês: "Cystic Fibrosis conductance Regulator"), que codifica um canal de Cl^- e bicarbonato (HCO_3^-) expresso na membrana apical de células epiteliais de vários tecidos, incluindo as vias respiratórias. Até à data, foram descritas mais de 2100 variantes neste gene, entre as quais a deleção de um resíduo de fenilalanina na posição 508 (F508del) é a mutação mais comum, que causa um defeito no processamento e tráfego da proteína para a membrana plasmática. Na ausência de CFTR funcional, a atividade do canal de Na^+ epitelial (ENaC) aumenta e, consequentemente, o volume do ASL e o conteúdo de água no muco diminuem. A redução do transporte de HCO_3^- é também prejudicial, visto que estes iões são essenciais para a correta expansão e hidratação das mucinas após secreção.

A ativação de canais alternativos de Cl^- é uma possível terapia para a FQ que pode ser aplicada a todos os doentes, independentemente da mutação no gene CFTR. Entre possíveis candidatos, destaca-se a TMEM16A (do inglês: Transmembrane protein 16A), um canal de Cl^- ativado por cálcio (Ca^{2+}) (CaCC) que é expresso na membrana apical de células epiteliais respiratórias. Contudo, alguns estudos descrevem a TMEM16A como regulador da produção e secreção de muco, defendendo que a sua ativação pode ser prejudicial para a FQ. De facto, a expressão da TMEM16A aumenta em condições inflamatórias, durante as quais esta se localiza na membrana apical de células caliciformes, juntamente com a MUC5AC. Assim, a inibição da TMEM16A foi proposta como uma possível estratégia para diminuir a produção e secreção de muco e melhorar o fenótipo da FQ. No entanto, não é ainda claro se a sobre-expressão da TMEM16A realmente causa a hiperprodução de muco ou se é apenas uma consequência do processo inflamatório.

O objetivo deste trabalho era o de determinar a importância dos canais de Cl^- para a produção de muco, diferenciação epitelial e inflamação das vias respiratórias. Para isso, utilizámos uma linha celular basal brônquica que se diferencia nas várias células epiteliais respiratórias ao fim de 30 dias (BCi-NS1.1, do inglês: “Basal Cell Immortalised Non-Smoker 1.1”).

No **primeiro capítulo** deste trabalho, pretendemos determinar o papel da TMEM16A na produção de muco. Para isso, começámos por analisar a expressão da TMEM16A e da MUC5AC durante a diferenciação das células BCi-NS1.1. Os nossos resultados mostram que as duas proteínas estão inversamente relacionadas – enquanto a TMEM16A é expressa em células basais não-diferenciadas e proliferativas, a expressão de MUC5AC é apenas detetável após diferenciação. Estes resultados foram também suportados por ensaios de imunofluorescência, onde é claro que a TMEM16A e a MUC5AC têm padrões de localização distintos no espaço e no tempo. Quando células diferenciadas foram expostas à interleucina-4 (IL-4), observámos não só um aumento significativo da expressão da TMEM16A e da MUC5AC, como também do marcador de proliferação Ki-67. O mesmo foi verificado quando a proliferação de células diferenciadas foi induzida através de uma “ferida” (em inglês: “wound healing”). Estes resultados levaram-nos a questionar se a proliferação celular regula a expressão da TMEM16A. De facto, quando a IL-4 é incubada na presença de um inibidor da proliferação, a sobre-expressão da TMEM16A é reduzida significativamente, apesar dos níveis da MUC5AC não serem alterados, demonstrando assim que a produção da MUC5AC é independente da TMEM16A. Resumidamente, neste trabalho identificámos o mecanismo pelo qual a expressão da TMEM16A é regulada durante a inflamação induzida pela IL-4. Os nossos resultados demonstram, que, apesar de essencial para a hidratação das vias respiratórias, a TMEM16A não é necessária para a produção de MUC5AC e, por isso, a sua ativação deve continuar a ser considerada uma terapia promissora para o tratamento da FQ.

No **segundo capítulo**, caracterizámos o papel da CFTR na diferenciação epitelial respiratória. Os nossos resultados mostram que a expressão de CFTR *wt* aumenta com a diferenciação epitelial, sendo esta funcional e localizada na membrana apical de células diferenciadas. Particularmente, observámos que a CFTR é expressa maioritariamente em células ciliadas e células em clava (em inglês: club cells), as quais protegem o epitélio através da secreção de proteínas e surfactantes. De seguida, através da técnica CRISPR/Cas9, introduzimos a mutação F508del no gene CFTR das células BCi-NS1.1, criando uma linha celular homozigótica (BCi-CF1.1) e outra heterozigótica (BCi-Hz1.1). Comparando as três linhas celulares, verificámos que as células BCi-CF1.1 têm reduzida expressão e função de CFTR, as quais são parcialmente corrigidas por moduladores que aumentam o tráfego da proteína mutada para a membrana plasmática (VX-661 e VX-445). Contrariamente às BCi-Hz1.1, as células homozigóticas exibem um defeito na diferenciação e polarização epitelial, o qual é mais evidente na frequência de células ciliadas. Paralelamente, não observámos nenhum defeito na diferenciação dos ionócitos pulmonares, um tipo de célula epitelial recentemente identificado que expressa elevados níveis de CFTR. Em suma, estes resultados mostram que a mutação F508del é suficiente para afetar a diferenciação epitelial, mesmo na ausência de infeções bacterianas ou inflamação. As linhas celulares produzidas neste trabalho são únicas porque permitem o estudo da diferenciação epitelial e relacioná-lo com a expressão, função e localização da CFTR.

No **terceiro capítulo**, estudámos a interação entre mediadores inflamatórios e o epitélio respiratório. Para isso, analisámos um painel de sete citocinas cujos níveis estão desregulados em doenças crónicas pulmonares (IL-8, IL-1β, TNF-α, IL-4, IL-17A, IL-10 e IL-22). Estas citocinas foram incubadas em células BCi-NS1.1 diferenciadas e foram analisados os seus efeitos na produção de muco, expressão de canais de Cl^- e diferenciação epitelial. Os nossos resultados mostram que todas as citocinas analisadas levam à hiperprodução de muco, através do aumento diferencial dos níveis de mRNA da MUC5AC e/ou MUC5B. Observámos também que, de entre todos os canais de Cl^- analisados, CFTR destaca-se por ser o mais modulado na inflamação, já que todas as citocinas aumentaram a sua expressão, à exceção de TNF-α que teve o efeito oposto. Adicionalmente, observámos também que algumas citocinas regulam a diferenciação epitelial, modulando a percentagem de células ciliadas (IL-1β, IL-4, IL-10, IL-22), células em clava (IL-1β, IL-4) ou ionócitos pulmonares (IL-10, IL-22). Ao comparar o efeito da IL-1β, IL-4 e IL-10, verificámos que a IL-4 é a única citocina que aumenta significativamente a função de CFTR, o que pode ser explicado pelo facto de aumentar também a quantidade de células em clava que expressam CFTR. Estes resultados sugerem que estas células são a principal fonte de função de CFTR na presença de IL-4. Resumidamente, estas descobertas vão contribuir para a elucidação dos mecanismos pelos quais a inflamação modula o transporte iónico, produção de muco e diferenciação epitelial, o

que pode ajudar no desenho de estratégias terapêuticas para solubilizar as secreções de muco no tratamento de doenças crónicas respiratórias obstrutivas.

Em suma, os resultados obtidos neste trabalho contribuíram para uma melhor compreensão do papel da TMEM16A na hiperprodução de muco, de CFTR na diferenciação epitelial e do efeito de diferentes estímulos inflamatórios no epitélio respiratório. Adicionalmente, os modelos *in vitro* gerados (BCi-Hz1.1 e BCi-CF1.1) são fisiologicamente relevantes, já que expressam CFTR endógeno e mantêm a capacidade de diferenciação durante várias passagens. Estas linhas celulares vão permitir o melhor entendimento da FQ, em particular dos mecanismos moleculares e celulares que regulam a diferenciação, imunidade inata do epitélio respiratório e resposta a alvos terapêuticos.

Palavras-chave: Fibrose Quística, Muco, Canais de cloreto, Diferenciação epitelial, Inflamação

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Abbreviations

ABC – ATP-binding cassette

ALI – Air-Liquid Interface

Amil – Amiloride

ANO1 – Anoctamin 1

APS – Ammonium Persulfate

ASL – Airway Surface Liquid

BCi-NS1.1 – Basal Cell Immortalised Non-Smoker 1.1 (cell line)

BCi-Hz1.1 – Basal cells heterozygous for the F508del mutation

BCi-CF1.1 – Basal cells homozygous for the F508del mutation

BSA – Bovine Serum Albumin

CaCC – Ca^{2+}-activated Cl^- channel

CC16 – Club Cell protein 16

cDNA – Complementary DNA

CF – Cystic Fibrosis

CFTR – Cystic Fibrosis conductance Transmembrane Regulator

COPD – Chronic Obstructive Pulmonary Disease

cRNA – CRISPR RNA

cryo-EM – Cryogenic Electron Microscopy

DAG – Diacylglycerol

DLL4 – Delta-Like Ligand 4

DMSO – Dimethyl Sulfoxide

DNAI1 – Dynein Axonemal Intermediate Chain 1

DTT – Dithiothreitol

EDTA – Ethylenediaminetetraacetic acid

ENaC – Epithelial Sodium Channel

ER – Endoplasmic Reticulum

ERQC – ER Quality Control

F508del – Deletion of phenylalanine at position 508

FITC – Fluorescein isothiocyanate

FOXI1 – Forkhead Box I1

Fsk – Forskolin

GAPDH – Glyceraldehyde 3-phosphate dehydrogenase

GCH – Goblet Cell Hyperplasia

GCM – Goblet Cell Metaplasia

gRNA – guide RNA

HBE – Human Bronchial Epithelial

HDR – Homology-Directed Repair

HRP – Horseradish Peroxidase

hTERT – Human Telomerase Reverse Transcriptase

IBMX – 3-isobutyl-1-methylxanthine

ICE – Inference of CRISPR Edits

I_{eq-sc} – Equivalent short circuit current

IF – Immunofluorescence

IL – Interleukin

IP_3 – Inositol triphosphate

IP_3R – IP_3 receptor

LPS – Lipopolysaccharide

MCC – Mucociliary Clearance

MCT – Mucociliary Transport

MUC – Mucin

mRNA – Messenger RNA

MSD – Membrane Spanning Domain

NAC – N-acetylcysteine

NBD – Nucleotide Binding Domain

NFM – Non-fat Milk

nhTMEM16 – *Nectria haematococca* TMEM16

PBS – Phosphate Buffered Saline

PCL – Periciliary Layer

PCR – Polymerase Chain Reaction

PFA – Paraformaldehyde

PFC – Perfluorocarbon

PHA – Pseudohypoaldosteronism

PI – Pancreatic Insufficiency

PIP_2 – Phosphatidylinositol 4,5-bisphosphate

PIP_2R – PIP_2 Receptor

PKA – Protein Kinase A

PLC – Phospholipase C

PM – Plasma Membrane

PMSF – Phenylmethylsulfonyl fluoride

PNECs – Pulmonary neuroendocrine cells

PTC – Premature Termination Codon

PVDF – Polyvinylidene difluoride

qRT-PCR – quantitative reverse-transcriptase PCR

RD – Regulatory Domain

RNP – Ribonucleoprotein

R_{te} – Transepithelial resistance

SDS – Sodium Dodecyl Sulfate

SDS-PAGE – SDS-polyacrylamide gel electrophoresis

SEM – Standard Error of the Mean

SLC26 – Solute carrier 26

ssODN – single-stranded donor oligonucleotides

STAT – Signal Transducer and Activator of Transcription

TAE – Tris-Acetate-EDTA

TBS – Tris-Buffered Saline

TEER – Transepithelial Electrical Resistance

TEMED – Tetramethylethylenediamine

Th – T-helper

TMEM16A – Transmembrane protein 16A

TNF-α – Tumor Necrosis Factor-α

TiJOR – Tight Junction Organization Rate

tracrRNA – Trans-activating cRNA

WB – Western Blot

V_{te} – Transepithelial voltage

ZO-1/TJP1 – Zonula occludens-1/tight junction protein 1

I. Introduction

1. Cystic Fibrosis: Overview

1.1 General Introduction

Cystic Fibrosis (CF) is the most common life-shortening autosomal recessive disease in Caucasians (Collins, 1992), affecting more than 90,000 individuals worldwide and more than 48,000 just in Europe (*European Cystic Fibrosis Society (ECFS)*, 2021). It is estimated to occur in 1 in 2500-6000 live births and to have a carrier frequency of 1 in 25 to 40 individuals, depending on the geographic region (Bobadilla et al., 2002; O'Sullivan & Freedman, 2009). Dorothy Andersen, who first described this disease in 1938, observed the destruction of the pancreatic exocrine function, introducing the term "Cystic Fibrosis of the pancreas" (Andersen, 1938). However, it was only in 1989 that the CF gene was identified and termed CF Transmembrane Conductance Regulator (CFTR) after the putative role of the respective encoded protein (Kerem et al., 1989; Riordan et al., 1989; Rommens et al., 1990)**.** CFTR protein is a chloride (Cl^-) and bicarbonate (HCO_3^-) channel expressed at the apical membrane of epithelial cells from different tissues, including the airways, pancreas, intestine and sweat glands (Sheppard & Welsh, 1999)**.**

1.2 Pathophysiology

Also known as mucoviscidosis, CF is a chronic disease with extensive respiratory involvement characterised by the build-up of highly viscous mucus that becomes an attractive environment for bacteria colonisation and propagation, especially by *Pseudomonas aeruginosa*. Mucus obstruction, recurrent respiratory infections and chronic inflammation ultimately lead to progressive loss of lung function that can only be solved by lung transplantation (Ehre et al., 2014; Kreda et al., 2012; O'Sullivan & Freedman, 2009).

While lung disease accounts for 80% of CF mortality, other organs are also affected, including the gastrointestinal system, sweat glands, biliary ducts and the male reproductive system (O'Sullivan & Freedman, 2009). Thus, classical CF is also manifested by pancreatic insufficiency (PI) that leads to malnutrition and failure to grow (in the absence of replacement enzymes), increased concentrations of Cl^- in the sweat and male infertility. Other common complications include intestinal obstruction, pancreatitis, liver disease and diabetes (Zielenski, 2000).

1.3 CFTR Protein Structure, Function, Biosynthesis and Processing

CFTR protein belongs to the ATP-binding cassette (ABC) transporter superfamily and is arranged in five domains: two membrane-spanning domains (MSD1/2) each with six transmembrane segments and forming the channel pore; two nucleotide-binding domains (NBD1/2) controlling channel gating via ATP binding and hydrolysis; and a regulatory domain (RD) that contains multiple phosphorylation sites essential for modulation of channel activity

(Amaral 2005; Riordan et al. 1989). Figure 1 illustrates a schematic representation of the CFTR topology (A) and the molecular structure of the human CFTR channel (B), resolved by electron cryogenic-electron microscopy (cryo-EM) (Liu et al., 2017). Activation of CFTR is dependent on the phosphorylation of serine residues at the RD by the cAMP-dependent Protein Kinase A (PKA). ATP binding to the NBDs and its subsequent hydrolysis allows the pore to open. Once phosphatases dephosphorylate the RD, CFTR returns to its closed status (Frizzell & Hanrahan, 2012).

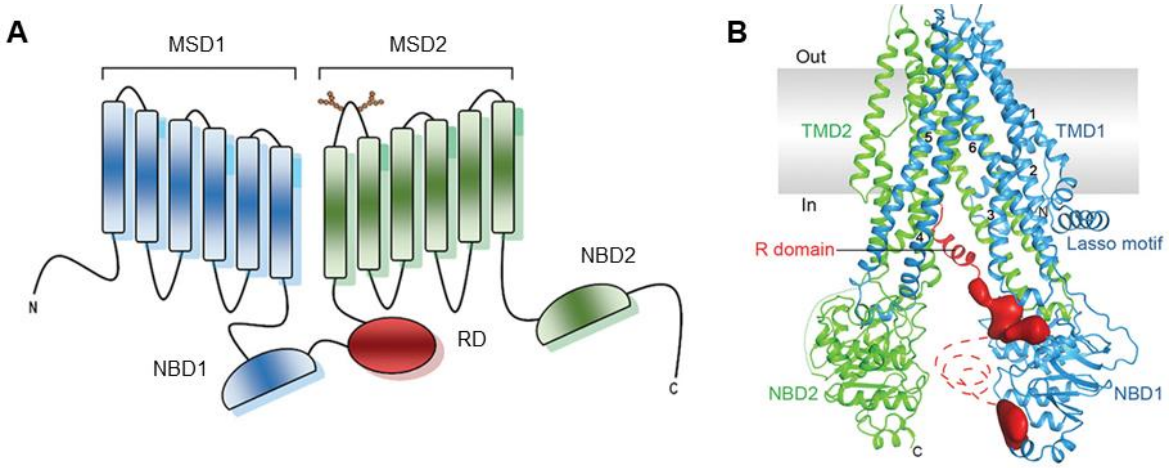


Figure 1 – CFTR protein structure. (A) CFTR is composed of two membrane-spanning domains (MSD1/2), two nucleotide-binding domains (NBD1/2) and one regulatory domain (RD). Adapted from (Lopes-Pacheco, 2020). **(B)** Molecular structure of the human CFTR channel in the dephosphorylated and ATP-free conformation, determined by electron cryomicroscopy (cryo-EM) (Liu et al., 2017).

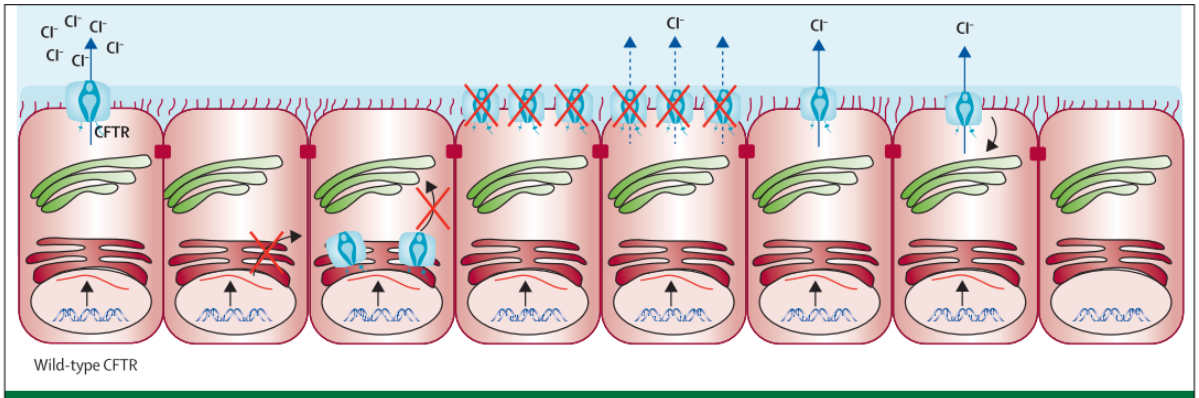
CFTR plays a key role in maintaining ion and fluid homeostasis, not only by functioning as a Cl^- and HCO_3^- channel but also by regulating other ion channels and transporters (Frizzell & Hanrahan, 2012). To carry out its function, CFTR needs to be localised at the apical plasma membrane (PM). CFTR synthesis, folding, and core-glycosylation occurs in the endoplasmic reticulum (ER), where it achieves its immature form with about 145 kDa. Once checked for proper folding by the ER quality control (ERQC), CFTR migrates to the Golgi complex where it is fully-glycosylated, reaching its mature form of about 170 kDa (Amaral 2005).

1.4 CFTR Mutations

Among the >2,100 variants that were so far reported in the CFTR gene, a deletion of a phenylalanine residue at position 508 (F508del) is the most common mutation, occurring in ~80% of CF alleles worldwide (*Cystic Fibrosis Mutation Database*, 2020).

Mutations in the CFTR gene are grouped into seven classes (Figure 2), based on the respective defect they cause on protein synthesis, trafficking, or function. Class I mutations terminate mRNA translation through the generation of premature termination codons (PTCs). Class II mutations (including F508del) result in a trafficking defect to the cell surface due to retention in the ER and premature proteasomal degradation. Class III and IV mutations impair

channel gating and reduce anion conductance, respectively. Class V mutations decrease protein synthesis, often due to alternative splicing. Class VI mutations reduce CFTR stability at the cell surface by increased exocytosis or reduced recycling. Lastly, class VII mutations result in the total absence of mRNA production and, therefore, are considered unrescuable by pharmacological agents (Amaral 2015).



	Class I	Class II	Class III	Class IV	Class V	Class VI	Class VII
CFTR defect	No protein	No traffic	Impaired gating	Decreased conductance	Less protein	Less stable	No mRNA
Mutation examples	GLy542X, Trp1282X	Phe508del, Asn1303Lys, Ala561Glu	Gly551Asp, Ser549Arg, Gly1349Asp	Arg117His, Arg334Trp, Ala455Glu	Ala455Glu, 3272-26A→G, 3849+10 kg C→T	c. 120del23, rPhe508del	dele2,3(21 kb), 1717-1G→A
Corrective therapy	Rescue synthesis	Rescue traffic	Restore channel activity	Restore channel activity	Correct splicing	Promote stability	Unrescuable
Drug (approved)	Read-through compounds (no)	Correctors (yes)	Potentiators (yes)	Potentiators (no)	Antisense oligonucleotides, correctors, potentiators? (no)	Stabilisers (no)	Bypass therapies (no)

Figure 2 – Functional classes of CFTR mutations and respective therapeutic strategies. Examples of the most common mutations and therapies for each class. Retrieved from (De Boeck & Amaral, 2016).

1.5 Therapeutic Approaches

Standard CF therapy is mainly based on mucolytics and chest physiotherapy to dissolve thick mucus and improve airway clearance, antibiotics to eradicate bacterial lung infections, anti-inflammatory agents to deal with chronic airway inflammation, a hypercaloric diet with good nutrition and replacement with pancreatic enzymes to compensate for food malabsorption. This set of 'symptomatic' therapies allowed significant advances in delaying disease progression and increasing life expectancy to more than 40 years of age. However, the quality of life of individuals with CF is still limited, and the disease needs to be addressed beyond its symptoms (De Boeck & Amaral, 2016; O'Sullivan & Freedman, 2009).

Remarkably, compounds that target mutant CFTR (i.e. CFTR modulators) showed significant improvements by either increasing F508del-CFTR processing and trafficking to the cell surface (correctors) or by stimulating the activity of CFTR gating mutants already at the PM (potentiators) and have been approved for the clinic (Ramsey et al., 2011; Rowe et al.,

2017; Wainwright et al., 2015). More recently, a triple combination (Trikafta™) of two correctors (VX-661/tezacaftor and VX-445/elexacaftor) and one potentiator (VX-770/ivacaftor) was approved for all individuals carrying the F508del mutation in at least one allele (Hoy, 2019). Nevertheless, this strategy is only suitable for F508del and mutations in classes III, IV and V (the latter two associated with residual function). Thus, different therapeutic strategies still need to be developed for CFTR mutants in the other classes (Figure 2), namely compounds that promote read-through of PTCs (class I), splicing factors that correct aberrant splicing (Class V) and molecules that stabilise CFTR at the cell surface (class VI). The only solution for the unrescuable mutations (class VII) is either gene therapy or the activation of alternative non-CFTR Cl^- channels/transporters that coexist at the apical membrane of epithelial cells (De Boeck & Amaral, 2016).

2. The human airway epithelium – Mucus, Mucins and Mucociliary Clearance

2.1 Structure of the human airway epithelium, mucus function and composition

The human airway epithelium is the interface of the human body with the inhaled environment, providing a complex physical barrier that maintains lung fluid homeostasis, mediates the clearance of invasive particles/pathogens and recruits immune cells in response to infection (Knight & Holgate, 2003).

Conducting airways are lined by a pseudostratified epithelium formed by different specialised epithelial cells that work together to maintain normal lung functions (Figure 3). The most common cells of the large conducting airways are ciliated cells that drive airway mucociliary clearance (MCC) through the coordinated beating of motile cilia (Braiman & Priel, 2008); goblet cells that secrete mucus to trap particles and pathogens (Curran & Cohn, 2010); club cells (formerly, Clara cells) that protect the epithelium by secreting surfactants and antiproteases (Rokicki et al., 2016); and basal progenitor cells that proliferate and differentiate into the other cell-types to renew the epithelium during both homeostasis and repair. Additionally, club cells can also be intermediate progenitors of ciliated and goblet cells during epithelial repair (Rock & Hogan, 2011a). Less frequent cell types include pulmonary neuroendocrine cells (PNECs), that sense oxygen levels and irritants and control lung immune response (Garg et al., 2019); tuft or brush cells that act as immune sensors (Ting & von Moltke, 2019); and the recently described pulmonary ionocytes that are enriched in CFTR and thus speculated to regulate fluid and solute concentrations (Montoro et al., 2018; Plasschaert et al., 2018), but in which CFTR seems to play no role in Cl^- secretion (Scudieri et al., 2020).

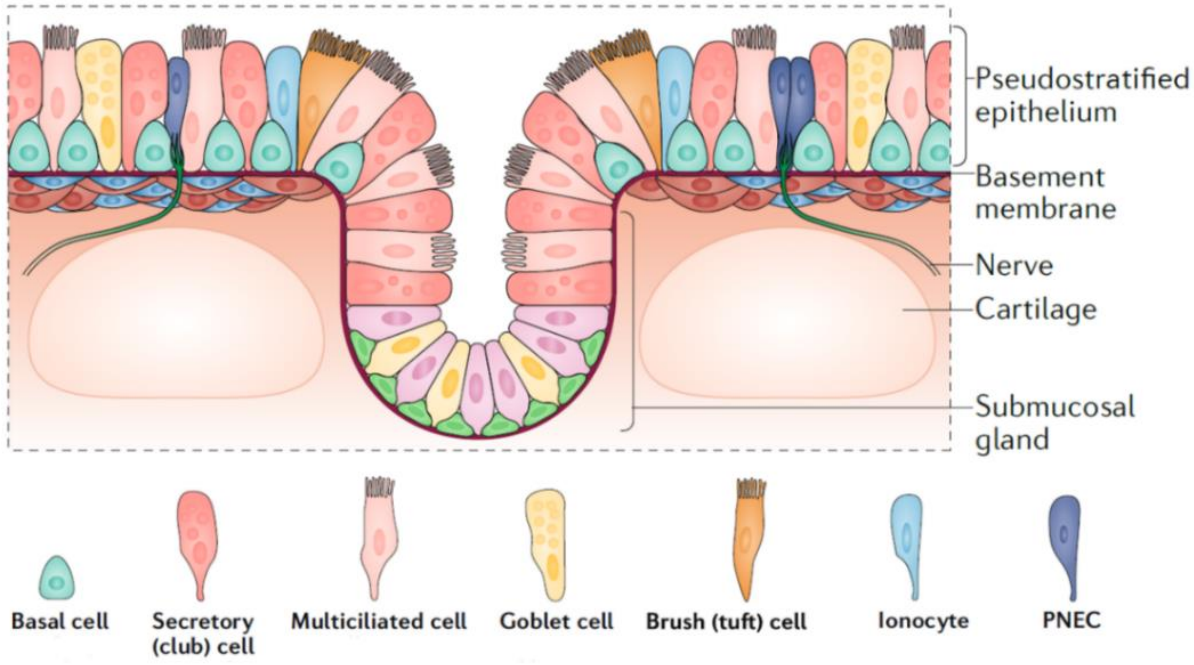


Figure 3 – Cellular composition of the surface airway epithelium. Schematic representation of the respiratory epithelium, including the pseudostratified epithelium and the submucosal glands. The pseudostratified epithelium is formed by a single layer of epithelial cells with different morphologies and functions that are all in contact with the basement membrane. Adapted from (Zepp & Morrisey, 2019).

Mucus provides the first line of innate lung defence, protecting the airway epithelium from the harmful effects of inhaled pollutants, allergens, and pathogens. Healthy airway mucus is a gel formed by 98% water and 2% solids, the latter including mostly mucins, but also non-mucin proteins, ions, lipids and antimicrobial peptides (Fahy & Dickey, 2010). Mucins (MUC) are a class of heavily (2 – 0.2 MDa) O-glycosylated proteins (50 – 90% glycan content) that have a tissue-specific expression and are classified as either membrane-tethered or gel-forming mucins (Thornton et al., 2008).

Mucin production and secretion comprise several biological processes from gene transcription to vesicle exocytosis that are tightly controlled both in homeostasis and in response to environmental challenges (Rose & Voynow, 2006; Symmes et al., 2018) (Figure 4). Mucus secretion occurs upon regulated exocytosis triggered either by a baseline mechanism or by stimulation of purinergic receptors (Davis & Dickey, 2008). Following secretion, mucins are hydrated and expand to form an airway mucus gel with optimal viscoelastic properties required for normal transport (Mehmet Kesimer et al., 2010; Pedro Verdugo, 1991).

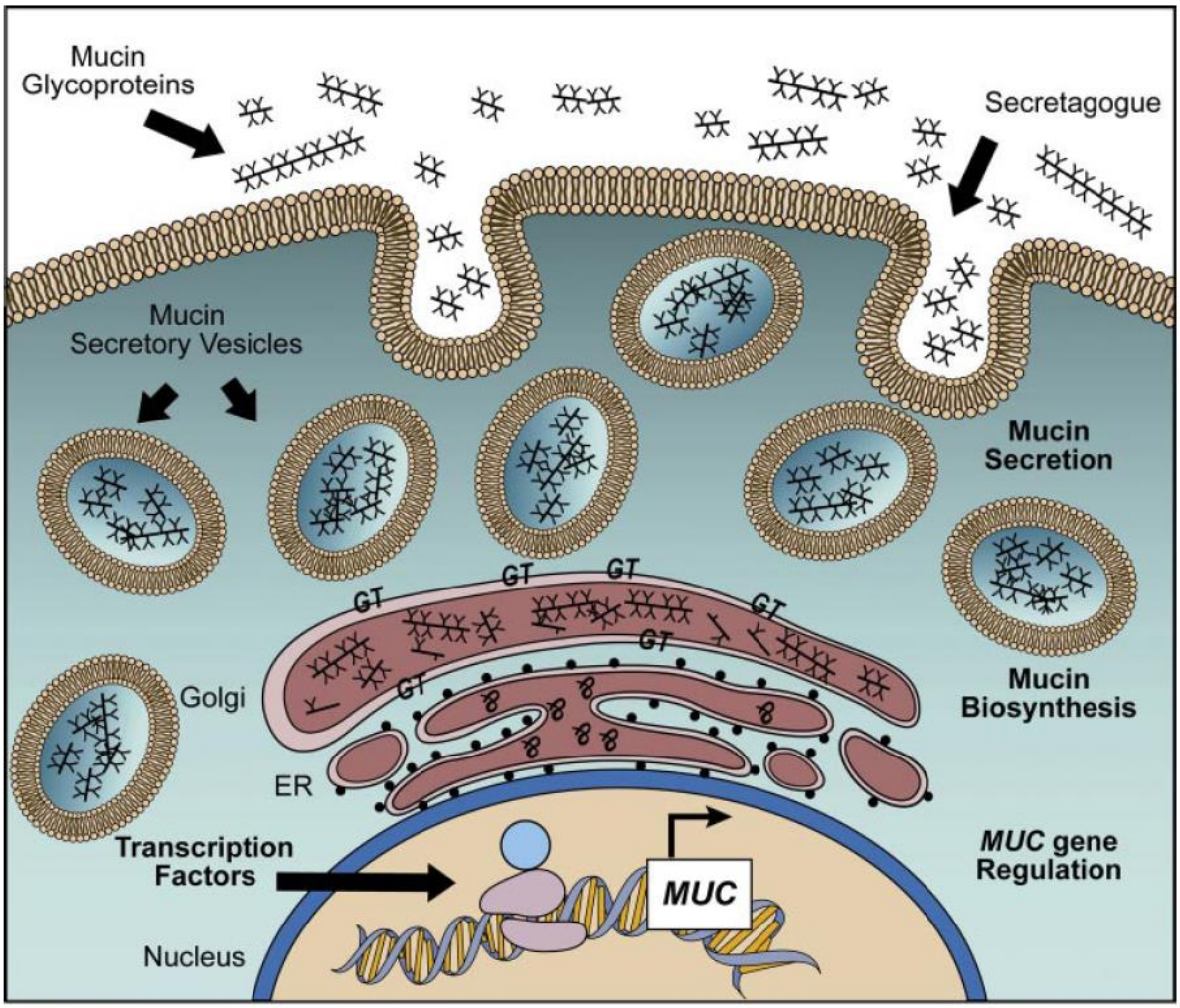


Figure 4 – Scheme illustrating mucin production and secretion in goblet cells. The expression of mucin genes (MUC) is modulated by different transcription factors. Following transcription and translation, the mucin protein backbone is transported from the Endoplasmic Reticulum (ER) to the Golgi complex, where it is O-glycosylated. Mature fully-glycosylated mucins are condensed and stored inside secretory granules/vesicles until mucin exocytosis is triggered by an external secretagogue. Retrieved from (Rose & Voynow, 2006).

Respiratory mucus comprises a component in the periciliary layer (PCL) (~ 10 µm) made up of extended membrane-tethered mucins, MUC1, 4, 16, 20 and then, surmounting this layer, is a mucus gel layer (~ 60 µm) containing MUC5AC and MUC5B, which are the main gel-forming mucins present in human airway mucus (Button et al., 2012; M. Kesimer et al., 2013). These two mucins are mostly secreted from goblet cells at the surface airway epithelium or by submucosal glands (Bonser & Erle, 2017; Buisine et al., 1999; Okuda et al., 2019). Interestingly, while MUC5B was reported to be essential to maintain normal MCC and to resolve inflammation following infection (Roy et al., 2014), recent studies also describe MUC5AC as a key controller of mucus transport by anchoring mucus bundles containing MUC5B that are secreted from the submucosal glands (Ermund et al., 2017; Xie et al., 2018).

2.2 Mucociliary clearance – Role of ion channels and transporters for airway hydration

Mucociliary Clearance (MCC) consists of the continuous transport of mucus from the airways through the coordinated beating of motile cilia (Button et al., 2008). Efficient MCC depends on the maintenance of a normal airway surface liquid (ASL) consisting of a heterogeneous fluid formed by ions, antimicrobial peptides/proteins, antioxidants, proteases, mucins and water (Webster & Tarran, 2018). In turn, electrolyte secretion by the airways is necessary to produce the ASL that allows MCC of the lungs. The ASL comprises the PCL that covers the cilia, containing membrane-tethered mucins to prevent mucus penetration (Button et al., 2012; M. Kesimer et al., 2013).

To maintain normal lung function, airway epithelial cells must control the volume, pH, composition and viscosity of the ASL (Webster & Tarran, 2018). Secretion is driven by the opening of Cl^- selective ion channels in the apical membrane of airway epithelial cells. To this end, cells are equipped with a variety of ion channels that are differentially distributed between the basolateral and apical membranes (Figure 5).

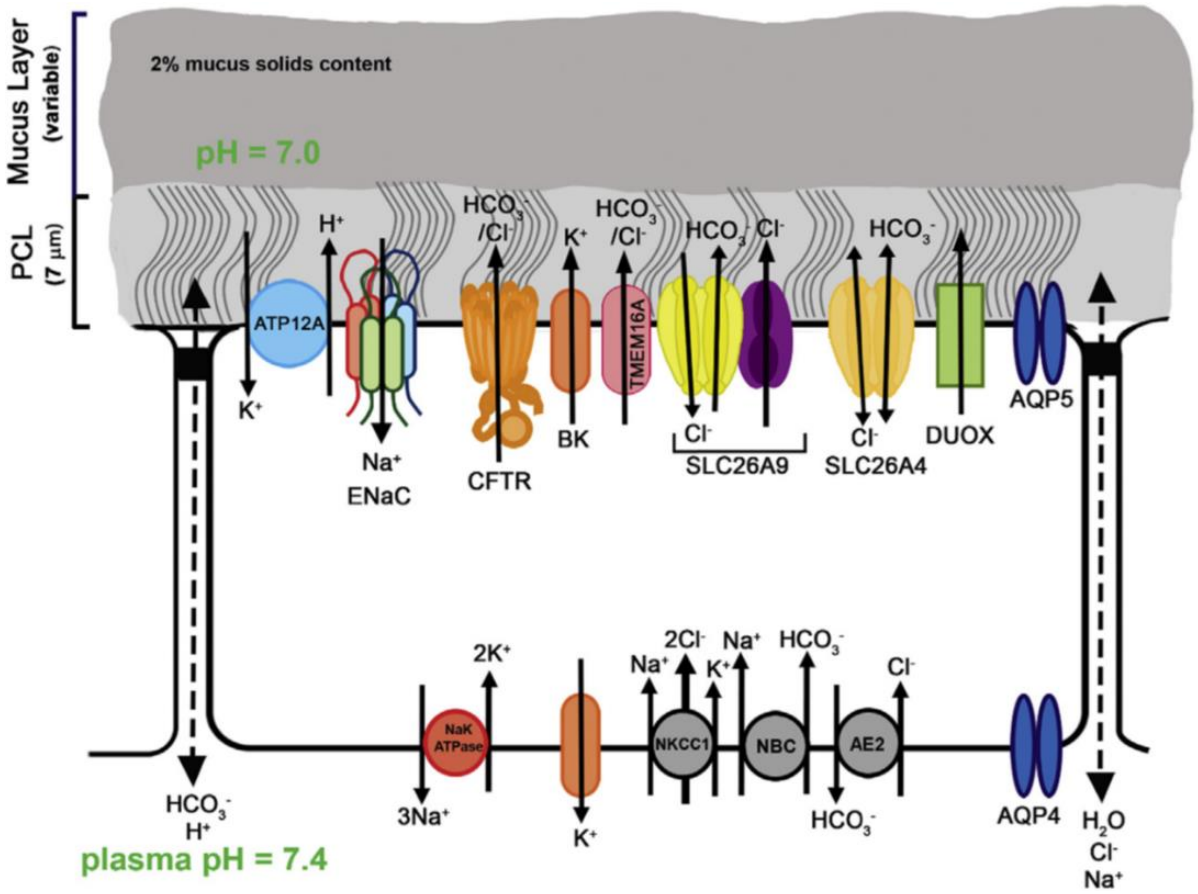


Figure 5 – Schematic representation of an airway epithelial cell with the specific distribution of ion channels, exchangers, and pumps in the apical and basolateral membranes. Particularly, transepithelial Cl^- transport is mediated by CFTR, TMEM16A, SLC26A4 and SLC26A9, while ENaC drives Na^+ absorption. Adapted from (Webster & Tarran, 2018).

Proper airway hydration is mainly achieved by the combination of apical sodium (Na^+) absorption through the epithelial sodium channel (ENaC) and cAMP-activated Cl^- secretion through CFTR, with water passively flowing according to the osmotic gradient (Hollenhorst et al., 2011). Additionally, other Cl^- channels and transporters coexist with CFTR at the apical membrane, namely the calcium (Ca^{2+})-activated Cl^- channel (CaCC) transmembrane protein 16 A (TMEM16A, also anoctamin 1 or ANO1) (Caputo et al., 2008; Schroeder et al., 2008; Yang et al., 2008) and the Cl^-/HCO_3^- exchangers of the solute carrier family 26 SCL26A9 and SLC26A4/Pendrin (Garnett et al., 2011). These channels, responding to either a receptor-mediated increase in intracellular cAMP or cytosolic Ca^{2+}, generate an ASL with approximately 7-8 µm, corresponding to the height of outstretched cilia (Button et al., 2012). Traditionally cAMP-dependent and Ca^{2+}-dependent secretory pathways were regarded as independent. However, this concept was challenged by the demonstration of cross-talk between CFTR and TMEM16A Cl^- channels (Kunzelmann & Mehta, 2013; Ousingsawat et al., 2011), thus further narrowing the close relationship of Cl^-/HCO_3^- and mucus secretion.

In CF, the defect in CFTR-mediated Cl^- secretion leads to ENaC hyperactivation and a consequent increase in Na^+ absorption. As these ions are essential for water flow, CF individuals have a dehydrated ASL and reduced water content in mucus, which consequently is abnormally thick and sticky (Boucher, 2007; Matsui et al., 2006). This leads to extensive small airway obstruction causing disseminated bronchiectasis. Thus, the airways of CF individuals become the perfect environment for bacteria colonisation and propagation, leading to recurrent respiratory infections and chronic inflammation that result in the progressive loss of respiratory function and ultimately lung failure (Amaral 2015) (Figure 6).

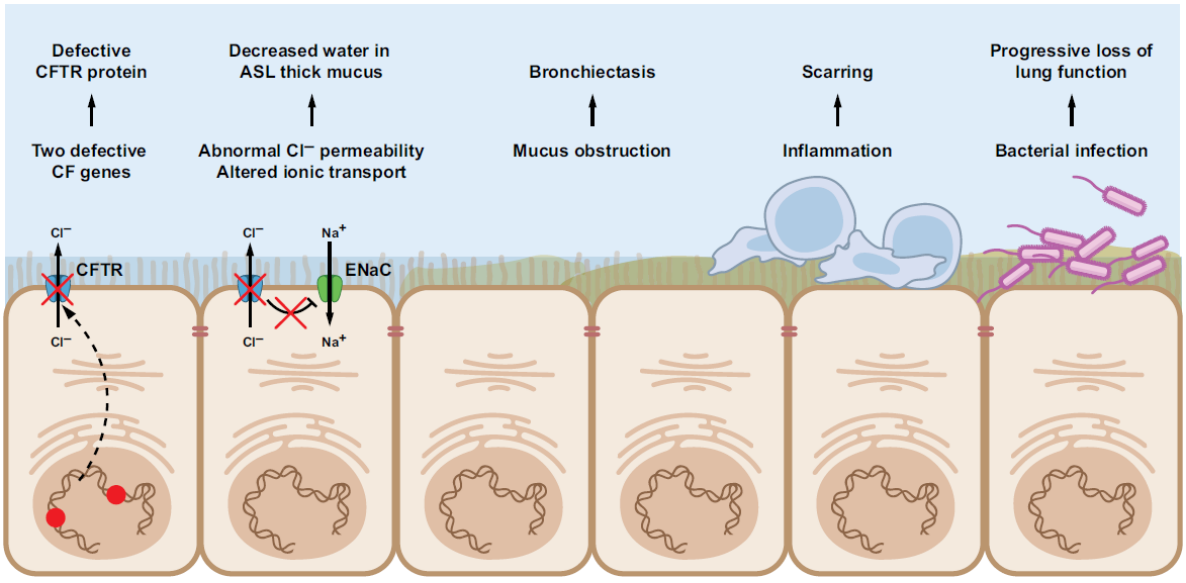


Figure 6 – The CF pathogenesis cascade in the lung. In the absence of functional CFTR, ENaC is hyperactive, the ASL becomes dehydrated, and the mucus very thick and sticky. Mucus obstruction of the small airways, continuous cycles of inflammation and bacterial infections leads to progressive loss of lung function. Retrieved from (Amaral 2015).

2.3 Mucus hyperproduction in airway obstructive diseases – focus on Cystic Fibrosis

Mucus hyperproduction and MCC dysfunction are common features in many chronic airway obstructive pulmonary diseases, such as asthma, Chronic Obstructive Pulmonary Disease (COPD) and CF. Both environmental *stimuli* and host response mediators (including allergens, viruses, bacterial lipopolysaccharide (LPS), neutrophil elastase, inflammatory cytokines, etc.) play a role in these conditions leading to upregulation of mucin production (Williams et al., 2006). Such an increase in airway mucus levels can result from mucus hypersecretion, proteolytic cleavage of membrane-tethered mucins and/or epithelial remodelling through goblet cell metaplasia and hyperplasia (GCM/H) (Ehre et al., 2014). Metaplasia is a trans-differentiation process by which a fully differentiated cell changes its phenotype (becoming a goblet cell in this case), while hyperplasia is caused by cell proliferation, and these *de novo* produced cells differentiate into a certain cell type (i.e., goblet cells in this situation) (Williams et al., 2006).

CF airways are thus clogged with a highly viscous mucus which is characterised by alterations in the concentration of several of its constituents, namely mucins, water, ions, pH and the degree of cross-linking between mucins (Ehre et al., 2014) (Figure 7).

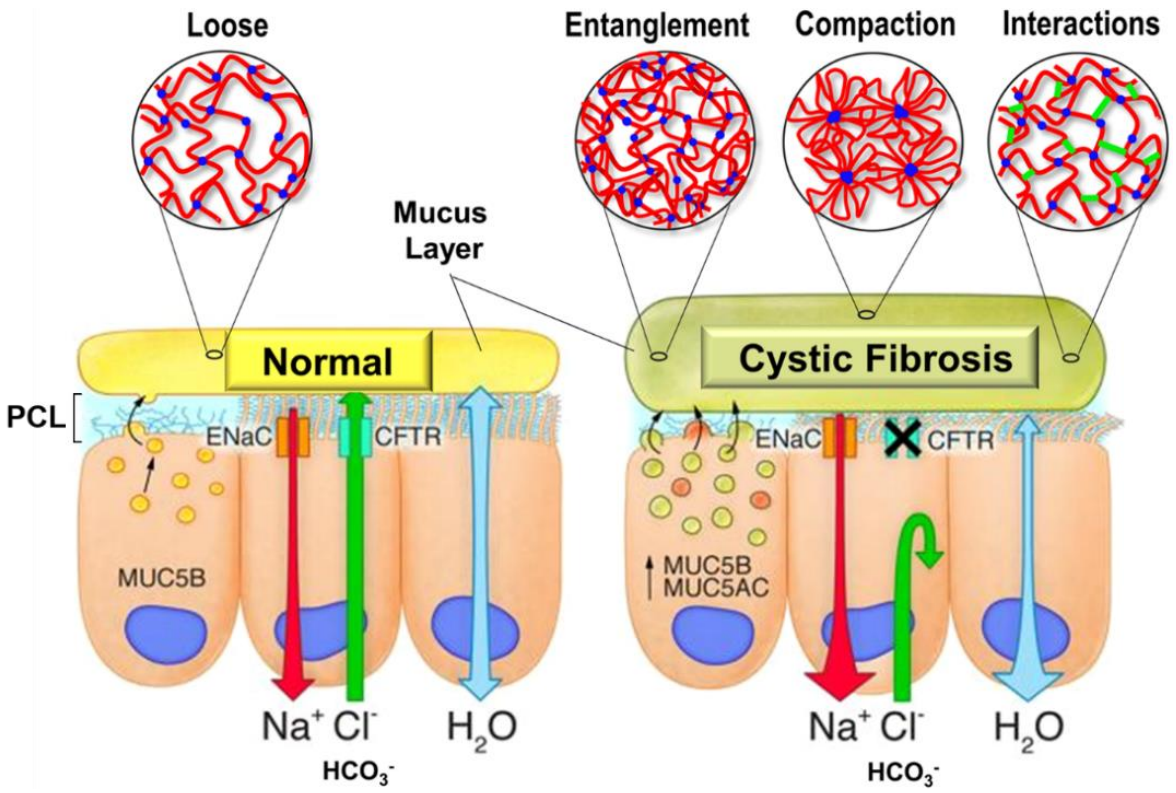


Figure 7 – Model illustrating the changes between non-CF and CF mucus. In non-CF airways, Cl^- secretion through CFTR and ENaC-mediated Na^+ absorption create a driving force for water movement across the epithelium, ensuring proper airway hydration. Goblet cells produce a thin layer of mucus that has the optimal rheological properties required for "loose," i.e. efficient, MCC. Conversely, in CF, the

absence of functional CFTR and the increased Na^+ absorption through ENaC impairs water transport, decreasing the PCL height. Airway dehydration and mucus hyperproduction compresses the cilia and leads to mucus entanglement. Reduced HCO_3^- secretion impairs mucin expansion, leading to mucus compaction. Changes in hydration, pH and oxidative stress introduce new permanent cross-links between mucin chains (interactions). Adapted from (Morrison et al., 2019).

CF mucus also has increased content of MUC5AC and MUC5B, especially during pulmonary exacerbations, being MUC5B the predominant mucin (Henderson et al., 2014; Henke et al., 2007; Kirkham et al., 2002). Nonetheless, a significant genetic association was found between the MUC5AC gene and lung disease, suggesting that this mucin may be a modifier gene in CF (Guo X. L. et al. 2011).

Mucus hyperproduction, coupled with ASL dehydration, increases the osmotic pressure on the mucus gel and collapses the PCL, compressing the cilia (Button et al., 2012; Kesimer et al., 2013). Under these conditions, the mucus layer adheres directly to the surface epithelium, the mucus becomes static, and clearance is stopped. Ultimately, mucus stasis leads to recurrent bacterial infections, chronic inflammation and structural lung damage through epithelial scarring (Boucher, 2007; Matsui et al., 2006). This phenotype can be partially reversed by the administration of osmotically active agents that rehydrate both ASL and mucus, such as hypertonic solutions or mannitol, improving clearance and lung function (Daviskas et al., 2010; Scott H. et al., 2006).

Additional factors contributing to the aberrant properties of CF mucus include neutrophil-mediated inflammation and oxidative stress, which induce permanent cross-links between mucin chains, thus stiffening the mucus gel (Yuan et al., 2015). Hence, reducing agents with mucolytic activity, such as N-acetylcysteine (NAC), also benefit airway clearance by breaking the disulphide bonds between mucin monomers and thus decreasing the size of mucus polymers (De Lisle et al., 2007).

Proper expansion and hydration of secreted mucus are facilitated by HCO_3^- ions that sequester the Ca^{2+} and H^+ ions present inside the mucin-containing granules to shield the negatively charged glycans (Chen et al., 2010; Kuver & Lee, 2004; Quinton, 2010; P. Verdugo et al., 1987) (Figure 8). Thus, in CF, the defective HCO_3^- transport and the consequent decrease of the ASL pH results in the impairment of mucin expansion (Chen et al., 2010; Gorrieri et al., 2016; Gustafsson et al., 2012; Quinton, 2008), increases mucus viscosity (Tang et al., 2016) and hampers bacterial killing (Pezzulo et al., 2012; Smith et al., 1996).

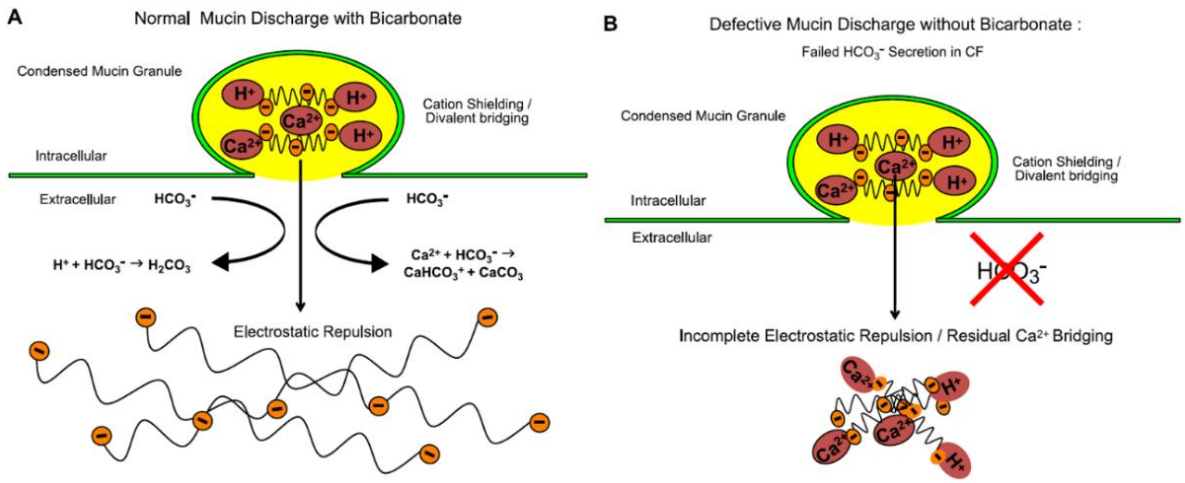


Figure 8 – Model illustrating the role of HCO_3^- on mucus expansion. Mucins are formed by negatively charged glycans that are shielded by Ca^{2+} and H^+ ions inside the mucin-containing granules. Upon mucus secretion, under normal conditions **(A)**, the HCO_3^- ions present in the extracellular space react with Ca^{2+} and H^+, forcing mucin expansion due to electrostatic repulsion. In contrast **(B)**, in CF, there is no competition of HCO_3^- ions and therefore, the mucin glycan chains remain bounded to Ca^{2+} and H^+ ions, which promotes the adherence of mucin chains, resulting in a highly condensed and viscous mucus. Adapted from (Quinton, 2010).

Overall, the abnormal rheological properties that define CF mucus lead to a cascade of events with mucus accumulation and adhesion directly to the surface epithelium, consequent small airway obstruction, recurrent bacterial infections, and chronic inflammation. This is a complex cyclic process, with bacterial infections stimulating goblet cell hyperplasia and neutrophil recruitment, which exacerbates mucus viscosity and clearance (Cash et al., 1979; Ehre et al., 2014). Consecutively, impaired mucus transport facilitates bacteria growth (Knowles & Boucher, 2002). Remarkably, the administration of CFTR modulators, such as the potentiator ivacaftor, improves MCC, indicating that partial CFTR function is sufficient to normalise mucus properties (Donaldson et al., 2018).

3. Chloride channels/transporters in airway inflammation and differentiation

3.1 General Introduction

Besides acting as a physical barrier and maintaining MCC, the airway epithelium also constitutes the primary host defence through a complex cross-talk with the immune system. In the presence of pathogens, the epithelium recruits immune cells that secrete inflammatory mediators, such as cytokines, chemokines and growth factors. These *stimuli* modulate the expression of several epithelial ion channels, transporters and regulators so as to increase ion transport, fluid secretion and thus promote MCC (Hiemstra et al., 2015; Sala-rabanal et al., 2015; Stanke, 2015). Different pathological conditions reflect the importance of this interaction, such as CF and pseudohypoaldosteronism (PHA) type I, both conditions characterised by an

increased susceptibility to pathogens associated with loss-of-function mutations in the CFTR or the ENaC gene, respectively (Stanke, 2015). In parallel, Cl^- ions have been described to function as signalling effectors in several physiological processes such as inflammation, cell differentiation and proliferation, modulating gene and protein expression. Therefore, Cl^- channels and transporters are crucial to maintaining the homeostasis of these processes by regulating the intracellular concentration of these ions (Valdivieso & Santa-Coloma, 2019).

3.2 Role of CFTR in airway inflammation and differentiation

The persistent inflammatory response that occurs in CF is excessive relative to the bacteria burden, being characterised by massive neutrophil recruitment and an imbalance in the secretion of inflammatory mediators (Roesch et al., 2018; Schnúr et al., 2016). This self-perpetuating inflammatory response occurs from an early age and contributes significantly to the pathogenesis cascade that leads to progressive loss of lung function (Chmiel & Davis, 2003; Sly et al., 2013).

Inflammatory mediators regulate the innate immunity of the airways by mediating the interaction between bacteria and epithelial cells (Nichols et al., 2008). Expectedly, CF airways thus contain elevated levels of pro-inflammatory mediators (e.g. IL-8, IL-1β, TNF-α) and decreased concentrations of anti-inflammatory cytokines (e.g. IL-10) (Bonfield et al., 1995; Courtney et al., 2004). This imbalance not only perpetuates the inflammatory response but also hampers its resolution (Nichols & Chmiel, 2015).

There is an extensive debate about whether the functional defect in CFTR directly causes airway inflammation, or instead, this is a consequence of bacterial infection. Different reports show that CF infants have inflammation in the absence of infection, characterised by increased concentrations of pro-inflammatory cytokines and neutrophils (Balough et al., 1995; Khan et al., 1995; Muhlebach et al., 1999). Moreover, CF primary cells secrete higher levels of IL-8, a potent neutrophil chemoattractant, compared to non-CF controls (Carrabino et al., 2006), and newborn CF pigs display pancreatic hyperinflammatory response in the absence of apparent infection (Abu-El-Haija et al., 2011). Indeed, mutations in the CFTR gene affect different signalling pathways that modulate cytokine production, leading, for example, to the activation of the pro-inflammatory transcription factor NF-kB (Perez et al., 2007; Weber et al., 2001). Nonetheless, other studies suggest that inflammation is a consequence of bacterial infection that is quickly eradicated in the early years of life and therefore cannot be detected (Armstrong et al., 1997; Dakin et al., 2002; Stoltz et al., 2010). Respiratory viruses are also abundant in CF airways resulting in an extensive inflammatory response (Billard et al., 2017).

Regardless of the cause, the elevated levels of pro-inflammatory mediators induce airway damage (Nichols et al., 2008). Under these conditions, epithelial cell migration, proliferation and differentiation are required for proper tissue repair and regeneration.

However, these processes are also impaired in CF not only due to the chronic hyperinflammatory environment with persistent bacterial infections but also due to CFTR dysfunction (Amaral et al. 2020).

The association between CFTR and epithelial cell differentiation was first reported after observing that CFTR mRNA levels increase significantly during polarisation and differentiation of intestinal epithelial cells (Buchwald et al., 1991; C. Montrose-Rafizadeh et al., 1992; Sood et al., 1992). Moreover, PM expression of wt-CFTR in well-differentiated epithelial cells is dependent on proper differentiation and polarisation (Dupuit et al., 1995; Morris et al., 1994; Puchelle et al., 1992) with the concomitant PM expression of mucins and aquaporins (Jun et al., 2001). Accordingly, genes involved in ciliogenesis (which are only expressed in a fully differentiated cell) have been found to be downregulated in nasal epithelial cells from CF individuals homozygous for F508del CFTR (Clarke et al., 2015).

Interestingly, and as proposed in the above studies, CFTR expression at the PM is not only dependent on proper folding but also on the differentiation status of the cell (Bebök et al., 1998). This is supported by the observation that some F508del CFTR protein has a normal expression and distribution in several CF tissues (Kälin et al., 1999), including nasal polyp tissues exposed to low levels of inflammation and airway damage (Dupuit et al., 1995). Nevertheless, this normal localisation of F508del-CFTR has been demonstrated to occur in a significantly lower number of epithelial cells than in cells from non-CF individuals or CF carriers (Penque et al., 2000), likely due to its intrinsic misfolding that leads to significant retention at the ERQC and premature degradation (Cheng et al., 1990; Farinha & Amaral, 2005; Loo et al., 1998; Thomas & Pedersen, 1993; Yang et al., 1993). Likewise, wt-CFTR has been shown to display an intracellular distribution in remodelling airway epithelia from non-CF individuals (Brézillon et al., 1997). Indeed, some studies suggest that the presence of CFTR at the PM is required to maintain epithelial polarity through the interaction with components of the actin cytoskeleton, tight and gap junctions (Chasan et al., 2002; Ruan et al., 2014). In turn, polarity integrity is required to maintain in place the trafficking machinery that allows CFTR to be transported to the PM (Amaral et al. 2020) (Figure 9). Moreover, a recent study suggests that the CFTR-mediated Cl^- secretion is equally important for epithelial polarity and that absence of functional CFTR in epithelial cells leads to transcriptionally reprogramming into a less differentiated partially mesenchymal phenotype (Quaresma et al., 2020).

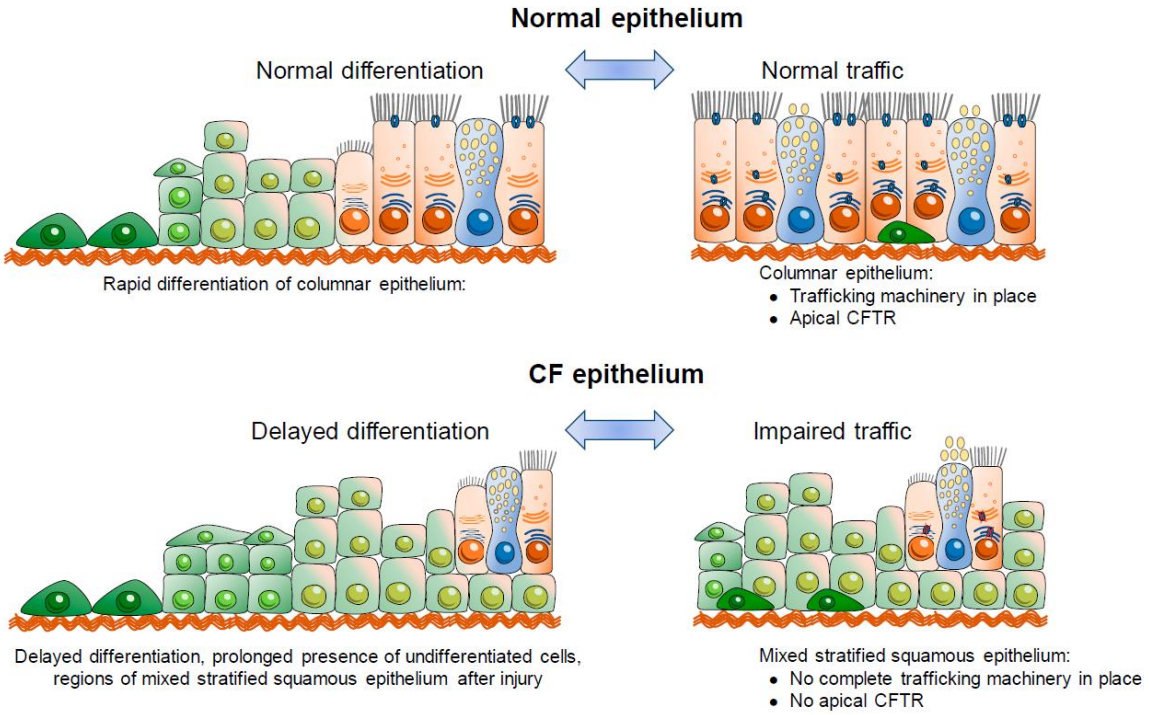


Figure 9 – Schematic representation of the relationship between airway epithelial differentiation and CFTR trafficking to the apical membrane. The normal epithelium has a pseudostratified columnar morphology with differentiated cells expressing apical CFTR (upper panel). In CF, there is delayed differentiation resulting in a stratified squamous epithelium that lacks the trafficking machinery to promote CFTR trafficking to the apical membrane (lower panel). Retrieved from (Amaral et al. 2020).

3.3 TMEM16A Protein Structure and Function

In 2008 three independent research teams identified TMEM16A/ANO1 as the primary channel responsible for the Ca^{2+}-activated Cl^- transport that is functionally separated from CFTR currents (Caputo et al., 2008; Schroeder et al., 2008; Yang et al., 2008). TMEM16A/ANO1 belongs to a family of 10 proteins (TMEM16A-K, ANO1-10) that function as anion channels or lipid scramblases, being the only member of this family with anion channel activity and relevant expression in secretory epithelia, namely in the airways (Mall & Galietta, 2015). Nevertheless, this protein has a broad expression in several tissues and cell types, including in smooth muscle, vascular endothelium, olfactory sensory neurons, cardiac muscles, and nociceptive neurons. Particularly, TMEM16A is overexpressed in several cancers, having been proposed to play a role in tumorigenesis through the regulation of cancer cells proliferation, invasion and migration (Ji et al. 2019).

The TMEM16A topology is predicted from the crystal structure of the fungal ortholog *Nectria haematococca* TMEM16 (nhTMEM16) that shares high homology with the mammalian TMEM16 family (Brunner et al., 2014). TMEM16A forms homodimers, with each subunit containing ten transmembrane domains and the N- and C-termini located in the cytosol (Tien et al. 2013) (Figure 10). There is still some debate regarding the composition of the conductive pore, with some studies considering that the channel contains lipids (Galietta, 2016; Whitlock

& Hartzell, 2016) and others showing that the pore is mostly shielded from the PM (Paulino et al., 2017).

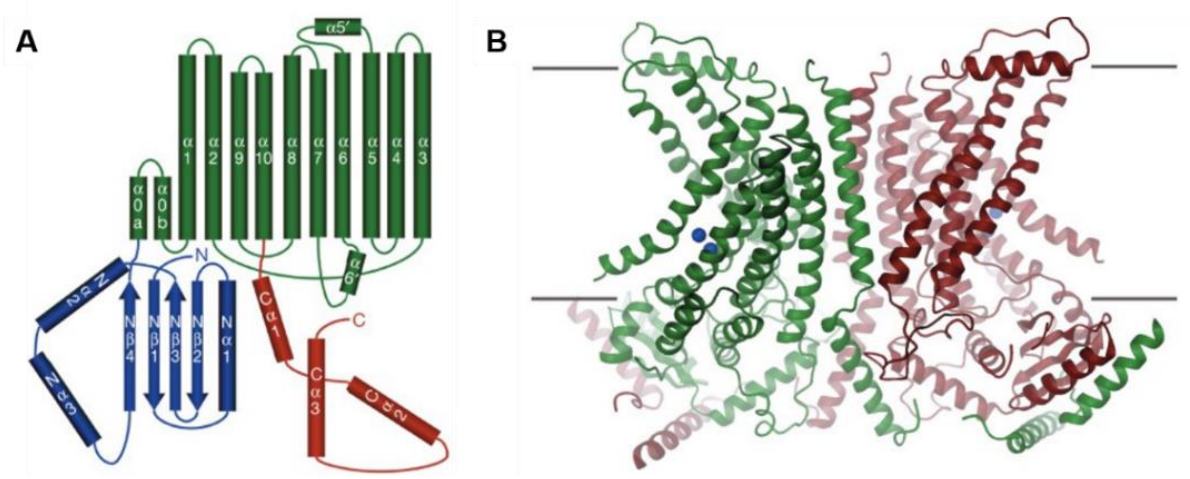


Figure 10 – Structure of the nhTMEM16 dimer. (A) Topology of the nhTMEM16 subunit, showing the transmembrane domains in green, the N-terminal in blue and the C-terminal in red. **(B)** Ribbon representation of the nhTMEM16 dimer. Adapted from (Brunner et al., 2014).

TMEM16A activation occurs through binding of ATP or UTP to the apical purinergic $P2Y_2$ receptors, which results in the stimulation of phospholipase C (PLC) and the release of inositol triphosphate (IP_3). Sequentially, IP_3 binds to its receptor in the ER, mediating the release of Ca^{2+} ions to the cytoplasm that will then activate TMEM16A (Yang et al., 2008) (Figure 11).

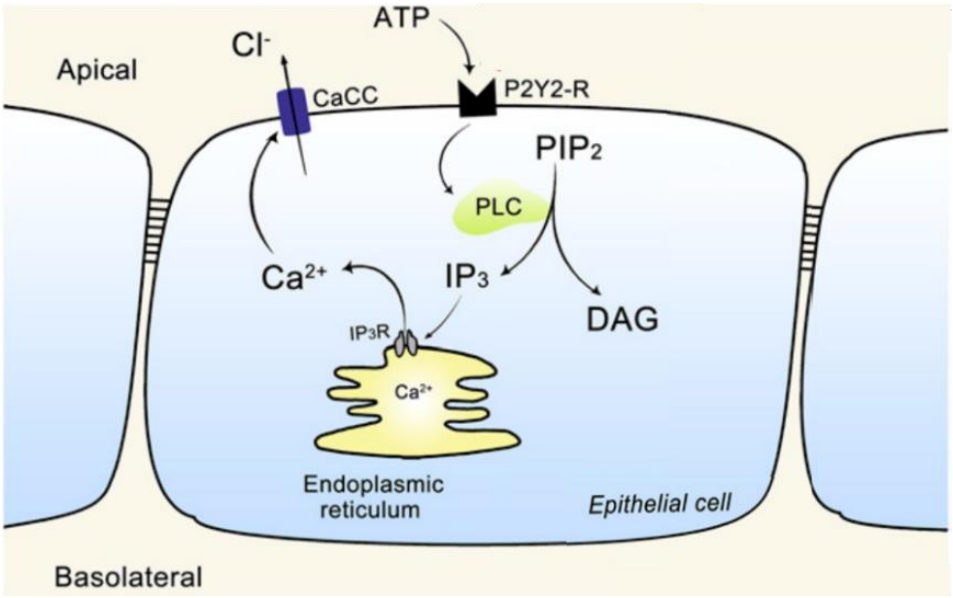


Figure 11 – Scheme illustrating the TMEM16A (CaCC) mechanism of activation in epithelial cells. Stimulation of apical $P2Y_2$ purinergic receptors ($P2Y_2$-R) with ATP (or UTP) results in the activation of phospholipase C (PLC) that mediates the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), producing two secondary messengers, inositol triphosphate (IP_3) and diacylglycerol (DAG). In turn, IP_3 binds to the IP_3 receptor (IP_3R) present in the endoplasmic reticulum, triggering a Ca^{2+} release in the cytoplasm. The Ca^{2+} ions will activate TMEM16A/CaCC, leading to efflux of Cl^- ions. Adapted from (Guo et al., 2015).

3.4 TMEM16A as an alternative therapeutic target for CF – Focus on the role of TMEM16A for inflammation, mucus production/secretion and epithelial cell differentiation.

The activation of non-CFTR Cl^- channels is an attractive CF therapeutic approach that can be applied to all individuals regardless of the mutations in the CFTR gene (Amaral, 2015). This strategy allows to compensate for the loss of CFTR-mediated anion conductance, improve airway hydration, ASL acidification, mucus fluidity and MCC (De Boeck & Amaral, 2016; Mall & Galietta, 2015). Among possible candidates, TMEM16A stands out (Sondo et al., 2014) as it functions as a CaCC expressed at the apical PM of airway epithelial cells (Huang et al., 2009; Pedemonte & Galietta, 2014) that is also permeable to HCO_3^- ions (Jung et al., 2013).

Apart from anion secretion, some studies describe that TMEM16A regulates mucus production and secretion in the airways, and its activation has been proposed to enhance mucus production, which would further worsen the increased mucus in CF (Benedetto et al., 2019; Cabrita et al., 2020; Huang et al., 2012; Lin et al., 2015). Indeed, this protein is upregulated by the pro-inflammatory cytokines IL-4 and IL-13 (Caputo et al., 2008; Lin et al., 2015; Scudieri et al., 2012), in CF (Caci et al., 2015) and other asthma-like conditions (Huang et al., 2012; Kang et al., 2017). Upon inflammation, TMEM16A is localised at the apical PM of goblet cells (Huang et al., 2012; Scudieri et al., 2012), where it might support goblet cell differentiation (Scudieri et al., 2012), mediate mucin exocytosis (Benedetto et al., 2019) or be essential for proper mucus expansion and hydration (Sondo et al., 2014).

As a corollary of such studies, more recently, TMEM16A inhibition was suggested as a way to reduce mucus production and secretion and thus to improve the CF phenotype (Benedetto et al., 2019; Cabrita et al., 2020; Huang et al., 2012; Miner et al., 2019). However, these studies should be taken precautiously, as some of them are carried out in animal models that do no mimic the CF airway phenotype (Benedetto et al., 2019; Huang et al., 2012) or use TMEM16A blockers that are rather unspecific and have low potency (Cabrita et al., 2020; Huang et al., 2012).

To be able to stimulate TMEM16A in CF, it is crucial to find activators that act independently of Ca^{2+} and are as much as possible specific to the airways (or epithelial cells) to avoid bronchoconstriction resulting from the stimulation of this channel in smooth muscle cells (Mall & Galietta, 2015). Remarkably, a recent and promising TMEM16A potentiator has been demonstrated to improve Cl^- secretion and MCC without affecting mucus production nor smooth muscle contraction (Danahay et al., 2020). Additionally, an independent study showed that TMEM16A stimulation reduces the secretion of pro-inflammatory cytokines, suggesting

that this approach might also reduce the chronic inflammation typical of CF airways (Veit et al., 2012).

Besides regulating fluid and mucus homeostasis, TMEM16A also has also been described to play an essential role in development, epithelial cell differentiation, regeneration, and proliferation. Interestingly, and similarly to CF pigs, mice, rats and humans (Bonvin et al., 2008; Meyerholz et al., 2010; Ornoy et al., 1987; Tuggle et al., 2014), TMEM16A mouse mutants show abnormal trachea morphology (Rock et al., 2008) and compromised airway differentiation with early signs of inflammation (He et al., 2020). Accordingly, these animals also mimic the CF airway phenotype with mucus obstruction and abnormal MCC (He et al., 2020; Ousingsawat et al., 2009; Rock et al., 2009). Interestingly, TMEM16A promotes PM remodelling during epithelial morphogenesis by controlling the intracellular Cl^- concentration (He et al., 2017). Nevertheless, the first human disorder caused by a TMEM16A deficiency was only recently reported (Park et al., 2020). Mutations in the TMEM16A gene were identified in two siblings and resulted in a potentially fatal neonatal disease, enhancing the importance of this protein for human physiology and development. Interestingly, these individuals were characterised by reduced CFTR-mediated currents (Park et al., 2020), an observation that agrees with previous studies describing that TMEM16A is essential for CFTR PM expression and function (Benedetto et al., 2017; Benedetto, et al., 2019).

In summary, all these discoveries describe Cl^- channels as critical to maintaining airway homeostasis by regulating many fundamental physiological processes beyond anion secretion. Nevertheless, despite all the knowledge obtained so far, some questions remained to be addressed. Particularly, there is now an extensive debate about whether TMEM16A activation is beneficial or harmful for CF individuals by either improving mucosal hydration or further enhancing mucus hypersecretion. The elucidation of this question is of extreme importance as modulation of TMEM16A is a potential therapeutic strategy that can be applied to all CF individuals independently of the mutations carried in the CFTR gene, contrarily to the current FDA-approved CFTR modulators.

Furthermore, an increasing number of studies show that, along with mucus production, Cl^- channels are regulators of epithelial differentiation and modulated by airway inflammation. Mucus hyperproduction, chronic inflammation and airway remodelling are features that not only characterise CF but nearly all obstructive airway diseases, including asthma and COPD. Therefore, it is crucial to understand how Cl^- channels are modulated in disease-like conditions so as to identify possible therapeutic targets that improve Cl^- secretion, airway barrier function and reduce inflammation.

Objectives

The present doctoral work aimed to understand the importance of chloride channels/transporters for mucus production, airway inflammation and epithelial cell differentiation to ultimately identify therapeutic strategies for obstructive airway diseases, namely Cystic Fibrosis. To achieve this general goal, three specific objectives were defined, each one corresponding to a chapter of this work:

1. Determine the role of TMEM16A for mucus production, assessing its potential as a drug target for Cystic Fibrosis.
2. Characterise the role of CFTR in epithelial cell differentiation by determining how mutant CFTR affects the differentiation of different airway epithelial cell types.
3. Understand the effect of different inflammatory mediators on mucus production, ion channels/transporters and epithelial cell differentiation.

II. Materials and Methods

1. Cell Culture

1.1 Cell lines and growth conditions

The human multipotent airway Basal Cell Immortalised Non-Smoker 1.1 (BCi-NS1.1) line was isolated from a bronchial brushing of a healthy non-smoker subject and immortalised using the retrovirus-mediated expression of human telomerase reverse transcriptase (hTERT) (Walters et al., 2013). BCi-NS1.1, BCi-Hz1.1 and BCi-CF1.1 cells were cultured with Pneumacult-Ex medium supplemented with Pneumacult-Ex 50x supplement (STEMCELL Technologies, 05008), 96 µg/mL hydrocortisone (Sigma-Aldrich, H0888) and 1% penicillin-streptomycin (10.000 U/mL, Gibco, 15140-148) in a 37ºC, 5% CO_2 humidified incubator. All experiments were performed with cells cultured bellow 25 passages.

Primary Human Bronchial Epithelial (HBE) cells were isolated from human lung tissues of healthy donors obtained from the paediatrics department (Motol University Hospital, Prague, Czech Republic) after receiving the patient's written consent and approval by the hospital ethics committee. Briefly, cells were dissociated from the bronchial tissue by treatment with protease/DNase and collected by centrifugation at 500 g for 5 min at 4°C, as previously described (M. Leslie Fulcher & Randell, 2013). Cells were cultured in BEpiCM (ScienceCell, 3211) on plates previously coated with PureCol (type I collagen) (30 µg/mL, Advanced Biomatrix, 5005) in a 37°C, 5% CO_2 humidified incubator.

1.2 Air-Liquid Interface Cultures

BCi-NS1.1, BCi-Hz1.1 and BCi-CF1.1 cells were seeded onto either 6.5- or 12-mm diameter-size transwells with 0.4 µm pore polyester membrane inserts (Corning Incorporated, #3470, #3460) previously coated with human type IV collagen (Sigma-Aldrich, C7521), at a density of 1.5 x 10^5 or 3 x 10^5 cells/transwell, respectively. Air-Liquid Interface (ALI) was established once cells reached full confluency by removing media from the apical chamber and replacing media in the basolateral chamber with Pneumacult-ALI maintenance medium (referred to as ALI day 0). Pneumacult-ALI maintenance medium (STEMCELL Technologies, #05001) was supplemented with Pneumacult-ALI 10x supplement, Pneumacult- ALI maintenance supplement, 96 µg/mL hydrocortisone, 2% heparin solution (STEMCELL Technologies, #07980), 1% penicillin/streptomycin, 0.5% amphotericin B (Gibco, 15290-026) and 0.1% gentamicin (Gibco, G1272). ALI cultures were grown in a 37ºC, 5% CO_2 humidified incubator, with media replacement every 2-3 days. Following 15 days under ALI conditions, the apical surface was washed with 1x Phosphate Buffered Saline (PBS) every media change to remove accumulated mucus.

Primary HBE cells were seeded onto 6.5 mm diameter-size transwells with 0.4 µm pore polyester membrane inserts at a density of 2 x 10^5 cells/transwell. The transwells were previously coated with human type IV collagen (Sigma-Aldrich, C7521). ALI was established once cells reached full confluency by removing media from the apical chamber (ALI day 0). ALI cultures were grown in a 37°C, 5% CO_2 humidified incubator for 21 days (ALI day 21) to generate a fully differentiated epithelium, as previously described (M. Leslie Fulcher & Randell, 2013).

1.3 Transepithelial Electrical Resistance measurements

Transepithelial Electrical Resistance (TEER) measurements were performed to monitor cell polarisation in differentiating BCi-NS1.1, BCi-Hz1.1, BCi-CF1.1 and primary HBE cells, using a volt-ohm meter (Millicell-ERS, Millipore, MER5000001). Measurements were carried out regularly before changing media and following equilibration of cultures with media in the apical chamber for 30 min in the incubator. ALI was restored by removing the apical medium. TEER ($\Omega.cm^2$) was calculated for each transwell by subtracting the empty insert's TEER value and multiplying by its area. Additionally, the TEER of fully differentiated cells was also measured in the Ussing chamber.

1.4 Treatment with compounds

To induce mucus production, differentiated BCi-NS1.1 cells (ALI day 28) were incubated with 5 ng/mL of Interleukin-4 (IL-4, eBioscience, BMS337) in the basolateral media for 48h. Cell proliferation was inhibited by 1 µg/mL mitomycin C (Roche, 10107409001) added to the basolateral media in the presence or absence of 5 ng/mL IL-4 at ALI day 28. Goblet cell metaplasia was induced by adding 400 ng/µL of the Delta-Like Ligand 4 (DLL4, Life Technologies, 10171-H02H-25) to the basolateral media since ALI day 0, after which the standard ALI method was employed. DLL4 stimulation was maintained until ALI day 30 by replacing it at each media change.

To compare the effect of various inflammatory mediators, fully differentiated BCi-NS1.1 cells (ALI day 27) were exposed from the basolateral media to 10 ng/mL of the following cytokines for 3 days: Interleukin-8 (IL-8, R&D Systems, 208), Interleukin-1β (IL-1β, STEMCELL Technologies, 78143), Tumor Necrosis Factor-α (TNF-α, STEMCELL Technologies, 78068.1), IL-4, Interleukin-17A (IL-17A, STEMCELL Technologies, 78032), Interleukin-10 (IL-10, STEMCELL Technologies, 78024.1) and Interleukin-22 (IL-22, STEMCELL Technologies, 78038.1).

To rescue F508del-CFTR, BCi-Hz1.1 and BCi-CF1.1 cells (ALI day 28) were incubated with 3 µM of the CFTR correctors VX-661/Tezacaftor (Selleckchem, S7059) and VX-

445/Elexacaftor (Med Chem Express, HY-111772) for 48h diluted in the basolateral media. Control cultures were incubated with the vehicle Dimethyl sulfoxide (DMSO).

2. Generation of BCi-NS1.1 cells expressing F508del CFTR by CRISPR/Cas9

[This protocol was performed with Lúcia Santos and is included here with permission].

2.1 Gene Editing Reagents

The guide RNA (gRNA) and homology-directed repair (HDR) single-stranded oligonucleotide donor (ssODN) sequences (Table 1) used for the introduction of the mutation F508del in the CFTR gene of BCi-NS1.1 cells were obtained from a previous study (Valley et al., 2019). The ssODN donor template (Ultramer® DNA Oligos) and the Alt-R® CRISPR Cas9 reagents (HiFi S.p. Cas9 Nuclease 3NLS; crRNA and tracrRNA) were purchased from Integrated DNA Technologies.

Table 1 – Sequences of the gRNA and ssODN HDR donor used for insertion of the F508del variant.

gRNA (5' – 3')	ssODN HDR donor template (5' – 3')
ATTAAAGAAAATATCATCTT	CACAGTGGAAGAATTTCATTCTGTTCTCAGTTTTCCTGGATTA TGCCTGGCACCATTAAAGAAAATATCATTGGTGTTTCCTATGA TGAATATAGATACAGAAGCGTCATCAAAGCATGCCAAC

2.2 Gene Editing Components Assembly and Electroporation

The 100 µM stocks of CRISPR RNA (crRNA) and trans-activating crRNA (tracrRNA) were annealed in equimolar concentration to a final duplex concentration of 44 µM, incubated at 95 ^{o} C for 5 min, and then cooled down to room temperature. The Ribonucleoprotein (RNP) complex was prepared by mixing the Cas9 protein with the previously assembled crRNA:tracrRNA mixture at a 1:1.2 M ratio and incubated at room temperature for 20 min. BCi-NS1.1 cells were electroporated with the Neon™ Transfection System (Thermo Fisher Scientific, MPK5000) at the following conditions: Voltage – 1400 V, Width – 20 ms, Pulses – 2 pulses. For each reaction, 2 x 10^5 cells were electroporated with the RNP complex in the presence of the ssODN HDR template.

2.3 Analysis of Gene Editing Efficiency in the Pool of Transfected Cells

Following 72 h of transfection, genomic DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen, 69504), according to manufacturer's instructions. DNA concentration was determined by spectrophotometry, measuring the absorbance at 260 nm. Gene editing events were analysed by a PCR reaction carried out with 250-500 ng of template DNA, using the Q5 Hot Start High-Fidelity 2x Master Mix (New England Biolabs, M0494S). The PCR

conditions and the primer set used for amplification and sequencing can be found in Table 2Table 3. PCR products were run in a 1% agarose gel, purified using the QIAquick PCR Product Purification Kit (Qiagen, 28104) and Sanger sequenced (Eurofins Genomics). Sequence analysis was performed using the Inference of CRISPR Edits (ICE) webtool (https://ice.synthego.com/#/).

Table 2 – PCR program for amplification of the gRNA target site in the CFTR gDNA sequence from the pool of transfected cells.

PCR Program		
Temperature (°C)	**Time**	**Number of cycles**
98	30 s	35
98	10 s	
57	30 s	
72	30 s	
72	2 min	
4	Pause	

Table 3 – Primers used for the amplification of the gRNA target site with the F508del variant.

Primers for gRNA target site amplification	
Name	**Sequence (5' – 3')**
Forward	TGTGCATAGCAGAGTACCTGAA
Reverse	ATTTGGGTAGTGTGAAGGGTT

2.4 Single-Cell Cloning and Identification of Edited Clones

The pool of transfected cells was divided into single-cell clones seeded at an average density of 0.5 cells/well in 96 well-plates. Following clone expansion, genomic DNA was extracted using the QuickExtract DNA Extraction Solution (Frilabo, QE09050). The target site with the F508del variant was amplified in a PCR reaction, using the conditions and primer present in Table 3, Table **4**. PCR products were run in a 1% agarose gel and Sanger sequenced (Eurofins Genomics). Sequence analysis confirmed the presence of a 3-nucleotide CTT deletion (F508del mutation) either in one or two alleles, matching the heterozygous (BCi-Hz1.1) or homozygous (BCi-CF1.1) cells, respectively.

Table 4 – PCR program used to amplify the gRNA target site in CFTR gDNA sequence from single-cell clones.

PCR Program		
Temperature (°C)	**Time**	**Number of cycles**
94	5 min	35
94	30 s	
57	30 s	
72	1 min	
72	10 min	
4	Pause	

3. Biochemical Assays

3.1 mRNA Extraction and cDNA Synthesis

Total RNA was isolated from BCi-NS1.1, BCi-Hz1.1 and BCi-CF1.1 cells using the NZY Total RNA Isolation kit (NZYTech, MB13402). Complementary DNA (cDNA) was generated from 1 µg of the messenger RNA (mRNA) sample using random primers and the M-MuLV reverse transcriptase (NZYTech, MB083), according to manufacturer's instructions.

3.2 Analysis of the CFTR Coding Sequence

RNA was collected from BCi-NS1.1 and BCi-CF1.1 cells after 30 days of ALI culture. Following reverse transcription, cDNA samples were used as a template to screen for possible alterations in the CFTR coding sequence, using nine different primer pairs designed to cover the CFTR coding region, as described (Felício et al., 2017). Primer sequences and respective annealing temperatures are listed in Table 5**Error! Reference source not found.**. PCR products were run in a 1% agarose gel and Sanger sequenced (Stabvida).

Table 5 – Sequences of the primer sets used for the nine PCR reactions, position in the CFTR mRNA sequence and expected PCR product size.

Reaction	Primer	Sequence (5'-3')	Annealing Temperature (°C)	cDNA Position	Fragment length (bp)
1	5'NC	GCATTAGGAGCTTGAGCCCA	62	77-96	743
	AC1L	GAAACCAAGTCCACAGAAGGC	64	819-799	
2	CF Ex5.F	CTCCTTTCCAACAACCTGAAC	62	679-699	751
	CF9AS	GTACTGAAGAAGAGGCTGTC	60	1429-1408	
3	B3R	AATGTAACAGCCTTCTGGGAG	52	1318-1338	431
	Ex11L	TATATTGTCTTTCTCTGCAAACTTGG	70	1724-1749	
4	Ex10N	GGCACCATTAAAGAAAATATCATCTT	58	1630-1655	207
	561AL	CAAATCAGCATCTTTGTATACTG	62	1814-1836	
5	C1R	GTGGAGGTCAACGAGCAAGA	62	1778-1797	904
	CF14a.R	AAGGAATGTGGTCCATGTAG	58	2663-2682	
6	D2R	GCAAACTTGACTGAACTGGA	58	2530-2549	730
	D2L	GTTGTTTGAGTTGCTGTGAG	58	3240-3259	
7	E2R	GATGACCTTCTGCCTCTTAC	54	3082-3101	700
	E2L	CTGTGAGATCTTTGACAGTC	58	3762-3781	
8	Ex18.F	AACTCCAGCATAGATGTGG	56	3574-3592	829
	Ex22.R	GCCAAGCACATCAACTGCA	58	4184-4202	
9	Ex21.F	CTATGAACAGTGGAGTGATC	58	4050-4069	549
	F2L	CATGTCAACATTTATGCTGC	56	4579-4598	

3.3 Quantitative reverse-transcriptase PCR

Quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR) was performed in a Bio-Rad CFX96 system. For each reaction, a mix was prepared with forward

and reverse primers (250 nM), cDNA (50 ng) and 1x Evagreen SsoFast PCR reagent (Bio-Rad, 172-5204). A standard qRT-PCR protocol was used for amplification: 1 min at 95°C followed by 40 cycles of 10 sec at 95°C and 30 sec at 60°C. Technical replicates were used in amplification. Melt curves were obtained with a temperature gradient from 65ºC to 95ºC to confirm amplification of specific products, and negative controls were confirmed to be free of amplification after 40 PCR cycles. Relative mRNA abundance was determined using the Bio-Rad CFX Manager 2.0 software (Bio-Rad, 1845000). Primer efficiency was determined for each primer set with the slope value of a standard amplification curve obtained with serial dilutions of cDNA (from 10 ng/µL to 0.016 ng/µL). Mean relative levels of expression were calculated by normalising the gene of interest against the housekeeping gene, using the ΔΔCT (threshold cycle) method, where Fold-Change = $2^{-\Delta\Delta CT}$. Primer sequences are available in Table 6. For all experiments, GAPDH was used as a housekeeping gene.

Table 6 – Sequences of primer sets used in qRT-PCR.

Target		Sequence (5'-3')
CFTR	Forward	ATGCCCTTCGGCGATGTTTT
	Reverse	TGATTCTTCCCAGTAAGAGAGGC
GAPDH	Forward	ATGGGGAAGGTGAAGGTCG
	Reverse	GGGGTCATTGATGGCAACAATA
MUC5AC	Forward	CCTGAGGGGACGGTGCTT
	Reverse	ACGAGGTGCAGTTGGTGC
MUC5B	Forward	GTGAGGAGGACTCCTGTCAAGT
	Reverse	CCTCGCAGAAGGTGATGTTG
SLC26A9	Forward	GACTACATCATTCCTGACCTGC
	Reverse	AGGAGTAGAGGCCATTGACTG
TMEM16A	Forward	ACTACCACGAGGATGACAAGC
	Reverse	CTCTGCACAGCACGTTCCA

3.4 Protein Extraction

Cells were washed twice with ice-cold 1x PBS and lysed with a buffer containing: 31.25 mM Tris-HCl (Sigma-Aldrich, 30721) pH 6.8, 1.5% (v/v) sodium dodecyl sulfate (SDS, Gibco, 15553), 10% (v/v) glycerol (Sigma-Aldrich, 92025), 50 mM dithiothreitol (DTT, Sigma-Aldrich, D0632) supplemented with protease inhibitor cocktail (Roche, 11697498001).

For mucin detection, cells were lysed with a non-denaturing lysis buffer containing: 1% (v/v) Triton X-100 (Amersham Biosciences, 17-1315-01), 50 mM Tris-HCl (pH 7.4), 300 mM NaCl (Calbiochem, 7760), 5 mM ethylenediaminetetraacetic acid (EDTA, Sigma-Aldrich, 60004), 0.02% (w/v) sodium azide (Sigma-Aldrich, S2002), 1 mM phenylmethylsulfonyl fluoride (PMSF, Roche, 10837091001), 1 mM Na_3VO_4 (Sigma-Aldrich, S6508), and protease inhibitor cocktail.

DNA was sheared by treatment with (5U) benzonase nuclease (Sigma-Aldrich, E1014) in the presence of 3 mM $MgCl_2$. Protein lysates were collected after cell scraping and stored at -20°C for further use.

3.5 Western Blot

Protein lysates were loaded onto polyacrylamide gels and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Samples were initially concentrated in a 4% stacking gel containing: 125 mM Tris-HCl pH 6.8; 4% (v/v) acrylamide:bisacrylamide (Bio-Rad, 1610154); 0.1% (v/v) glycerol; 0.1% (v/v) SDS; 0.075% (v/v) ammonium persulfate (APS) (Bio-Rad, 1610700); 0.08% (v/v) tetramethylethylenediamine (TEMED) (Sigma, T9281). Protein was then separated in 5%, 7%, 10% or 12.5% separating gels containing: 375 mM Tris-HCl pH 8.8; 5%/ 7%/ 10%/ 12,5% (v/v) acrylamide:bisacrylamide; 0.1% (v/v) glycerol; 0.1% (v/v) SDS; 0.075% (v/v) APS; 0.06% (v/v) TEMED.

Electrophoresis was run on Tris-Glycine-SDS buffer (Bio-Rad, 161-0772) for 90 to 210 min at 60 to 120V. Transfer onto polyvinylidene difluoride (PVDF) membranes (Merck Millipore, IPVH00010) was performed using a wet-transfer system at 400 mV for 90 min with a Tris-Glycine buffer (Bio-Rad, 161-0771). Additionally, 0.0375% SDS was added to the Tris-Glycine buffer to facilitate mucin transfer to the PVDF membrane. Membranes were blocked for 1h with either 5% (w/v) non-fat milk (NFM, Nestlé, Molico) prepared in PBS with Tween (Fisher BioReagents, BP337-100) or with 1% (w/v) NFM diluted in Tris Buffered Saline (TBS) with Tween (only for TMEM16A, CFTR and FOXI1 detection). Primary antibodies were incubated overnight at 4°C with gentle shaking (40-60 rpm). On the following day, membranes were washed and incubated with horseradish peroxidase (HRP)-conjugated goat secondary antibodies for 1 h at room temperature. All antibodies were diluted in the respective blocking buffer.

Chemiluminescence was detected on a Chemidoc XRS+ system (Bio-Rad, 170-8265) using a 1:1 mixture of peroxide: luminol/enhancer solution (Bio-Rad, 170-5061). Quantification of band intensity was performed using the Image Lab software (Bio-Rad, 170-9690). Levels of protein expression were normalised against the loading controls (GAPDH, Vinculin, Tubulin and Calnexin). Primary and secondary antibodies and respective conditions are listed in Table 7 andTable **8**.

3.6 Mucin Agarose Electrophoresis

Alternatively to the protocol described above, MUC5AC was also detected by agarose electrophoresis. To this end, protein samples loaded into a 0.8% 1x TAE (Tris-Acetate-EDTA) – 0.1% SDS agarose gel, which was run at 80 V for 90 min. Transfer into a PVDF membrane

was performed using the capillary transfer method (Gnanapragassam et al., 2013) with a Tris-Glycine buffer containing 0.0375% SDS. Membranes were blocked for 1h with 5% (w/v) NFM. Incubation with primary and secondary antibodies was performed as described in the western blot section.

3.7 Immunofluorescence

BCi-NS1.1 cells grown on transwell inserts were rinsed three times with ice-cold 1x PBS and fixed by adding 4% (v/v) paraformaldehyde (PFA, Merck Millipore, 104003) to both apical and basolateral chambers for 15 min at 4°C. After washing, cells were permeabilised for 15 min with 0.1% (v/v) Triton X-100 and blocked for 20 min with 1% (w/v) bovine serum albumin (BSA, Sigma-Aldrich, A9647). Transwell inserts were then incubated overnight at 4°C with primary antibodies. After incubation, cells were rinsed three times with 1x PBS and incubated for 1 h at room temperature with a solution containing secondary antibodies and the nuclear dye Hoechst 33258 (1 µg/mL, Sigma-Aldrich, 94403). Transwells were removed from their supports with a scalpel and mounted in glass slides with N-propyl-gallate in glycerol–PBS mounting medium. Primary and secondary antibodies and respective conditions are listed in Table 7Table 8.

Images were acquired with a Leica TCS SP8 confocal microscope and analysed using the ImageJ software by the following approach: images are represented as average or maximum-intensity projections; brightness and contrast are adjusted (being the same criteria applied for each group of images); the background was subtracted (being the same value subtracted for each group of images). Quantification of the proportion of cells expressing CFTR and different cell-type markers was determined by averaging values obtained from at least five different randomly acquired images in each transwell. Cells positive for two antibodies were counted manually using the Fiji software by generating maximum intensity projections and confirming co-staining with xz images (z-stack).

Table 7 – List of primary antibodies and conditions used in Western Blot (WB) and Immunofluorescence (IF) assays.

Target	Application	Dilution	Host	MW (kDa)	Company	Reference
β-Tubulin IV	IF	1:100	Mouse	55	Abcam	ab11315
			Rabbit			ab222822
Calnexin	WB	1:3000	Mouse	90	BD Biosciences	610523
CC16	WB	1:500	Rabbit	16	BioVendor	RD181022220-01
	IF	1:100				
CFTR	WB	1:500	Mouse	120-180	CFF	596
	IF	1:100				570
					RD Biotech	MATG1104
FOXI1	WB	1:500	Rabbit	40		HPA071469

	IF	1:100			Sigma-Aldrich	
GAPDH	WB	1:10000	Mouse	37	Abcam	ab8245
DNAI1	WB	1:1000	Rabbit	79	Sigma-Aldrich	HPA021649
Ki-67	WB	1:250	Rabbit	250	Abcam	ab16667
MUC5AC	WB	1:1000	Mouse	400-600	Invitrogen	MA1-38223
	IF	1:100				
P63	WB	1:1000	Rabbit	77	Abcam	ab124762
SLC26A4	WB	1:1000	Rabbit	86	Abcam	ab98091
TMEM16A	WB	1:500	Rabbit	90-120	Abcam	ab64085
	IF	1:100				
Tubulin	WB	1:10000	Mouse	50	Sigma-Aldrich	SAB3501072
Vinculin	WB	1:1000	Mouse	130	Santa Cruz	sc-73614
ZO-1	WB	1:2000	Mouse	250	Invitrogen	33-9100
	IF	1:100				

Table 8 – List of secondary antibodies and conditions used in Western Blot (WB) and Immunofluorescence (IF) assays.

Antibody	Target	Application	Dilution	Host	Company	Reference
(H+L)-HRP Conjugate	Mouse IgG	WB	1:3000	Goat	Bio-Rad	170-6516
	Rabbit IgG					170-6515
Alexa Fluor 488	Mouse IgG	IF	1:500	Donkey	Life Technologies	A21202
	Rabbit IgG					A21206
Alexa Fluor 568	Mouse IgG					A10037
	Rabbit IgG					A10042

3.8 ZO-1 Quantification

Zonula occludens-1/tight junction protein 1 (ZO-1) staining was used to quantify the tight junction organisation. The analysis was performed using Fiji software together with a Tight Junction Organization Rate (TiJOR) macro, which is an index for the organisation level of the tight junction network (Terryn et al., 2013). Z-stack images obtained by confocal microscopy were Z-projected using the maximum intensity projection. A white top-hat segmentation was applied before using the TIJOR macro where parameters (100 polygons, five steps) of the evaluation were kept constant for all conditions analysed.

4. Functional Assays

4.1 Ussing Chamber

Monolayers of polarised BCi-NS1.1, BCi-Hz1.1 and BCi-CF1.1 cells were mounted in a perfused micro-Ussing chamber and analysed under open-circuit conditions at 37°C. The basolateral side was continuously perfused with Ringer solution (145 mM NaCl, 0.4 mM KH_2PO_4, 1.6 mM K_2HPO_4, 5 mM D-glucose, 1 mM $MgCl_2$, 1.3 mM Ca-gluconate, pH 7.4) and

the apical surface with a low Cl^- Ringer solution (32 mM NaCl; 0.4 mM KH_2PO4; 1.6 mM K_2HPO_4; 5 mM D-glucose; 1 mM $MgCl_2$; 5.7 mM Ca-gluconate and 112 mM Na-gluconate, pH 7.4). Following an equilibrium period, 30 µM amiloride (Sigma-Aldrich, A7410) was added apically to block ENaC. TMEM16A activity was measured by applying 100 µM ATP (Sigma-Aldrich, 1852) in the presence or absence of the pre-incubated 30 µM CaCCAO1 inhibitor (Tocris, 4877). CFTR activity was measured by the simultaneous addition of the CFTR agonists Forskolin (Fsk, 2 µM, Sigma-Aldrich, 66575-29-9) and 3-isobutyl-1-methylxanthine (IBMX, 100 µM, Sigma-Aldrich, 28822-58-4), followed by incubation with the CFTR inhibitor 172 (30 µM, Sigma-Aldrich, 307510-92-5). To test the effect of CFTR modulators, the potentiator VX-770 (50 µM, Selleckchem, 873054-44-5) was added in the presence of IBMX and Fsk. All compounds were applied in the presence of 30 µM amiloride. Values for transepithelial voltages (V_{te}) were referenced to the basal surface of the epithelium. Transepithelial resistance (R_{te}) was determined by applying short current pulses (1 s) of 0.5 µA (5-s period). The equivalent short circuit current (I_{eq-sc}) was calculated according to Ohm's law ($I_{eq-sc} = V_{te}/R_{te}$).

4.2 Measurements of Airway Surface Liquid Height

Differentiated BCi-NS1.1 cells were washed with PBS for 30 min before performing experiments to remove the excess mucus. ASL was labelled with Fluorescein isothiocyanate (FITC) conjugated to 70 kD Dextran (Sigma-Aldrich, 46945). Cells were apically loaded with 20 µL of a FITC-Dextran solution (in PBS) containing either DMSO or 10 µM Ani9 (Tocris, 6076) to block TMEM16A. Before imaging, cultures were transferred to Ringer's solution, and 100 µL perfluorocarbon (PFC, FC-770) (Sigma-Aldrich, F3556) was added apically to avoid ASL evaporation. ASL was imaged using an XZ scan on a Leica TCS SP8 confocal microscope with a 63x water immersion lens and the 488 nm laser. For each experiment, images were acquired at five different points on the transwell insert. ASL height was measured using the ImageJ software.

4.3 Wound Healing Assays

Following 20 days under ALI, a sterile P100 pipette tip was used to scratch the BCi-NS1.1 cell monolayer in three different areas, inducing injury. The unattached cells were removed by washing the apical surface twice with PBS. Cells were then returned to the standard ALI culture protocol, and protein was collected from different transwells at the indicated time-points.

5. Data Analysis

All data are presented as mean ± standard error of the mean (SEM). The number of replicates is described in the legend of each figure (“n”) and represents independent experiments performed with cells from different passages. Statistical comparisons were calculated using an unpaired student's t-test, with a p-value < 0.05 considered as the level of statistical significance.

Experimental results were also represented in a heatmap generated in Rstudio, using the heatmap.2 function from the package gplots and rescaling data between 0 and 1.

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III. Results and Discussion

Chapter I – TMEM16A chloride channel does not drive mucus production

Data presented in this chapter was included in the following work:

Simões, F. B., Quaresma, M. C., Clarke, L. A., Silva, I. A. L., Pankonien, I., Railean, V., Kmit, A., & Amaral, M. D. (2019). TMEM16A chloride channel does not drive mucus production. Life Science Alliance, 2(6), 1–13. https://doi.org/10.26508/LSA.201900462

Abstract

Airway mucus obstruction is the main cause of morbidity in Cystic Fibrosis, a disease caused by mutations in the CFTR Cl^- channel. Activation of non-CFTR Cl^- channels like TMEM16A can likely compensate for defective CFTR. However, TMEM16A was recently described as a key driver in mucus production/secretion. Here, we examined whether indeed there is a causal relationship between TMEM16A and MUC5AC production, the main component of respiratory mucus. Our data show that TMEM16A and MUC5AC are inversely correlated during differentiation of human airway cells. Furthermore, we show for the first time that the IL-4-induced TMEM16A upregulation is proliferation-dependent, which is supported by the correlation found between TMEM16A and Ki-67 proliferation marker during wound healing. Consistently, the notch signalling activator DLL4 increases MUC5AC levels without inducing changes in TMEM16A nor Ki-67 expression. Moreover, TMEM16A inhibition decreased airway surface liquid height. Altogether, our findings demonstrate that upregulation of TMEM16A and MUC5AC is only circumstantial under cell proliferation but with no causal relationship between them. Thus, although essential for airway hydration, TMEM16A is not required for MUC5AC production.

Introduction

Mucus clearance or mucociliary transport (MCT) consists of the coordinated integration of ion transport, water flow, mucin secretion, cilia action, and coughing, resulting in the continuous flow of fluid and mucus on airway surfaces (Button & Boucher, 2008). Mucus is thus an efficient system for protecting the epithelium from the deleterious effects of inhaled pollutants, allergens, and pathogens, namely bacteria, by promoting their clearance and separating them from the epithelial cells, thereby inhibiting inflammation and infection (Hansson, 2012; Roy et al., 2014). Mucus is a gel formed by 97% water and 3% solids (mucins, non-mucin proteins, ions, lipids and antimicrobial peptides) (Fahy & Dickey, 2010). Mucins are heavily (2-20 x 10^5 Da) glycosylated proteins (50-90% glycan content) that constitute the main structural components of mucus (1%). The main mucins present in human airway mucus are MUC5AC and MUC5B, which are mostly secreted from goblet cells at the surface airway epithelium and by submucosal glands, respectively (Bonser & Erle, 2017; Buisine et al., 1999).

Mucus hyperproduction and mucociliary dysfunction are major features of many airway obstructive pulmonary diseases, such as Chronic Obstructive Pulmonary Disease (COPD), asthma and Cystic Fibrosis (CF) (Adler et al., 2013). Specific inflammatory/immune response mediators lead to mucus hyperproduction in each of these chronic airway diseases through activation of mucin gene expression and airway remodelling, including goblet cell metaplasia or hyperplasia [GCM/H: reviewed in (Rose & Voynow, 2006)]. While metaplasia implies a change in the phenotype of a fully differentiated cell, hyperplasia is caused by cell proliferation (Williams et al., 2006). Importantly, mucin overproduction and GCM/H, although linked, are not synonymous and may result from different signalling and gene regulatory pathways (Rose & Voynow, 2006).

CF, also known as mucoviscidosis, is a disease with major respiratory involvement characterised by clogging of the airways by a highly viscous mucus (Ehre et al., 2014), which is its most prominent hallmark and cause of morbidity and mortality (Boucher, 2007). This genetic condition is caused by mutations in CFTR (CF Transmembrane conductance Regulator), a cAMP-gated chloride (Cl^-) and bicarbonate (HCO_3^-) channel expressed at the apical membrane of epithelial cells in different tissues, including the airways (Kreda et al., 2012). CFTR is also a negative regulator of the epithelial Na^+ channel (ENaC) (König et al., 2001). As these ions are essential to drive the water flow, CF patients have a dehydrated airway surface liquid (ASL) and reduced water content in mucus (Matsui et al., 2006), impaired MCT and extensive mucus plugging (Boucher, 2007). This is further exacerbated because of CFTR permeability to HCO_3^-, which is essential in the extracellular space for proper mucus release, probably by promoting Ca^{2+} and H^+ exchange from the mucin-containing intracellular granules, thus facilitating mucin expansion (Garcia et al., 2009; Gustafsson et al., 2012).

Individuals with CF have not only mucus plugging in the airways (and in the ducts of several organs) but also mucus stasis. This has been proposed to result from dehydration of both ASL and mucus, leading to abnormally high mucus viscosity and deficient MCT (Kreda et al., 2012). Nevertheless, according to other authors, impaired MCT in CF is not due to ASL depletion but rather to the fact that secreted mucus strands remain tethered to submucosal gland ducts (Hoegger et al., 2014). Moreover, it was shown that inhibition of anion secretion in non-CF airways replicates these CF abnormalities (Hoegger et al., 2014). More recently, based on data obtained in newborn CFTR knock-out piglets, it was proposed that MUC5AC (produced by goblet cells) anchors the mucus bundles, mostly composed of MUC5B (produced by submucosal glands), thus being the key controller of mucus transport (Ermund et al., 2017; Xie et al., 2018). Furthermore, the number of MUC5AC-mediated anchorage points in CF mucus is much higher than in non-CF mucus and without sufficient HCO_3^- the mucus cannot detach from its goblet cell anchor, initiating CF lung disease (Ermund et al., 2017; Xie et al., 2018). Altogether these data indicate that MUC5AC is the key responsible for mucus stasis in CF.

Clearance of these secretions is a major objective of CF care, typically involving daily chest physiotherapy (C. Castellani et al., 2018). Notwithstanding, measurements performed in individuals with CF during stable CF disease found that the vol/vol quantity of MUC5AC protein was ~90% less than in normal mucus, and the mucin-associated sugars were about half of those present in non-CF mucus. However, during exacerbations, levels of MUC5AC protein significantly increased by 9-fold in comparison to periods of stable disease in the same individual. Levels of MUC5B also increased but far less than MUC5AC (Henke et al., 2004).

One long-sought way to compensate for the absence of functional CFTR and thus benefit individuals with CF has been the activation of non-CFTR Cl^- channels (Li et al., 2017; Mall & Galietta, 2015; Verkman & Galietta, 2009). Among possible candidates, transmembrane protein 16A (TMEM16A), also known as Anoctamin 1 (ANO1), stands out (Mall & Galietta, 2015; Pedemonte & Galietta, 2014; Sondo et al., 2014), a Ca^{2+}-activated Cl^- channel (CaCC) which is expressed at the apical membrane of airway epithelial cells (Huang et al., 2009; Scudieri et al., 2012). Indeed, its higher expression levels in goblet cells suggest that it is important for the release and hydration of mucins and thus may circumvent the primary defect in CF (Sondo et al., 2014). TMEM16A expression is controlled by pro-inflammatory stimuli, namely by the T-helper (Th) 2 cytokines IL-4 and IL-13 (Huang et al., 2012; Kang et al., 2017; Lin et al., 2015; Scudieri et al., 2012) and is shown to be induced by asthma-like conditions, i.e., in ovalbumin-challenged mice (Benedetto, et al., 2019; Huang et al., 2012), in pig airway tissues treated with histamine (Kang et al., 2017) and in biopsies from asthmatic patients (Huang et al., 2012). Moreover, recent reports have suggested that TMEM16A plays a positive critical role in mucus production/secretion (Benedetto et al., 2019; Huang et al.,

2012; Kang et al., 2017; Kondo et al., 2017; Lin et al., 2015; Scudieri et al., 2012). Indeed, when upregulated, TMEM16A was reported to co-localise with MUC5AC at the apical membrane of goblet cells (Huang et al., 2012; Scudieri et al., 2012). Should TMEM16A stimulation indeed cause MUC5AC hyperproduction, applying such a treatment in CF would further enhance a major symptom in this condition, with a harmful instead of a beneficial result. Nonetheless, the mere fact that TMEM16A and MUC5AC are co-activated by Th-2 cytokines stimulation does not imply a causal relationship between them. So, the true role of TMEM16A upregulation in mucus production/secretion is still unclear, but its elucidation is of elevated importance. Moreover, pathways occurring in asthma have been shown to significantly diverge from those in CF (Clarke et al., 2015).

Our goal here was thus to examine whether there is a causal relationship between upregulation of TMEM16A and mucus production, namely MUC5AC, as previously suggested. To that end, we used a recently described human respiratory basal cell line (BCi-NS1.1) that differentiates into the various respiratory cell types (Walters et al., 2013). Our data show that there is an inverse correlation between TMEM16A and MUC5AC expression levels during differentiation of these multipotent basal cells into different human airway cell types. Indeed, at early differentiation stages, when cells are still proliferating, TMEM16A levels are high, and MUC5AC are low, and as cells differentiate into various cell types, the opposite is observed. Moreover, TMEM16A and MUC5AC also have distinct spatio-temporal localisations in these differentiated cell types. Furthermore, we show that under stimulation with IL-4 (a strong inducer of both TMEM16A and MUC5AC), TMEM16A is only upregulated when the proliferation of differentiated cells occurs. Indeed, IL-4 stimulation in the presence of a proliferation blocker no longer induces TMEM16A, in contrast to MUC5AC levels, which still go up. These results demonstrate that MUC5AC production is independent of TMEM16A. Additionally, we find that during wound healing, the expression levels of TMEM16A rise concomitantly with the proliferation marker Ki-67, further corroborating that proliferation triggers TMEM16A upregulation.

Altogether, our findings clearly show for the first time in human airways that TMEM16A upregulation by IL-4 is proliferation-dependent and that this channel is not essential for MUC5AC production, thus remaining a good target for activation in CF and likely other obstructive airway diseases.

Results

1. Analysis of TMEM16A and MUC5AC during differentiation of airway basal epithelial cells

An association between the expression of TMEM16A and MUC5AC was previously reported by several authors (Benedetto et al., 2019; Huang et al., 2012; Kang et al., 2017; Kondo et al., 2017; Lin et al., 2015; Qin et al., 2016; Scudieri et al., 2012). We thus first examined the expression levels of these two proteins during differentiation of BCi-NS1.1 cells. To this end, protein was collected from cells every 5 days during 30 days of differentiation in ALI culture and TMEM16A and MUC5AC were detected by WB with respective antibodies (see Methods). In parallel, RNA was also extracted at 3 different time-points (0, 15 and 30 days). Our data clearly show that TMEM16A expression decreases during differentiation at both transcript and protein levels (Figure 1.1A-C). In contrast, this TMEM16A levels decrease occurs simultaneously with an increase in the expression of cell-type-specific markers and TEER (**Figure S1.1**), including MUC5AC, which was found to increase significantly during differentiation (Figure 1.1D-F). These data show an inverse correlation between TMEM16A and MUC5AC expression during differentiation (Figure 1.1G).

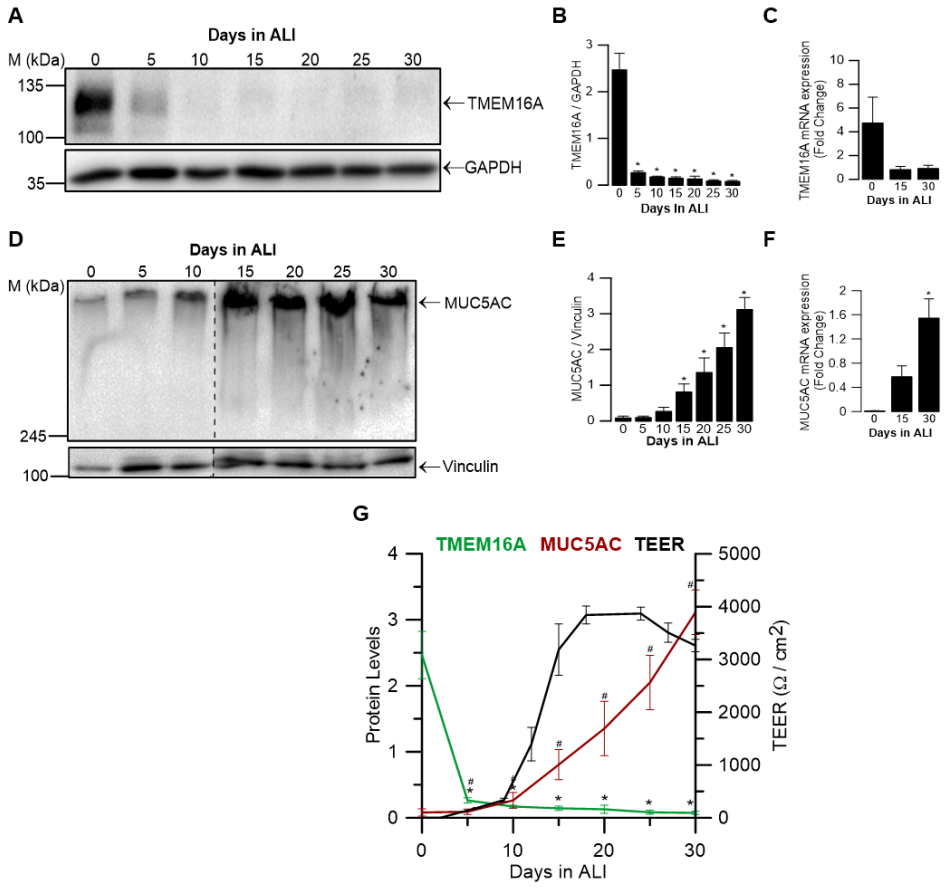

Figure 1.1 – TMEM16A and MUC5AC expression levels are inversely correlated during differentiation of BCi-NS1.1 cells. (A) Time-course levels of endogenous expression of TMEM16A

protein during differentiation of BCi-NS1.1 cells grown at ALI (days 1-30) detected by WB, showing both its non-glycosylated (~100 kDa) and glycosylated forms (~120 kDa). GAPDH (~36 kDa) was used as a loading control. **(B)** Quantification by densitometry of TMEM16A total protein levels from (A) normalised to the loading control shown as mean ± SEM (n = 3). **(C)** Fold-change in TMEM16A mRNA expression levels as a time-course of differentiation of BCi-NS1.1 cells grown at ALI (days 0, 15 and 30), determined by qRT-PCR. Fold-change values are mean ± SEM, relative to the mean value of day 30 (n = 3). **(D)** Time-course levels of endogenous expression of MUC5AC (> 300 kDa) analysed by WB during differentiation of BCi-NS1.1 cells. Vinculin was used as a loading control (~120 kDa). Dashed line indicates lanes run on the same gel but noncontiguous. **(E)** Quantification by densitometry of MUC5AC protein levels from (D) normalised to the loading control shown as mean ± SEM (n = 4). **(F)** Fold-change in MUC5AC mRNA expression levels as a time-course of differentiation of BCi-NS1.1 cells grown at ALI (days 0, 15 and 30), determined by qRT-PCR. Fold-change values are mean ± SEM, relative to the mean value of day 30 (n = 4). **(G)** Correlation of TMEM16A and MUC5AC normalised protein levels during differentiation and TEER measurements. Asterisks and cardinals indicate significant difference compared to day 0 (p-value < 0.05, unpaired t-test). [RT-qPCR experiments were performed with Luka A Clarke and are included here with permission].

Next, we assessed by immunofluorescence the cellular localisation of TMEM16A and MUC5AC in non-differentiated (day 0) and differentiated (day 30) BCi-NS1.1 cells by confocal microscopy (Figure 1.2). Consistently with WB and qRT-PCR results, our data show a decrease in TMEM16A and an increase in MUC5AC staining densities between day 0 and 30. Furthermore, TMEM16A localisation changes as cells differentiate: while it is present in all non-differentiated (basal) cells with a predominant intracellular distribution at day 0, it is only expressed in some cells and apically localized at day 30 (Figure 1.2A, B). To distinguish the apical from the basolateral membrane, we stained the apical membrane with an antibody against the tight junction barrier protein ZO-1 (Figure 1.2A, B). In contrast, MUC5AC is not present on day 0 but is apically secreted at day 30 and only from specific cells (Figure 1.2C, D).

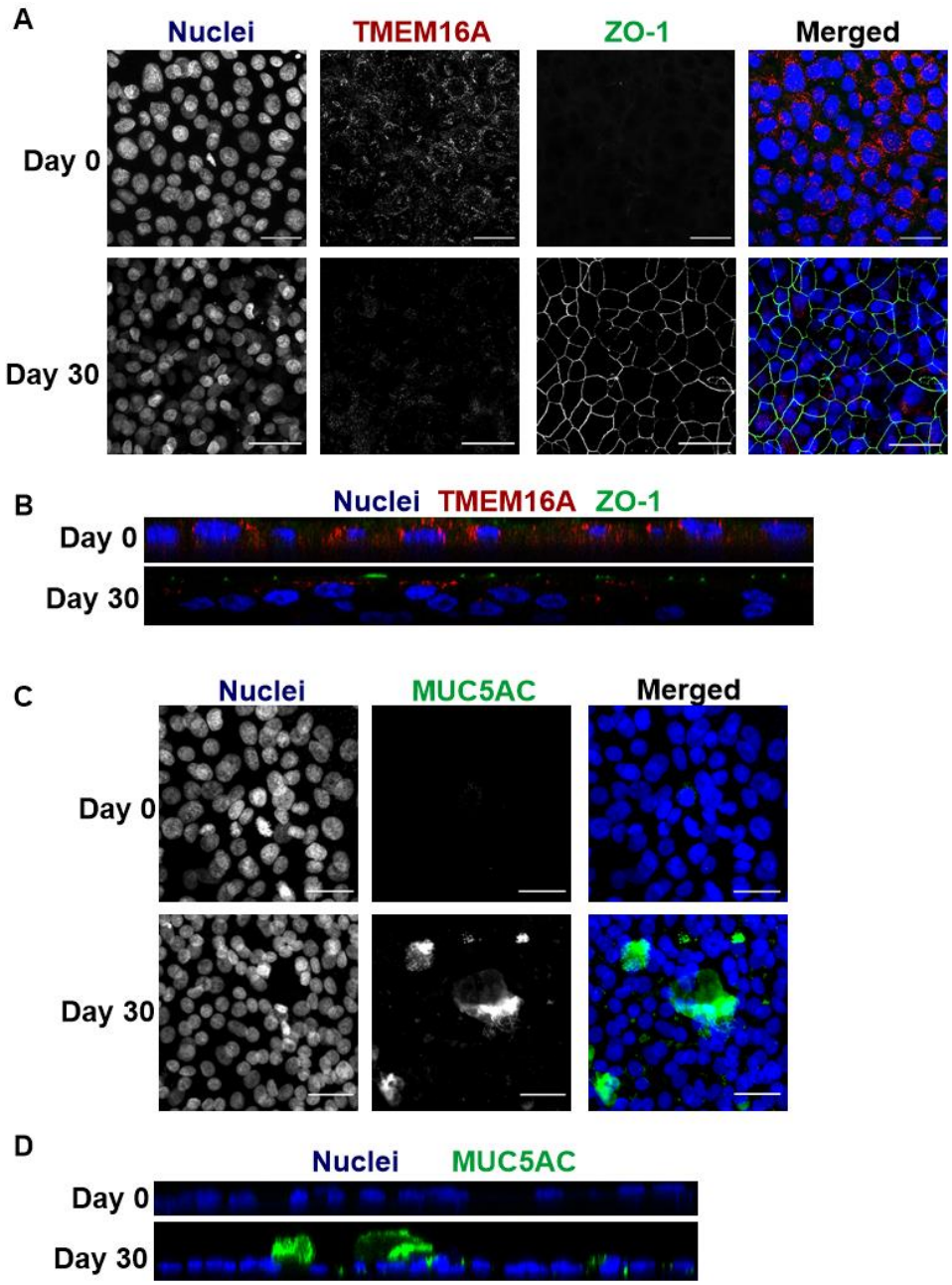

Figure 1.2 – TMEM16A and MUC5AC have distinct spatio-temporal localisations in differentiating BCi-NS1.1 cells. **(A)** Confocal immunofluorescence microscopy images showing TMEM16A localisation in permeabilised BCi-NS1.1 cells on days 0 and 30 (upper and lower rows, respectively) of differentiation. Left panels: Nuclei stained with Hoechst. Middle panels: Endogenous TMEM16A, detected by Alexa Fluor® 568 fluorescence. Endogenous ZO-1, detected by Alexa Fluor® 488 fluorescence. Right panels: Merged image of the three fluorescent channels: Blue – Alexa 647; Red – Alexa 568; Green – Alexa 488. Images were acquired with a Leica TCS SP8 confocal microscope (objective 63 x oil, NA 1.4). Scale bar = 30 µm. (n = 3) **(B)** Z-stack of a representative group of cells showing TMEM16A and ZO-1 staining on days 0 and 30 (upper and lower panels, respectively). **(C)** Confocal immunofluorescence microscopy images showing MUC5AC expression in permeabilised BCi-NS1.1 cells on days 0 and 30 of differentiation (upper and lower rows, respectively). Left panels: Nuclei stained with metal green, represented by Alexa Fluor® 647 fluorescence. Middle panels: Endogenous MUC5AC, detected by Alexa Fluor® 488 fluorescence. Right panels: Merged image of the two fluorescent channels: Green – Alexa 488; Blue – Hoechst. Images were acquired with a Leica TCS SP8 confocal microscope (objective 63 x oil, NA 1.4). Scale bar = 30 µm. (n = 3) **(D)** Z-stack of a representative group of cells showing MUC5AC stained apically on days 0 and 30 (upper and lower panels, respectively).

2. TMEM16A is upregulated in proliferating cells

Our next goal was to further explore this inverse correlation found between TMEM16A and MUC5AC during differentiation of BCi-NS1.1 cells (Figure 1.1Figure 1.2). Notably, TMEM16A has for long been associated with cell proliferation and tumour growth, being upregulated in several cancer types (Jia et al., 2015; Wang et al., 2017; Wanitchakool et al., 2014). Therefore, we hypothesised that the increased expression levels of TMEM16A observed at the early stages of differentiation could be related to cell proliferation.

We thus determined by WB the expression levels of the proliferation marker Ki-67 as a time-course of differentiation of BCi-NS1.1 cells grown at ALI (days 1-30) and observed, as expected, its significant decrease during differentiation (Figure 1.3A, B), paralleling that of TMEM16A (Figure 1.1A-CFigure 1.3C). These data thus support the concept that high levels of TMEM16A expression correlate with cell proliferation.

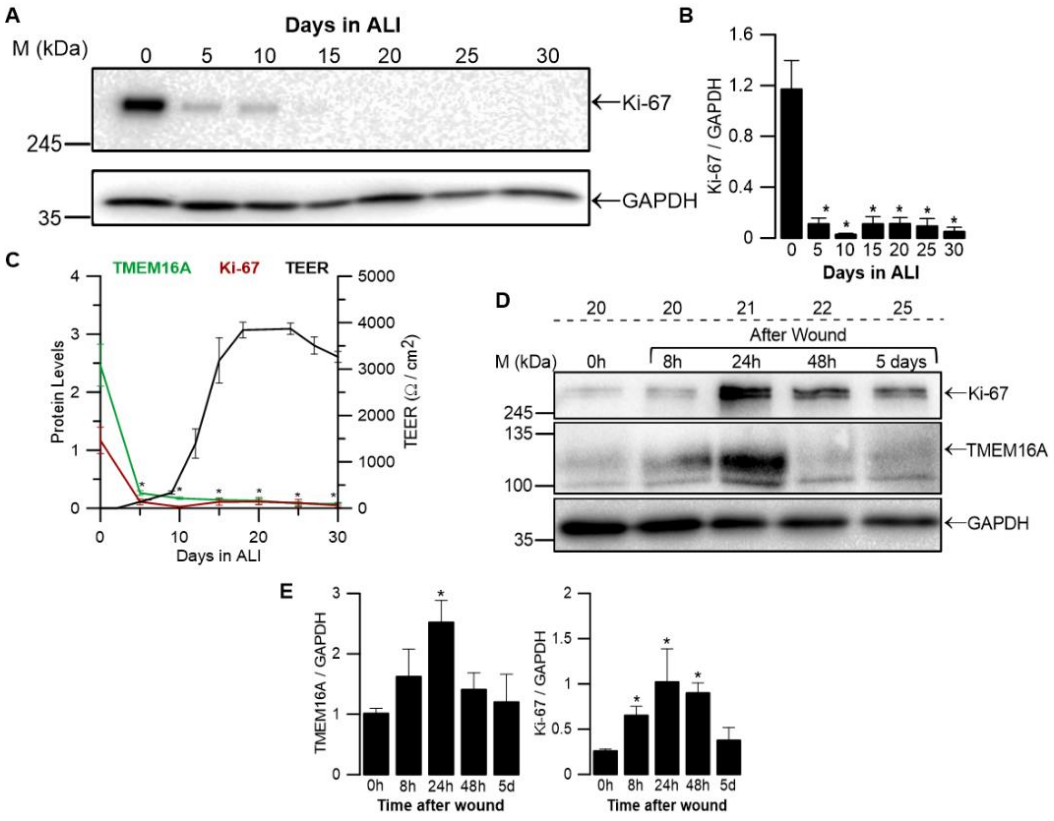

Figure 1.3 – High TMEM16A expression levels correlate with cell proliferation in BCi-NS1.1 cells after wound healing. **(A)** Time-course levels of endogenous expression of Ki-67 protein (~300 kDa) during differentiation of BCi-NS1.1 grown at ALI (days 0-30) analysed by WB. GAPDH (~36 kDa) was used as loading control. **(B)** Quantification by densitometry of Ki-67 expression detected by WB and normalised to the loading control shown as mean ± SEM (n = 3). **(C)** Correlation of TMEM16A and Ki-67 normalised protein levels and TEER measurements during differentiation. Summary of TMEM16A total protein levels represents data in Figure 1. **(D)** Time-course (0h to 5 days) of expression levels of endogenous expression of TMEM16A and Ki-67 during wound closure (0h, 8h, 24h, 48h and 5 days after injury) on the 20th day of differentiation at ALI analysed by WB. GAPDH (~36 kDa) was used as loading control. **(E)** Quantification by densitometry of total TMEM16A and Ki-67 expression detected by WB and normalised to the loading control shown as mean ± SEM (n = 3-5). Asterisks indicate significant

difference compared to day 0 (in graph B) or before wound (in graph E) (p-value < 0.05, unpaired t-test).

3. TMEM16A is induced by wound-healing in differentiated cells in BCi-NS1.1 cells

To further confirm this hypothesis, monolayers of differentiated BCi-NS1.1 cells were subjected to a wound-healing experiment (by scratching) on ALI day 20, i.e., a time point when expression levels of both TMEM16A (Figure 1.1A-C) and Ki-67 are low (Figure 1.3A, B). During the healing process, the levels of these two proteins were determined by WB at 8h, 24h, 48h and 5 days after injury. Wound-healing results indicate that the expression levels of both TMEM16A and Ki-67 increase dramatically after wounding and decrease significantly during the healing process (Figure 1.3D, E). These results further support the association between TMEM16A induction and cell proliferation.

4. BCi-NS1.1 results are confirmed in primary cultures of human bronchial epithelial cells

Since BCi-NS1.1 is an immortalised cell line derived from the bronchi of a healthy subject, we next aimed to validate the above data in primary cultures of human bronchial epithelial (HBE) cells. Remarkably, we also found that TMEM16A and Ki-67 protein expression decrease during differentiation of HBE cells (Figure 1.4A, B), while MUC5AC levels increase (Figure 1.4C, D), confirming the experiments performed with BCi-NS1.1 cells and indicating that this cellular system is robust and recapitulates the physiological properties of human airways.

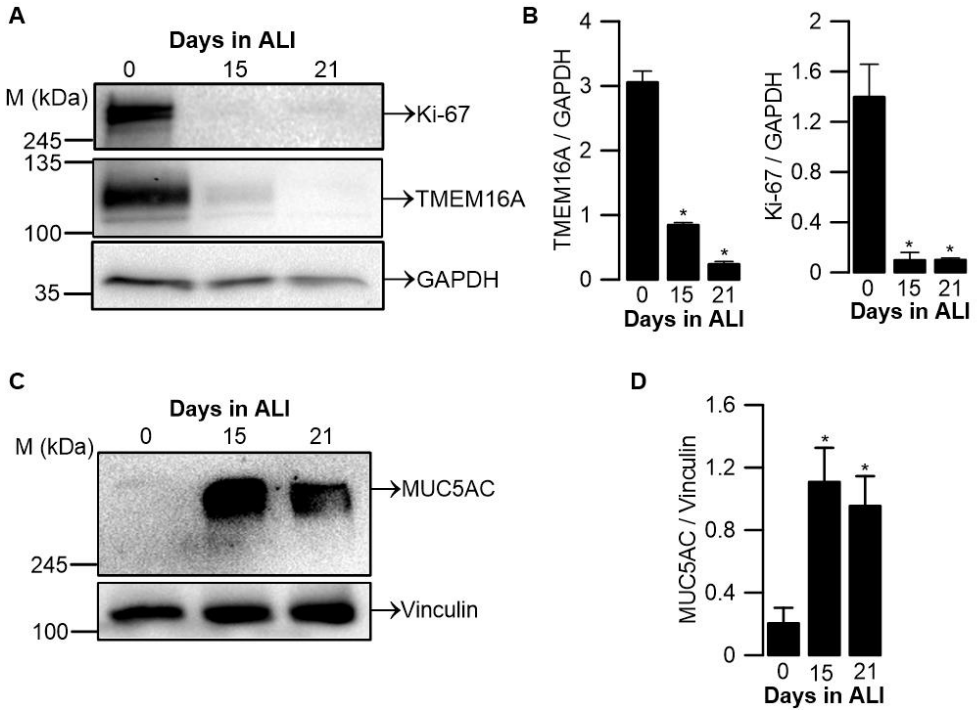

Figure 1.4 – TMEM16A and Ki-67 protein expression during differentiation of primary human bronchial epithelial cells (HBE). (A) WB of endogenous TMEM16A and Ki-67 proteins during differentiation of primary HBE cells. GAPDH was used as loading control. **(B)** Quantification by densitometry of total TMEM16A and Ki-67 expression detected by WB and normalised to the loading control shown as mean ± SEM (n = 3). **(C)** WB of endogenous MUC5AC during differentiation of primary HBE cells. Vinculin was used as loading control. **(D)** Quantification by densitometry of MUC5AC detected by WB and normalised to the loading control shown as mean ± SEM (n = 3). Asterisks indicate significant difference compared to day 0 (p-value < 0.05, unpaired t-test).

5. Upregulation of TMEM16A induced by IL-4 is driven by cell proliferation

Altogether, the above expression and localisation data on TMEM16A and MUC5AC strongly suggest that MUC5AC production is independent of TMEM16A, not just in BCi-NS1.1 cells but also in primary HBE cells. Nevertheless, these data appear contradictory to previous reports suggesting that TMEM16A plays a key role in mucus production, namely in asthma, where both TMEM16A and MUC5AC are concomitantly upregulated by IL-4 or IL-13 induction (Huang et al., 2012; Kang et al., 2017; Lin et al., 2015; Scudieri et al., 2012).

Thus, given that TMEM16A is upregulated under proliferation (Figure 1.3), we next investigated whether the conditions previously shown to significantly induce TMEM16A expression levels, such as the pro-inflammatory cytokine IL-4 (Caputo et al., 2008), also affect cell proliferation. We thus postulated that perhaps IL-4 also triggers cell proliferation. Since at day 30 (at ALI) in fully differentiated BCi-NS1.1 cells, TMEM16A expression levels were already undetectable by WB (Figure 1.1A, B), we chose this time point to test IL-4 induction. As expected, upon treatment with IL-4 for 48h, a significant upregulation of this channel occurred (Figure 1.5A, B, left panels). Consistently, Ussing chamber experiments show that this cytokine also enhances the ATP-activated currents, which are inhibited by the TMEM16A blocker, CaCC-AO1 (Figure 1.5C, D). Moreover, after removing ATP and CaCC-AO1 solution,

the third application of ATP was still effective (Figure S1.2). Concomitantly, and also as previously described (Gorrieri et al., 2016; Scudieri et al., 2012; Temann et al., 1997), MUC5AC levels also increased upon IL-4 stimulation (Figure 1.5A, B, middle panels). Notwithstanding, and confirming our hypothesis, IL-4 also led to a significant increase in Ki-67 expression in these fully differentiated cells, thus indicating that cells underwent proliferation (Figure 1.5A, B, right panels).

In order to understand whether proliferation drives TMEM16A upregulation (or the other way around), we performed IL-4 stimulation in the presence of the proliferation blocker mitomycin C and then determined protein levels of Ki-67 and TMEM16A by WB (Figure 1.5E, F). Remarkably, our data clearly show that under blockage of cell proliferation, TMEM16A is not upregulated, despite the presence of IL-4, concomitantly with the expected lack of increase in Ki-67 expression levels (Figure 1.5E, F). As a control, we could observe that the expression levels of the Na^+/K^+ ATPase pump remain constant (Figure 1.5E, F). These data strongly favour that the driver of TMEM16A upregulation is cell proliferation. However, under proliferation arrest, the expression levels of MUC5AC still go up upon IL-4 stimulation (Figure 1.5G, H), as previously reported for this cytokine (Gorrieri et al., 2016; Scudieri et al., 2012; Temann et al., 1997). These data further support that MUC5AC production is not dependent on TMEM16A.

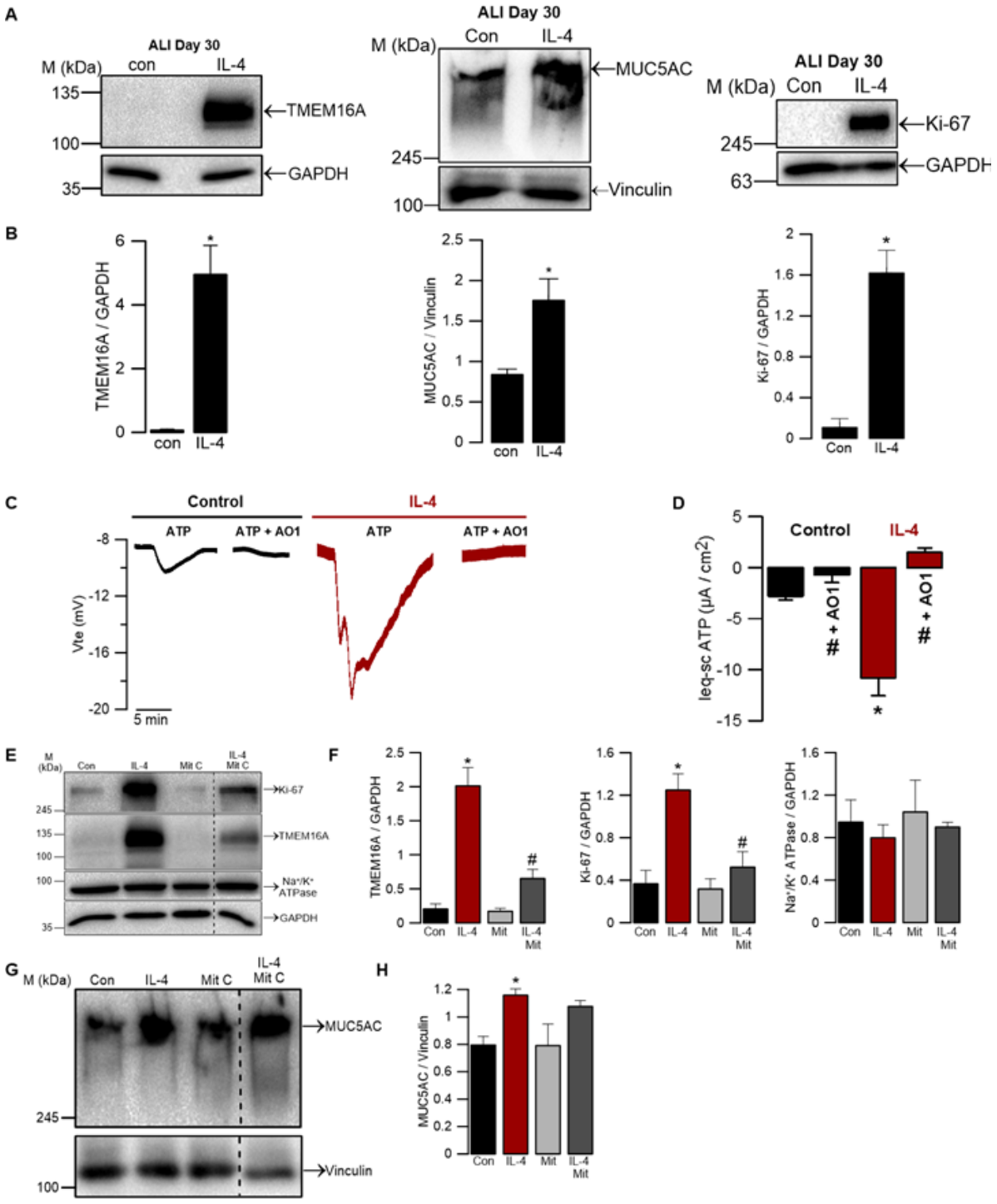

Figure 1.5 – TMEM16A and MUC5AC upregulation induced by IL-4 are associated with cell proliferation. (A) WB indicating upregulation of TMEM16A, MUC5AC and Ki-67 by stimulation with 5 ng/mL IL-4 for 48h in BCi-NS1.1 cells. GAPDH was used as a loading control for TMEM16A and Ki-67 WB. Vinculin was used as a loading control for MUC5AC WB. **(B)** Quantification by densitometry of WB for total TMEM16A, MUC5AC and Ki-67 expression normalised to the loading control, data shown as mean ± SEM (n = 3). **(C)** Original Ussing chamber tracings +/- IL-4, obtained for ATP-induced Cl^- currents (100 μM) in the presence of the epithelial Na^+ channel (ENaC) inhibitor, amiloride (30 μM). A reduction of the ATP-activated Cl^- currents was observed under the CaCC-AO1 TMEM16A inhibitor (30 μM). [Ussing Chamber measurements were performed with Arthur Kmit and are included here with permission]. **(D)** Summary of $I_{sc\text{-}eq}$ ATP currents in the presence or absence of IL-4. Values are mean ± SEM (n = 3 - 7). Asterisk indicates significant difference compared to control; Cardinal indicates significant difference compared to ATP alone (p-value < 0.05, unpaired t-test). **(E)** WB showing downregulation of TMEM16A and Ki-67 expression by treatment with the proliferation blocker mitomycin

C (1 µg/mL) and IL-4 (5 ng/µL) for 48h. Na^+/K^+ ATPase (~ 100 kDa) - another protein expressed in the membrane - was detected as a control. GAPDH was used as loading control. Dashed line indicates lanes run on the same gel but noncontiguous. **(F)** Quantification by densitometry of total TMEM16A, Ki-67 and Na^+/K^+ ATPase expression detected by WB and normalised to the loading control, shown as mean ± SEM (n = 5-7). **(G)** WB showing MUC5AC levels upon treatment with mitomycin C (1 µg/mL) +/- IL-4 (5 ng/mL) for 48h. Vinculin was used as loading control. Dashed line indicates lanes run on the same gel but noncontiguous. **(H)** Quantification by densitometry of MUC5AC detected by WB and normalised to the loading control, shown as mean ± SEM (n = 4-5). Asterisks indicate significant difference compared to control (p-value < 0.05, unpaired t-test).

6. TMEM16A is not essential for mucus production in BCi-NS1.1 cells

Increasing evidence accumulated in support of MUC5AC and TMEM16A being uncoupled. Thus, our next goal was to determine that indeed there is no causal relationship between TMEM16A and MUC5AC production/secretion. To this end, we looked into conditions that, despite leading to mucus hypersecretion, do not lead to hyperplasia (i.e., no cell proliferation), unlike IL-4/IL-13 stimulation. One such situation that positively regulates mucus hypersecretion is stimulation by activators of the notch signalling pathway, such as DLL4 (Delta-Like Ligand 4), which directs epithelial differentiation into secretory cells by metaplasia, but no hyperplasia, i.e. without proliferation (Guseh et al., 2009; Rock & Hogan, 2011b). Strikingly, our results obtained in BCi-NS1.1 cells treated (at day 0 of ALI) with DLL4 for 30 days did not show any elevation in TMEM16A levels, despite the significant increase in MUC5AC levels (Figure 1.6). These data strongly suggest that the concomitant rise of TMEM16A and MUC5AC levels under IL-4/IL-13 stimulation reported by many groups (Huang et al., 2012; Kondo et al., 2017; Lin et al., 2015; Qin et al., 2016; Scudieri et al., 2012) is coincidental but not causal: while TMEM16A is upregulated because of goblet cell *hyperplasia* (i.e., proliferation), that of MUC5AC results from the higher number of *mucus-producing cells*.

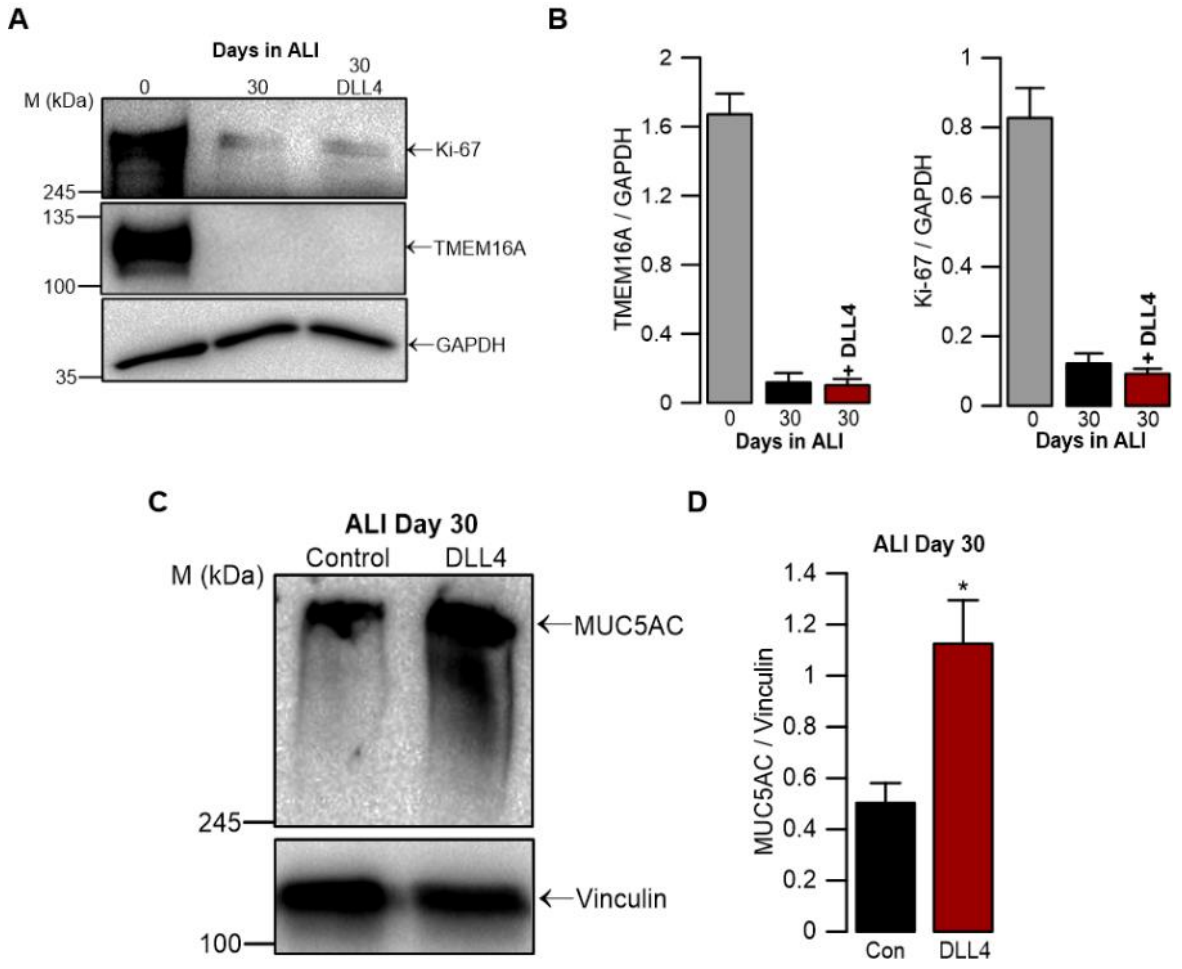

Figure 1.6 – TMEM16A is not essential for mucus production in BCi-NS1.1 cells. (A) WB of endogenous TMEM16A and Ki-67 expression in the presence or absence of 400 ng/µL DLL4 for 30 days. Samples from undifferentiated cells were used as a positive control for TMEM16A and Ki-67 expression. GAPDH was used as loading control. **(B)** Quantification by densitometry of total TMEM16A and Ki-67 expression detected by WB and normalised to the loading control shown as mean ± SEM (n = 3). **(C)** WB of endogenous MUC5AC in the presence or absence of 400 ng/µL DLL4. Vinculin was used as a loading control. **(D)** Quantification by densitometry of MUC5AC expression detected by WB and normalised to the loading control shown mean ± SEM (n = 3). Asterisks indicate significant difference compared to control (p-value < 0.05, unpaired t-test).

7. Effect of regulating TMEM16A activity on airway surface liquid (ASL) height

Since our data so far have uncoupled TMEM16A from a possible causal effect on mucus, this channel seems to remain a good potential drug target for CF through its activation (not inhibition) to compensate for the absence of CFTR-mediated Cl^-/HCO_3^- secretion. So next, we aimed to observe the effects of inhibiting this channel on ASL height. Indeed, by inhibiting TMEM16A with the specific inhibitor Ani9 (Seo et al., 2016), we observed a decrease in ASL height compared to control cultures which was significant at all time points (Figure 1.7). Thus, inhibiting TMEM16A causes significant airway dehydration by reducing fluid secretion, and TMEM16A potentiation remains a good target for hydrating CF airways.

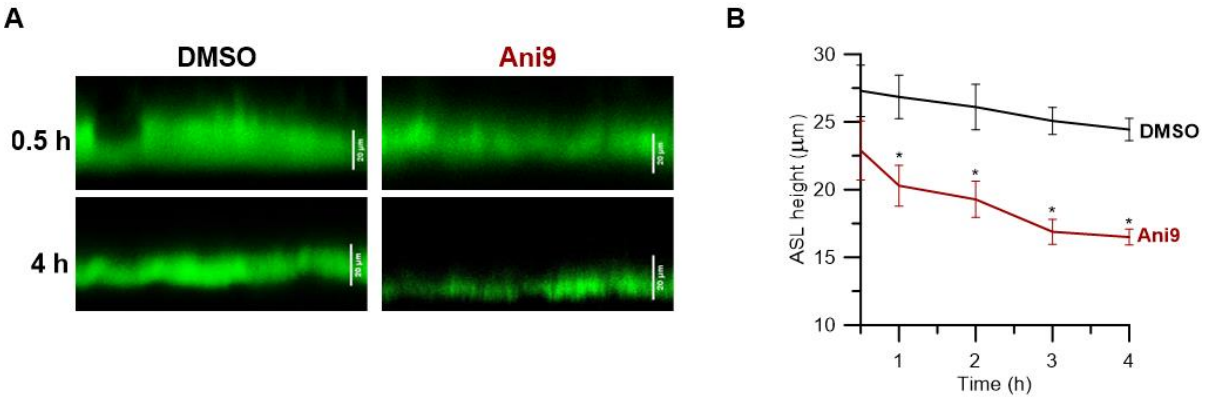

Figure 1.7 – TMEM16A contributes to fluid secretion in differentiated BCi-NS1.1 cells. (A) Representative confocal images of ASL labelled with FITC-Dextran obtained 0.5 and 4h after apical exposure to DMSO or 10 µm Ani9. **(B)** Quantification of over-time (0.5, 1, 2, 3, 4h) ASL height measurements shown as mean ± SEM (n = 7 - 9). Asterisks indicate significant difference compared to 0.5 h (p-value < 0.05, unpaired t-test).

Discussion

Mucus hyperproduction is a feature that characterises nearly all airway obstructive pulmonary diseases, including CF, asthma and COPD (Adler et al., 2013; Williams et al., 2006). Therefore, understanding the molecular mechanisms behind mucus synthesis and/or release is key to developing disease-specific therapies (Rogers & Barnes, 2006). In particular, individuals with CF suffer from extensive mucus plugging and stasis, resulting from airway dehydration and mucus hyperproduction, which leads to impaired MCT and breathing due to malfunctioning of a Cl^-/HCO_3^- channel, CFTR (Kreda et al., 2012). In this regard, the Ca^{2+}-activated Cl^- channel TMEM16A has for long been considered an attractive alternative therapeutic target for these individuals (Li et al., 2017; Verkman & Galietta, 2009). Pharmacological activation of TMEM16A would thus be expected to compensate for the absence of defective CFTR by improving airway hydration through an increase in ASL height (Haq et al., 2016) and mucin solubilisation through TMEM16A-dependent HCO_3^- secretion (Gorrieri et al., 2016; Jung et al., 2013).

Nevertheless, recent reports suggest that not activation but rather inhibition of this channel could improve the CF phenotype (Benedetto et al., 2019; Kunzelmann et al., 2019; Lin et al., 2015) by decreasing mucus production and/or secretion (Huang et al., 2012; Kondo et al., 2017; Lin et al., 2015; Qin et al., 2016). However, these studies are only based on the observation that TMEM16A and mucus/MUC5AC are concomitantly upregulated during inflammation and/or are carried out in animal models, namely mice (Benedetto, Cabrita, et al., 2019; Huang et al., 2012; Lin et al., 2015), that do not mimic the CF airway disease (Benedetto, Cabrita, et al., 2019; Huang et al., 2012; Lin et al., 2015).

The main goal of this study was to examine whether TMEM16A drives mucus production (MUC5AC) or if it is just upregulated by the same stimuli that trigger mucus to contribute to better airway hydration. These findings will contribute to broadening our knowledge of the role that TMEM16A plays in the airways, with an impact on how it may be used as a drug target for CF and also for other airway obstructive diseases (Brett, 2015; Sala-Rabanal, Yurtsever, Nichols, et al., 2015; Sondo et al., 2014).

The experiments in this study were performed using a recently described human multipotent airway basal cell line (BCi-NS1.1) isolated from a bronchial brushing of a healthy non-smoker subject (Walters et al., 2013). This cell line is particularly relevant for the current study because it retains the capacity to multi-differentiate into all airway epithelial cell types (ciliated, goblet, club) being thus a good model to represent the cellular diversity that characterises the human airway epithelium (Tam et al., 2011, Figure S1.1). We have first examined the relationship between TMEM16A and MUC5AC during differentiation of BCi-NS1.1 cells, and then we challenged these cells with a pro-inflammatory stimulus, namely the

Th2-cytokine IL-4, and finally, we have induced goblet cell metaplasia mediated by the Notch1 activator DLL4. Importantly, as this is a novel cell line in the field, crucial experiments were also validated in primary cultures of human bronchial epithelial cells collected from the lungs of healthy donors. As results obtained in BCi-NS1.1 and primary HBE cells are consistent (Figure 1.4), we conclude that this cell line is a good model to recapitulate human airway physiology.

Our results show that cell proliferation is the driver for TMEM16A upregulation during goblet cell hyperplasia through several lines of evidence. Firstly, TMEM16A and MUC5AC are inversely correlated as human airway epithelial cells differentiate under control conditions: while TMEM16A expression levels are high in non-differentiated (basal) cells and almost undetectable (by WB) in differentiated cells, MUC5AC is very low in the former and abundantly expressed in the latter (Figure 1.1). The fact that TMEM16A expression is high in basal cells is not totally surprising, given its inducibility by Th2 cytokines (e.g. IL-4, IL-13). Indeed, these cytokines promote the proliferation of basal cells, which will then differentiate into goblet cells due to activation of the Notch signalling pathway (Williams et al., 2006). Moreover, those results already suggest that mucus production does not require high levels of TMEM16A. Interestingly, even though TMEM16A protein expression was almost undetectable by WB, immunofluorescence showed apical staining of TMEM16A in differentiated cells (Figure 1.2). This is consistent with the pattern that can be found in human bronchi, in which TMEM16A staining in the surface epithelium is apical but at very low levels (Caci et al., 2015; Lérias et al., 2018; Ousingsawat et al., 2009).

Secondly, our data show that TMEM16A is positively correlated with the proliferation marker Ki-67. This occurs during differentiation under control conditions: fully differentiated cells which do not proliferate also have low levels of TMEM16A (Figure 1.3AFigure 1.1A-C). But strikingly, this correlation also occurs when a fully differentiated cell monolayer is subjected to wound healing: the two proteins are upregulated ((Figure 1.3D, E). This result is in agreement with previous reports in cancer research that link TMEM16A with cell proliferation, migration and metastasis (Ayoub et al., 2010; Duvvuri et al., 2012; Jia et al., 2015; Ruiz et al., 2012). Additionally, another study has shown that knockdown of TMEM16A in primary cultures of human airway epithelial cells of CF patients impairs wound closure (Ruffin et al., 2013).

Thirdly, our data show that induction of TMEM16A by IL-4 is dependent on cell proliferation, while mucus hyperproduction is not. Indeed, our data show that when differentiated BCi-NS1.1 cells were exposed to this cytokine, both TMEM16A and MUC5AC protein expression were upregulated as others reported (Gorrieri et al., 2016; Scudieri et al., 2012). This finding was also corroborated by functional data, as IL-4 also enhanced the TMEM16A-mediated ATP-induced current in Ussing chamber recordings (Figure 1.5C, D). In

fact, IL-4 was reported to modulate the ion transport in the human bronchial epithelium, increasing Cl^- secretion and decreasing Na^+ absorption, thus promoting airway hydration and mucus clearance (L. J. Galietta et al., 2002). Remarkably, we show that incubation with IL-4 induced proliferation of fully differentiated cells (Figure 1.5A, B, right panels), confirming the hypothesis that TMEM16A is upregulated by IL-4 due to cell proliferation. Indeed, when proliferation is arrested by mitomycin C, TMEM16A expression induced by IL-4 is significantly reduced (Figure 1.5E, F), despite MUC5AC still being upregulated (Figure 1.5G, H), and thus independent of cell proliferation.

Finally, our data also show that when mucus production is induced by goblet cell metaplasia (through modulation of the Notch signalling pathway with the Notch1 activator DLL4), MUC5AC production is significantly increased, but neither TMEM16A nor Ki-67 are upregulated (Figure 1.6).

In conclusion, all data presented here clearly show that TMEM16A is only present at high levels in proliferating cells in situations like goblet cell hyperplasia (Figure 1.8).

Since these data seem to contradict the conclusion of several studies in the literature, we attempt to conciliate our data with those reports (Benedetto et al., 2019; Huang et al., 2012; Kondo et al., 2017; Lin et al., 2015). It is of relevance to note that mucus synthesis (production) and release (secretion) are controlled by different signalling pathways and that the respective regulators do not entirely correlate in the different airway obstructive pulmonary diseases (Fahy & Dickey, 2010; Rogers & Barnes, 2006). Moreover, the conclusion by previous studies that TMEM16A inhibition improves mucus hypersecretion is strongly based on the use of nonspecific inhibitors (Benedetto, Cabrita, et al., 2019; Huang et al., 2012; Kondo et al., 2017; Lin et al., 2015; Qin et al., 2016), which were already shown to act on other chloride channels/transporters, including CFTR (Benedetto et al., 2017; Dienna et al., 2007; Scott-Ward et al., 2004) and other members of the TMEM16 family (Namkung et al., 2011; Sirianant et al., 2016; Wanitchakool et al., 2014) and many, like niclosamide, were also shown to inhibit cell proliferation (Han et al., 2019; Mazzone et al., 2012).

Other studies have shown that the signalling pathways behind TMEM16A upregulation induced by IL-4 binding to its membrane receptors activate the transcription factor STAT6, which will then bind to the TMEM16A promoter (Mazzone et al., 2015). Interestingly and consistently, knockdown of STAT6 also impairs cell proliferation (Salguero-Aranda et al., 2019).

Importantly, none of these studies addressed the impact of TMEM16A inhibition on ASL height. In fact, mice lacking TMEM16A exhibit a CF-like phenotype (Ousingsawat et al., 2009; Rock et al., 2009), suggesting that this protein is essential for chloride secretion (Gianotti et al., 2016) and for maintaining a proper airway surface liquid thickness in mouse airways.

Data in the current study also clearly show that TMEM16A inhibition results in a significant decrease in the ASL height in human airway epithelial cells (Figure 1.7).

Chloride secretion is essential in the airway epithelium to maintain the ASL with a proper thickness, allowing MCT (Haq et al., 2016). Just like CFTR, TMEM16A is expressed at the apical membrane of differentiated airway epithelial cells (Huang et al., 2009; Scudieri et al., 2012), contributing to the alternative route for Cl^- secretion in individuals with CF (Sondo et al., 2014). Interestingly, two recent reports show that TMEM16A is required for CFTR expression and activity (Benedetto et al., 2017; Benedetto, Ousingsawat, et al., 2019), suggesting that inhibition of TMEM16A would compromise airway hydration. In fact, we show that by blocking TMEM16A with a specific inhibitor (Ani9), the ASL height is significantly reduced, demonstrating that inhibition of this protein would further dehydrate the airways of CF patients.

Taking together all data in this study, we propose a novel model for the mechanism of the relationship between TMEM16A, cell proliferation and mucus production (Figure 1.8). In highly proliferating (undifferentiated) basal cells (Figure 1.8A), TMEM16A expression levels are high. However, as basal cells differentiate (stopping proliferation, Figure 1.8B), TMEM16A expression levels decrease, and its localisation becomes specifically apical, while MUC5AC levels increase due to the appearance of differentiated mucus-producing cells (Figure 1.8B). Whenever there is inflammation associated with cell proliferation (goblet cell hyperplasia), MUC5AC production is increased due to enrichment in mucus-producing cells, while concomitantly, TMEM16A is also upregulated due to cell proliferation (Figure 1.8C). As some TMEM16A inhibitors are also inhibitors of cell proliferation (Han et al., 2019; Mazzone et al., 2012), it is very likely that these molecules are reducing mucin levels by inhibiting goblet cell hyperplasia, albeit through a TMEM16A-independent mechanism.

Given the data presented here, we conclude that finding TMEM16A activators, i.e., stimulators of the channel activity acting specifically in non-proliferating differentiated cells, remains a bona fide goal for drug discovery in CF and likely other chronic obstructive airway diseases.

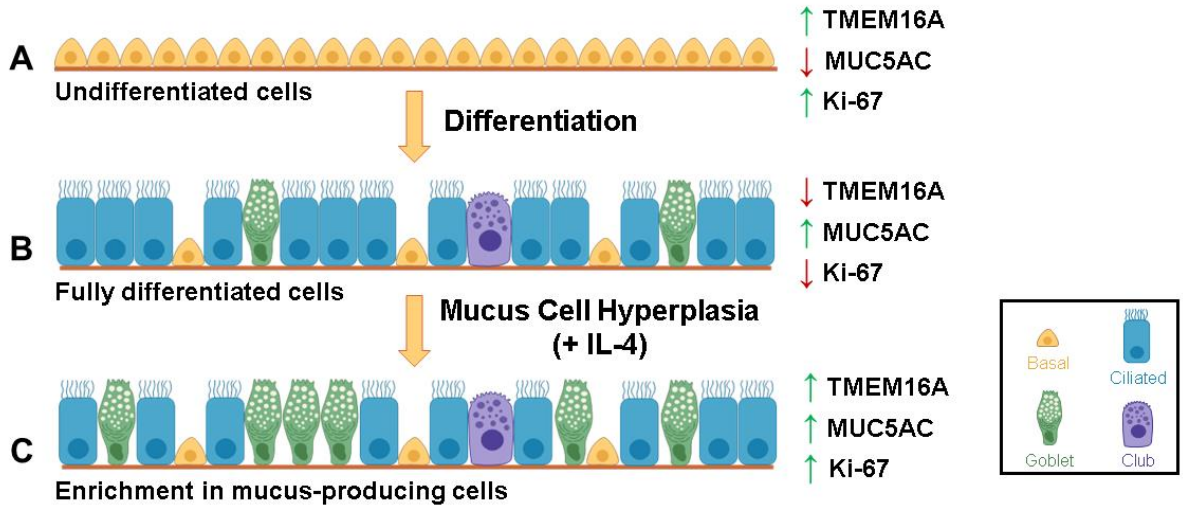

Figure 1.8 – Relationship between TMEM16A, MUC5AC and Ki-67 during differentiation and goblet cell hyperplasia. (A) Undifferentiated basal cells are proliferating and thus have high levels of TMEM16A and Ki-67. On the contrary, due to the absence of goblet cells, MUC5AC is not expressed. **(B)** When cells are differentiated, proliferation stops (low TMEM16A and Ki-67), and mucus production is high, mostly secreted from goblet cells. **(C)** Induction of goblet cell hyperplasia by pro-inflammatory cytokines such as IL-4/13 switches the human airway epithelium from a non-proliferative to a proliferative state, inducing TMEM16A and Ki-67 expression. As a consequence of inflammation, basal cells proliferate and differentiate into mucus-producing cells, increasing MUC5AC levels.

Supplementary Data

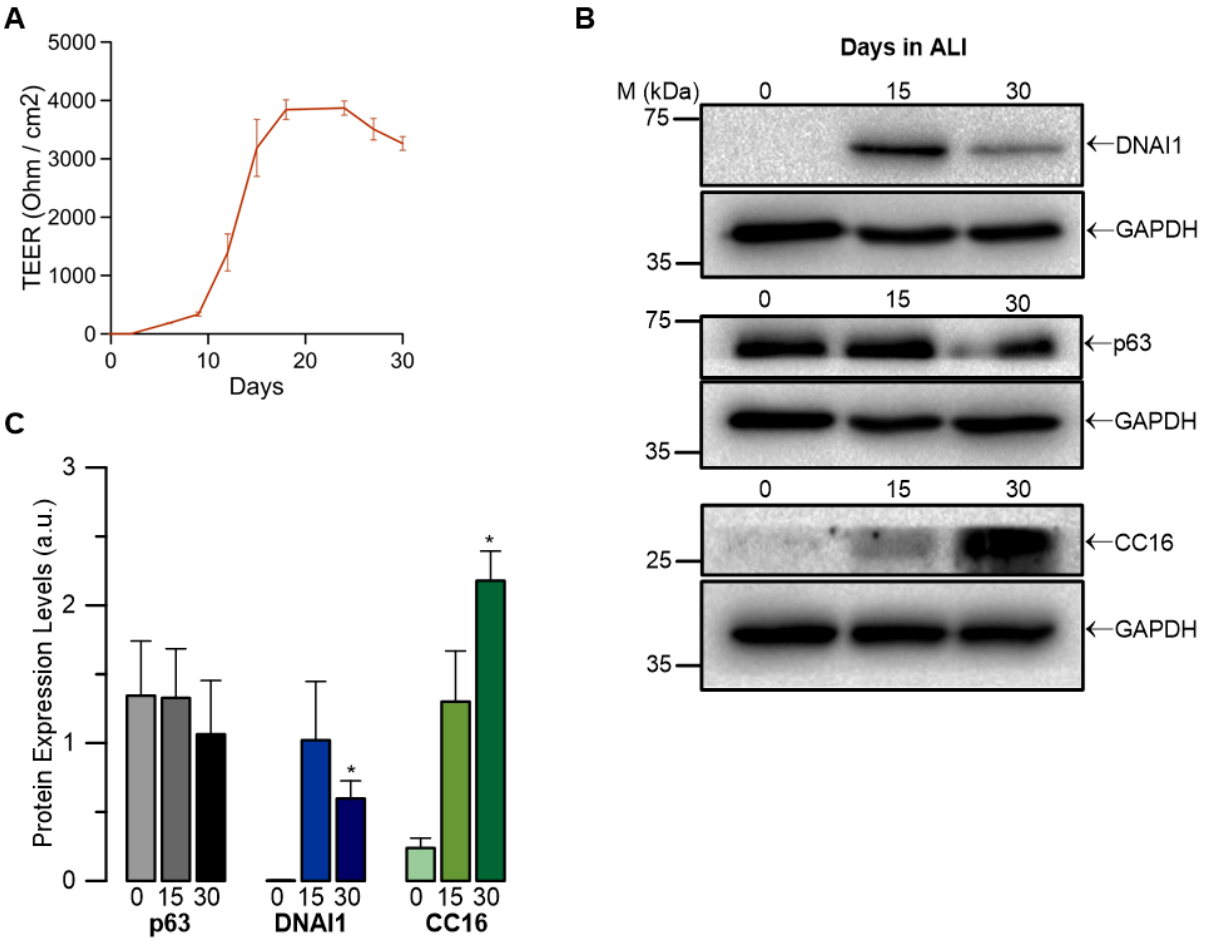

Figure S1.1 – Tight junction formation of BCi-NS1.1 cells and expression of specific cell-type markers for basal, club and ciliated cells in BCi-NS1.1 cells cultured at Air-Liquid Interface (ALI). **(A)** Time-course values of transepithelial electrical resistance (TEER) measurements of BCi-NS1.1 cells cultured at ALI for 30 days. Resistance (Ohm / cm^2) is indicated as an average ± SEM of n = 5 independent experiments from day 0 to day 30 of ALI culture. **(B)** WB showing endogenous expression of different cell-type specific markers during differentiation of BCi-NS1.1 cells – basal cell marker (p63 ~75 kDa), Club cell marker (CC16 ~25 kDa) and ciliated cell marker (DNAI1 ~75 kDa). GAPDH (~36 kDa) was used as a loading control. **(C)** Quantification by densitometry of p63, DNAI1 and CC16 WB normalised to the loading control shown as mean ± SEM (n = 3). Asterisks indicate significant difference compared to day 0 (*p-value* < 0.05, unpaired t-test). [WB data were performed by Margarida Quaresma and are reproduced here with permission].

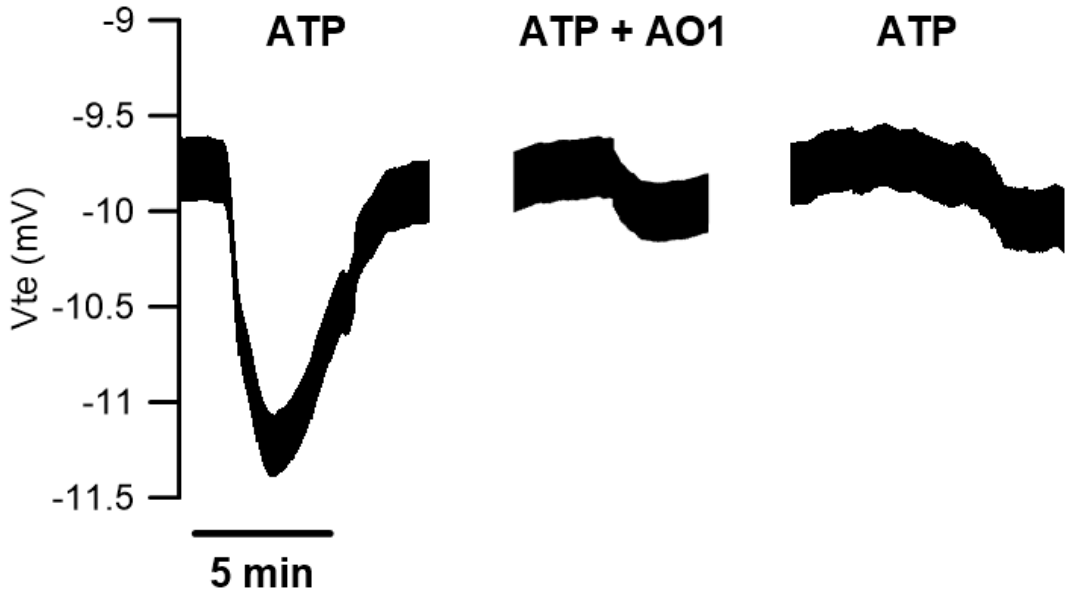

Figure S1.2 – Original Ussing chamber tracing from differentiated control cells (ALI day 30) obtained for ATP-induced Cl^- currents (100 µM) in the presence of the epithelial Na^+ channel (ENaC) inhibitor, amiloride (30 µM) +/- CaCC-AO1 TMEM16A inhibitor (30 µM). [Ussing Chamber measurements were performed with Arthur Kmit and are included here with permission].

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Chapter II – Loss of CFTR leads to Impairment of Epithelial Cell Differentiation, but with no Impact on ionocytes

Data presented in this chapter was included in the following work:

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Abstract

Cystic Fibrosis (CF) is a monogenic disease caused by mutations in the CF Transmembrane conductance Regulator (CFTR) gene that encodes an anion channel expressed at the apical membrane of airway epithelial cells. CF lungs are highly susceptible to bacterial infections, leading to chronic inflammation and progressive airway remodelling. Recent studies also describe that dysfunctional CFTR impairs the process of epithelial differentiation, even though the mechanisms behind this regulation are still unclear.

Here, we assessed whether mutant CFTR directly impacts airway epithelial cell differentiation in the absence of bacterial infection and inflammation. To this end, we introduced the most common CF-causing (F508del) mutation into the endogenous CFTR gene of human airway basal cells (BCi-NS1.1), generating one homozygous (BCi-CF1.1) and one heterozygous cell line (BCi-Hz1.1).

Our data show that normal (wt) CFTR expression increases during differentiation, being functional and apically localised mainly in both ciliated and club cells. Importantly, BCi-CF1.1 cells display reduced F508del-CFTR expression and function, as well as an epithelial polarisation and differentiation defect, particularly towards ciliated cells but with no effect on pulmonary ionocytes. Exposure to CFTR modulators partially rescued the expression and function of F508del-CFTR and ciliated-cell differentiation. Altogether, we show that dysfunctional CFTR is enough to impair epithelial differentiation, even in the absence of bacterial infection or inflammation. This novel and unique CF cell line is the first in vitro model allowing the study of CFTR-dependent differentiation along with its expression, function and localisation, thus contributing to the understanding of the CF pathogenesis.

Introduction

Cystic Fibrosis (CF) is the most common life-shortening autosomal recessive disease among Caucasians (De Boeck & Amaral, 2016). It is caused by mutations in the CF Transmembrane conductance Regulator (CFTR) gene that encodes a chloride (Cl^-) and bicarbonate (HCO_3^-) channel expressed at the apical membrane of epithelial cells (Sheppard & Welsh, 1999). The most common CF-causing mutation is a deletion of the phenylalanine residue at the position 508 (F508del), occurring in approximately 80% of CF alleles worldwide. This mutation results in a processing and trafficking defect to the cell surface, causing retention in the endoplasmic reticulum (ER) and premature proteasomal degradation (Farinha & Amaral, 2005).

While CF affects multiple organs, lung disease represents the primary cause of morbidity and mortality (O'Sullivan & Freedman, 2009), characterised by the extensive clogging of the airways with highly viscous mucus, chronic inflammation and recurrent bacterial infections (Ehre et al., 2014; O'Sullivan & Freedman, 2009). Thus, CF airways are continuously exposed to infectious and inflammatory mediators, such as proteases, bacterial virulence factors and oxidants (Nichols & Chmiel, 2015; Regamey et al., 2011) that induce airway damage and tissue remodelling. Under these conditions, epithelial cell proliferation, migration and differentiation are required for proper tissue repair and regeneration (Beers & Morrisey, 2011; Crosby & Waters, 2010). However, these processes are impaired in CF airways not only due to the chronic hyperinflammatory environment with persistent bacterial infections but also due to CFTR dysfunction (reviewed in Amaral et al., 2020). This differentiation defect further deteriorates lung function, increasing the susceptibility of the CF epithelium to bacterial colonisation and thus enhancing inflammation (O'Grady, 2017).

Although it is generally accepted that CFTR is expressed at the apical membrane of airway epithelial cells, the identity of the cells expressing this channel has been controversial (Hawkins & Kotton, 2018). Ciliated cells have for long been considered the predominant CFTR-expressing cell-type (Kreda et al., 2005; Penque et al., 2000; Puchelle et al., 1992; Scudieri et al., 2012), where not only CFTR secretes Cl^-, but also regulates other ion channels, such as the epithelial Na^+ channel (ENaC), together contributing to proper airway hydration (Sheppard & Welsh, 1999). However, recent single-cell RNA sequencing studies identified pulmonary ionocytes as a very rare cell-type (frequency < 1%) highly enriched in CFTR, expressing approximately 50% of the total CFTR transcripts in the whole epithelium (Montoro et al., 2018; Plasschaert et al., 2018). These findings challenged the paradigm of CF, and while some studies corroborate these observations (Goldfarbmuren et al., 2020; Vieira Braga et al., 2019) also in the nasal epithelium (Scudieri et al., 2020), others report a broader CFTR

expression in the epithelium, particularly in club cells and in basal cells that are transitioning into a secretory phenotype (Carraro et al., 2020; M. He et al., 2020; Okuda et al., 2020).

Hence, to improve the CF phenotype, it is essential to define where CFTR is expressed and to understand how CFTR is involved in epithelial cell differentiation and regeneration at the molecular and cellular levels (O'Grady, 2017). Previous studies on this topic mostly relied on primary cells isolated from biopsy samples or lung explanted tissue, which are however limited resources and hard to manipulate (Awatade et al., 2018). Furthermore, the expansion and differentiation of primary cells are highly limited by the number of passages, and results can dramatically vary according to donors and culture conditions (M. L. Fulcher et al., 2009). As an alternative model, researchers usually resort to cell lines that allow an infinite amount of passages but have less physiological relevance and lack differentiation capacity (Gruenert et al., 2004). Therefore, there is an unmet need for accessible and representative *in vitro* models of CF airways that not only differentiate into a mucociliated epithelium representative of all respiratory cell types but also maintain this ability for a significant number of passages.

In the present study, we used a recently established basal cell immortalised non-smoker 1.1 line (BCi-NS1.1) that was isolated from a bronchial brushing of a healthy subject and immortalised using a retrovirus-mediated expression of human telomerase reverse transcriptase (hTERT) (Walters et al., 2013). These cells retain the characteristics of the original primary basal cells, being able to differentiate into all airway epithelial cell types for more than 40 passages when cultured under Air-Liquid Interface (ALI) conditions (Simões et al., 2019; Walters et al., 2013). While this model was already well characterised in terms of differentiation and polarisation capacity (Durgan et al., 2015; Haas et al., 2019), modulation of notch signalling (Gomi et al., 2015) and response to environmental or inflammatory stimuli (Wu et al., 2020), it remains unknown whether BCi-NS1.1 cells express functional CFTR and whether this model can be used to clarify the role that CFTR plays in epithelial cell differentiation.

The main goal of this study was to determine the direct role of CFTR in epithelial cell differentiation, in the absence of airway infection and inflammation, by comparing differentiation in two isogenic BCi cell lines expressing normal (wt) or mutant (F508del) CFTR. To this end, we 1) assessed wt-CFTR expression, localisation and function in BCi-NS1.1 cells; 2) introduced the F508del mutation by CRISPR/Cas9 into BCi-NS1.1 cells to generate both F508del-homozygous (BCi-CF1.1) and F508del-heterozygous (BCi-Hz1.1) isogenic cell lines, corresponding to the CF and carrier genotype, respectively; 3) determined how mutant CFTR affects epithelial cell differentiation into pulmonary ionocytes, ciliated and club cells. Our results demonstrate that the expression of wt-CFTR increases during epithelial cell differentiation, being localised at the apical membrane and fully functional

in completely differentiated BCi-NS1.1 cells, with a higher prevalence in ciliated and club secretory cells. Gene editing with the mutation F508del in homozygosity (BCi-CF1.1 cells) results in a decrease in CFTR protein expression and function that was partially rescued by the combination of the recently approved correctors VX-661/Tezacaftor and VX-445/Elexacaftor (Hoy, 2019). Furthermore, BCi-CF1.1 cells show a compromised epithelial cell differentiation, particularly in terms of ciliated cells, but with no effect on pulmonary ionocytes. Accordingly, exposure of BCi-CF1.1 cells to VX-661 and VX-445 slightly increased ciliated-cell differentiation. Altogether, our findings demonstrate that disruption of CFTR expression and function is enough to impair epithelial cell differentiation. Moreover, the novel BCi-CF1.1 and BCi-Hz1.1 cells constitute a valuable resource to overcome the lack of physiologically relevant *in vitro* models needed for the understanding of CF pathogenesis, in particular, the molecular and cellular mechanisms regulating epithelial differentiation.

Results

1. Fully differentiated BCi-NS1.1 cells are a suitable *in vitro* model to study CFTR expression, localisation and function

Since an association between CFTR and epithelial cell differentiation was previously reported by different authors in various cellular systems (reviewed in Amaral et al. 2020), here, we first examined the CFTR expression pattern in differentiating BCi-NS1.1 cells. To this end, protein was collected every five days along a 30-day differentiation period under ALI conditions, and CFTR protein levels were determined by Western Blot (WB). In parallel, RNA was extracted at three different time points (0, 15 and 30 days), and CFTR transcript abundance was assessed by quantitative reverse-transcriptase PCR (qRT-PCR). Our results clearly show that CFTR expression increases significantly during differentiation of BCi-NS1.1 cells at both transcript and protein levels (Figure 2.1). This increase coincides with an increase in the expression of cell type-specific markers, namely ciliated (βTubulin-IV), club (CC16, Club Cell protein 16) and goblet cells (MUC5AC), confirming differentiation into various cell types (Figure S2.1).

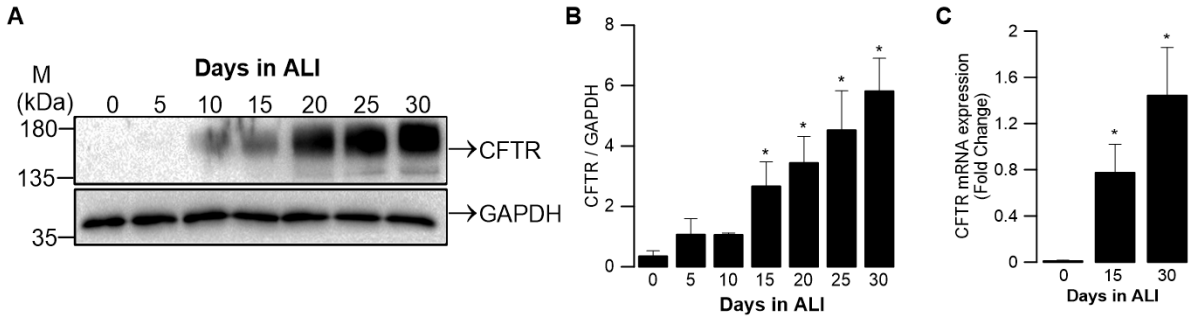

Figure 2.1 – CFTR expression levels increase during the differentiation of BCi-NS1.1 cells. (A) Western Blot (WB) showing the time-course (days 1-30) levels of CFTR endogenous protein expression (~180 kDa) during differentiation of BCi-NS1.1 cells grown at Air-Liquid Interface (ALI). GAPDH (~36 kDa) was used as a loading control. **(B)** Quantification by densitometry of total CFTR protein levels from (A) normalised to the loading control and shown as mean ± SEM (n = 5). **(C)** Fold-change in CFTR mRNA expression levels as a time-course (days 0, 15 and 30) of BCi-NS1.1 cells differentiation grown at ALI, determined by qRT-PCR. Fold-change values are mean ± SEM, relative to the mean value of day 30 (n = 4). Asterisks indicate significant difference compared to day 0 (p-value < 0.05, unpaired t-test). [RT-qPCR experiments were performed with Luka A Clarke and are included here with permission].

Furthermore, as there is no available information about the CFTR genotype in BCi-NS1.1 cells, we collected RNA from differentiated cells (ALI day 30) and amplified the CFTR cDNA sequence using the previously described 9-reaction PCR protocol (Felício et al., 2017). Sequence analysis showed no pathogenic alterations in the CFTR coding sequence, which harboured two heterozygous synonyms variants in exons 14a and 24 and one homozygous missense variant in exon 11 (V470M) (Figure S2.2).

Next, we determined the cellular localisation of CFTR in fully-differentiated BCi-NS1.1 cells (ALI day 30) by confocal microscopy and observed the expected apical distribution (Figure 2.2A, B). Moreover, CFTR function was also assessed in differentiated monolayers by Ussing chamber. This data showed a typical amiloride-sensitive Na^+ absorption followed by a CFTR-mediated Cl^- secretion resulting from the simultaneous application of IBMX and Forskolin, which was inhibited by CFTR inhibitor-172 (Figure 2.2C, D).

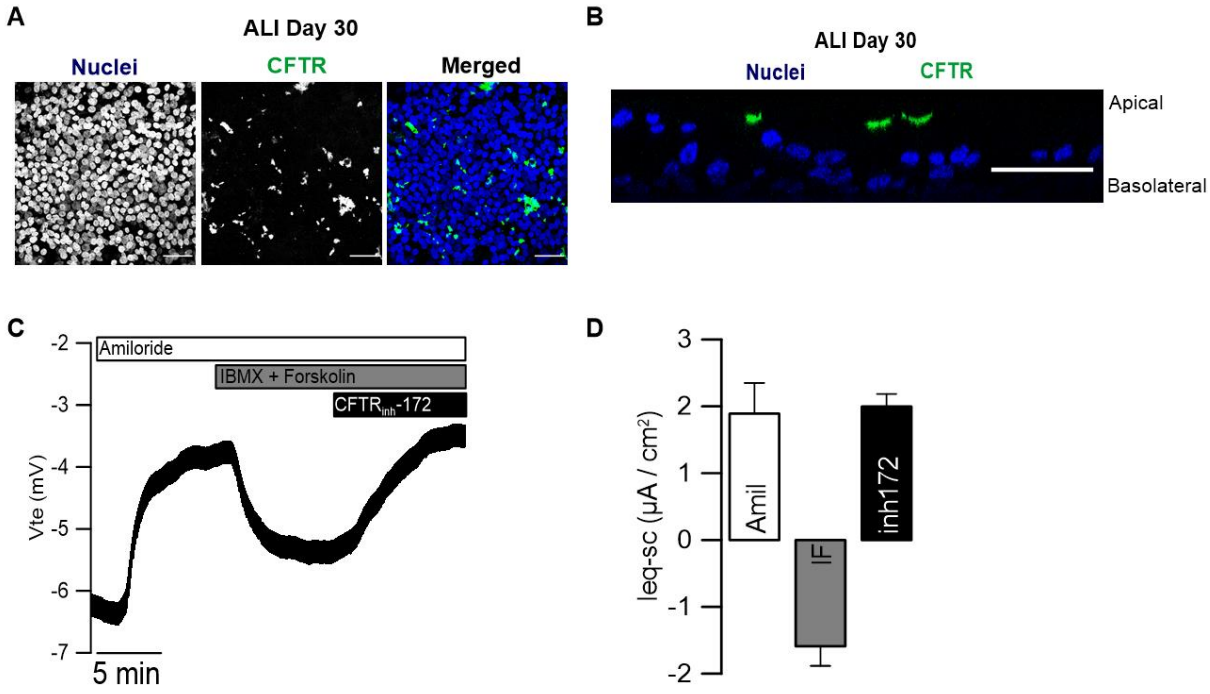

Figure 2.2 – CFTR has an apical distribution and is functional in fully-differentiated BCi-NS1.1 cells. **(A)** Confocal immunofluorescence microscopy images showing CFTR localisation in permeabilised BCi-NS1.1 cells after 30 days of ALI culture. Left: nuclei stained with Hoechst (blue); middle: endogenous CFTR, (green); right: merged images of the two fluorescent channels. Images were acquired with a Leica TCS SP8 confocal microscope (objective 63 x oil, NA 1.4). Scale bar = 40 µm. **(B)** Z-stack of a representative group of cells showing CFTR staining at the apical membrane of fully-differentiated cells (ALI day 30). Scale bar = 40 µm. **(C)** Original Ussing chamber tracing showing transepithelial voltage measurements (V_{te}) in differentiated BCi-NS1.1 cells (ALI day 30). A negative deflection is observed after stimulation with apical IBMX (100 µM) and Forskolin (2 µM) in the presence of the epithelial Na^+ channel (ENaC) inhibitor, amiloride (30 µM). This effect was reverted upon the addition of CFTR inhibitor-172 (30 µM). **(D)** Summary of the $I_{sc\text{-}eq}$ (µA / cm^2) calculated for the response to amiloride (Amil), IBMX + Forskolin (IF) and CFTR inhibitor-172 (inh172). Values are mean ± SEM (n = 4). [Ussing Chamber measurements were performed with Arthur Kmit and are included here with permission].

2. CFTR is mainly expressed in ciliated and club secretory cells in fully differentiated BCi-NS1.1 cells

Our next aim was to determine the distinctive expression of CFTR across distinct respiratory cell types, namely in ciliated, club cells and pulmonary ionocytes. As it is currently unknown whether BCi-NS1.1 cells differentiate into pulmonary ionocytes, we started by immunostaining a fully-differentiated culture (ALI day 30) with an antibody

against the pulmonary ionocytes-specific transcription factor Forkhead box protein I1 (FOXI1) (Montoro et al., 2018; Plasschaert et al., 2018). Our data (**Error! Reference source not found.**A) clearly show the nuclear expression of FOXI1 in approximately 2% of differentiated BCi-NS1.1 cells, indicating that they are indeed able to differentiate into ionocytes. Next, we co-stained CFTR with markers specific for pulmonary ionocytes, ciliated and club cells (see above), respectively (Figure 2.3B, C). By quantifying the proportion of cells expressing CFTR and each one of these markers, we observed that CFTR is mainly localised in ciliated cells, representing approximately 60% of the CFTR-positive cells. Club cells were approximately 30% of CFTR expressing cells, while pulmonary ionocytes only accounted for 5% (Figure 2.3B, C).

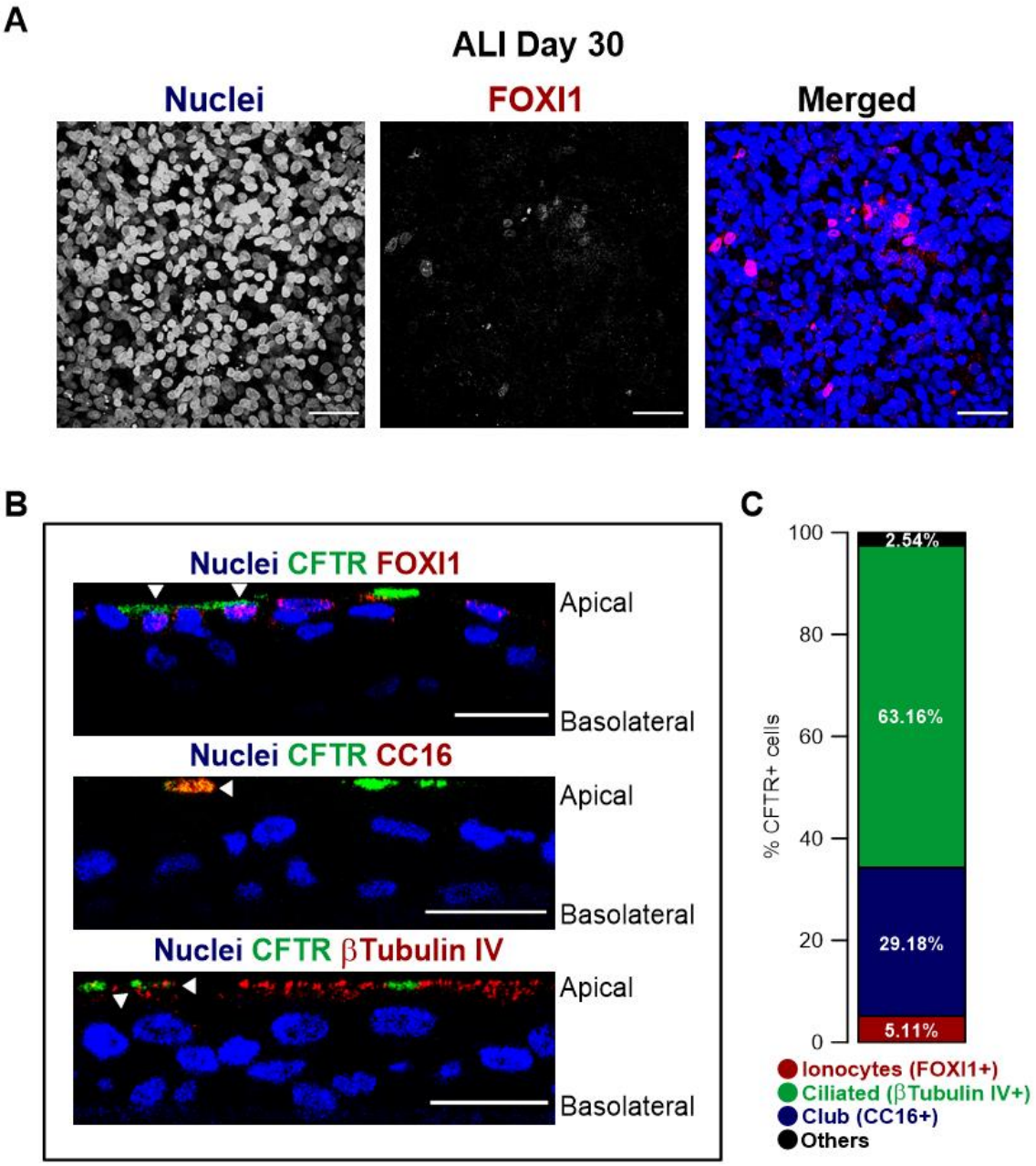

Figure 2.3 – Detection of pulmonary ionocytes in BCi-NS1.1 cells and distinctive CFTR expression across different epithelial cell types. (A) Confocal immunofluorescence microscopy images showing the pulmonary ionocyte-specific transcription factor FOXI1 in permeabilised fully-differentiated BCi-NS1.1 cells (ALI, day 30). Left: nuclei stained with Hoechst (blue); middle: endogenous FOXI1 (red); right: merged images of the two fluorescent channels. Images were acquired with a Leica TCS SP8 confocal microscope (objective 63 x oil, NA 1.4). Scale bar = 40 µm. **(B)** Representative confocal xz images in BCi-NS1.1 cells, cultured for 30 days under ALI, showing CFTR (green) and top: ionocyte marker FOXI1 (red); middle: club cell marker CC16 (red); and bottom: ciliated cell marker β Tubulin-IV (red). Nuclei are stained with Hoechst (blue) in all panels. Arrows show cells co-expressing CFTR and FOXI1/CC16/βTubulin-IV. The apical and basolateral sides are identified in all images. Images were acquired with a Leica TCS SP8 confocal microscope (objective 63x oil, NA 1.4). Scale bar = 20 µm. (n = 3). **(C)** Graph representing the percentage of cells co-expressing CFTR and: FOXI1 (Ionocytes, red), CC16 (Club cells, blue) and βTubulin-IV (Ciliated cells, green). The remaining percentage of $CFTR^{+}$ cells is represented in black (Others). Values represent the average number of cells co-expressing CFTR and the different markers quantified in 15 different images randomly acquired in three independent experiments (5 images/experiment).

3. Characterisation of novel basal cell lines with endogenous expression of F508del-CFTR

Next, we introduced the most common CF-causing mutation F508del in the endogenous CFTR gene of BCi-NS1.1 cells, either in homozygosity (BCi-CF1.1), corresponding to the CF genotype, or in heterozygosity (BCi-Hz1.1), corresponding to the carrier genotype (Figure S2.3).

To characterise the two novel cell lines in terms of CFTR expression and localisation, RNA and protein were collected after 30 days of ALI culture. RNA analysis by RT-qPCR showed no significant differences in CFTR expression at the transcript level between the BCi-Hz1.1 and BCi-CF1.1 cell lines vs BCi-NS1.1, relative to the housekeeping gene GAPDH (Figure 2.4A). Nevertheless, at the protein level, a significant reduction of CFTR expression was observed in BCi-Hz1.1 and BCi-CF1.1 cells vs BCi-NS1.1, approximately 2- and 6-fold, respectively (Figure 2.4B, C). Immunofluorescence data after 30 days of ALI culture show that CFTR localises to the apical membrane of BCi-Hz1.1 as in BCi-NS1.1 cells (Figure 2.4D, E top and middle panels). In striking contrast, the very low levels of CFTR protein present in BCi-CF1.1 cells (Figure 2.4B, C) are only detected in a few cells with a non-apical distribution (Figure 2.4D, E lower panels).

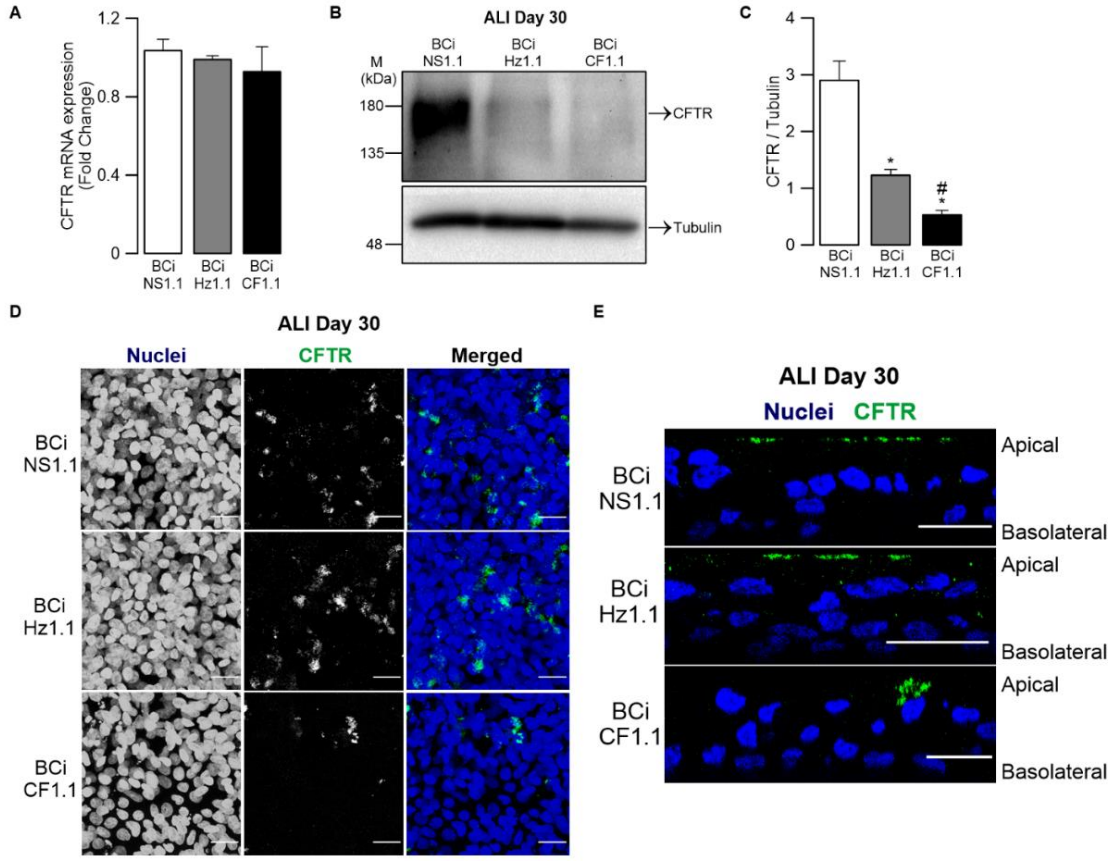

Figure 2.4 – Characterisation of novel basal cell lines with endogenous expression of F508del-CFTR. [BCi-Hz1.1 and BCi-CF1.1 cells were generated with Lúcia Santos and are used in this study with permission]. **(A)** Fold-change in CFTR mRNA expression levels in BCi-Hz1.1 and BCi-CF1.1 cells

compared to BCi-NS1.1, at ALI day 30, determined by qRT-PCR. Fold-change values are mean ± SEM, relative to the housekeeping gene GAPDH (n = 3). [RT-qPCR experiments were performed with Lúcia Santos and are included here with permission]. **(B)** Western Blot (WB) showing the endogenous expression of CFTR (~180 kDa) in BCi-Hz1.1 and BCi-CF1.1 cells in comparison to BCi-NS1.1 at ALI day 30. α-Tubulin (~50 kDa) was used as a loading control. **(C)** Quantification by densitometry of total CFTR protein levels from (B) normalised to the loading control and shown as mean ± SEM (n = 4). Asterisks and cardinals indicate significant differences vs BCi-NS1.1 and BCi-Hz1.1 cells, respectively (p-value < 0.05, unpaired t-test). **(D)** Confocal immunofluorescence microscopy images showing CFTR localisation in permeabilised BCi-Hz1.1 and BCi-CF1.1 cells in comparison to BCi-NS1.1 at ALI day 30 days. Left: Nuclei stained with Hoechst (blue); middle: endogenous CFTR (green); right: merged image of the two fluorescent channels. Scale bar = 20 µm. (n = 3) **(E)** Z-stack of a representative group of cells showing subcellular localisation of CFTR (green) in BCi-Hz1.1 and BCi-CF1.1 cells in comparison to BCi-NS1.1 at ALI day 30. Scale bar = 20 µm. Images were acquired with a Leica TCS SP8 confocal microscope (objective 63x oil, NA 1.4).

4. Expression of mutant F508del CFTR in human airway basal cells impairs epithelial differentiation and polarisation

Our main aim was to study the direct impact of the F508del mutation on epithelial cell polarisation and differentiation in the absence of infection and inflammation. Indeed, we observed a significant reduction in the transepithelial electrical resistance (TEER) of BCi-CF1.1 cells compared to either BCi-NS1.1 or BCi-Hz1.1 (Figure 2.5A) suggesting impaired polarisation and barrier function. As CFTR was previously reported to be essential for epithelial tightness by regulating tight junctions (S. Castellani et al., 2012; LeSimple et al., 2010; Quaresma et al., 2020), we next quantified the protein expression of the tight junction protein 1/zonula occludens-1 (TJP1/ZO-1) in the three basal cell lines. Analysis of the WB data showed no significant differences between BCi-Hz1.1 and BCi-CF1.1 vs BCi-NS1.1 cells, demonstrating that F508del-CFTR does not affect ZO-1 protein expression (Figure 2.5B, C). Furthermore, immunofluorescence staining for ZO-1 shows a network that is characteristic of functional tight junctions in BCi-NS1.1 and BCi-Hz1.1 cells. However, the staining pattern in BCi-CF1.1 cells shows gaps without any ZO-1 labelling in the epithelial monolayer (Figure 2.5D). Thus, calculating the Tight Junction Organisation Rate (TIJOR) (Terryn et al., 2013) revealed a significant decrease of TIJOR for BCi-CF1.1 cells, whereas BCi-Hz1.1 cells do not show a significant difference in this parameter compared to BCi-NS1.1 (Figure 2.5E).

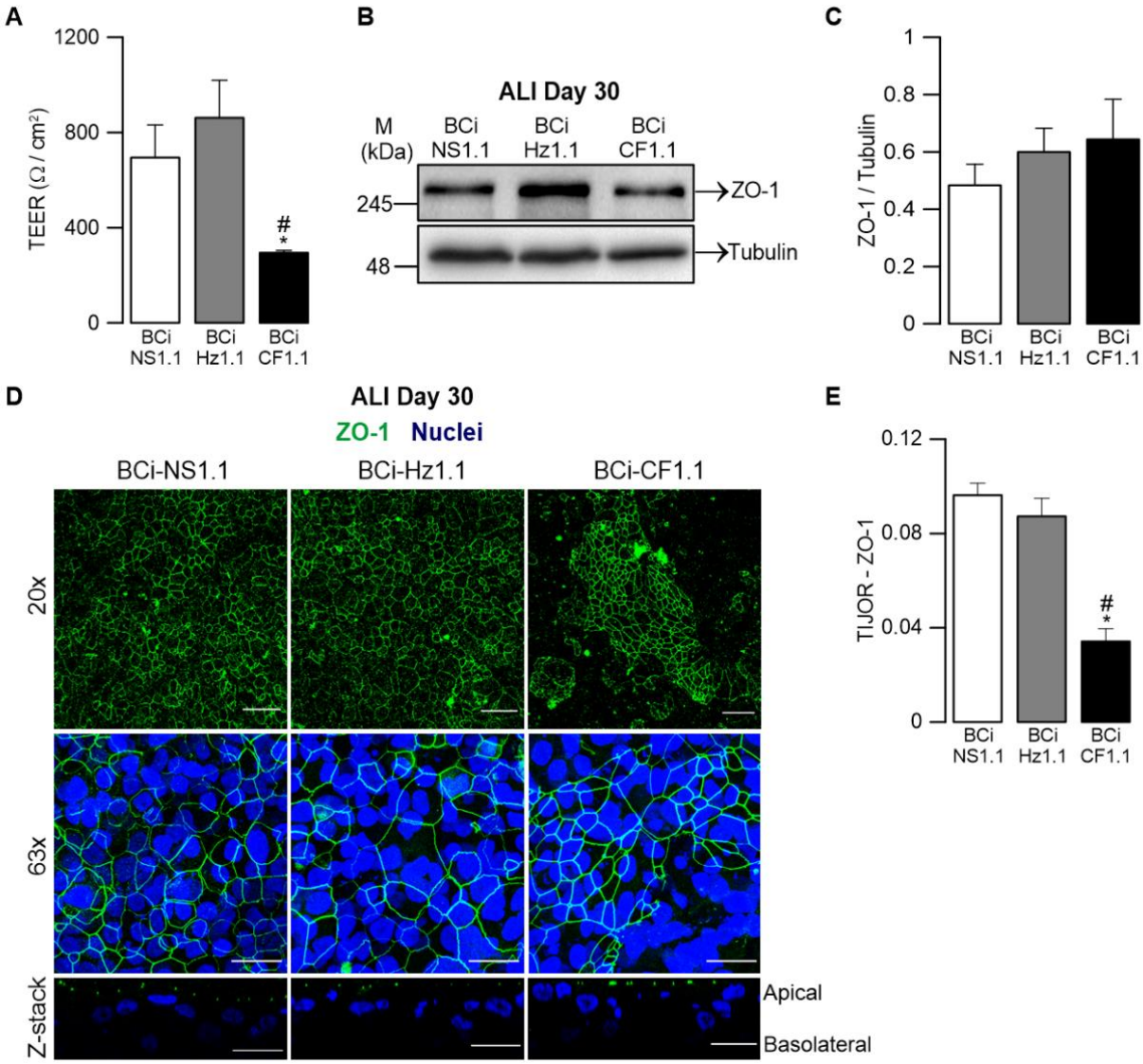

Figure 2.5 – BCi-CF1.1 cells show impaired polarisation. (A) Transepithelial Electrical Resistance (TEER) of BCi-NS1.1, BCi-Hz1.1 and BCi-CF1.1 cells measured in the Ussing chamber before carrying out electrophysiological measurements. Asterisks and cardinals indicate significant differences vs BCi-NS1.1 and BCi-Hz1.1, respectively (p-value < 0.05, unpaired t-test). **(B)** Western Blot (WB) comparing the endogenous expression of the tight junctions marker ZO-1 (~220 kDa). α-Tubulin (~50 kDa) was used as the loading control. **(C)** Quantification of ZO-1 expression levels by densitometry normalised to the loading control and shown as mean ± SEM (n = 4). **(D)** Confocal immunofluorescence microscopy images showing ZO-1 localisation in permeabilised BCi-NS1.1, BCi-Hz1.1 and BCi-CF1.1 cells at ALI day 30. Tight junctions were stained with the ZO-1 antibody (green) and nuclei with Hoechst (blue). Top: images acquired with the 20x objective (scale bar = 40 µm); middle: images acquired with the 63x objective (scale bar = 40 µm); bottom: Z-stack of a representative group of cells showing ZO-1 localisation in tight junctions of BCi-NS1.1, BCi-Hz1.1 and BCi-CF1.1 cells. Scale bar = 20 µm. Images were acquired with a Leica TCS SP8 confocal microscope. **(E)** Tight junction organisation rate (TIJOR) of ZO-1 in BCi-NS1.1, BCi-Hz1.1 and BCi-CF1.1 cells, calculated from 5 different 20x images (D, top panels) for each cell line. [TiJOR measurements were performed by Ines Pankonien and are included here with permission].

Since CFTR is expressed in ciliated cells, club cells and pulmonary ionocytes (Figure 2.3B, C), we then asked whether the F508del mutation affects epithelial cell differentiation into

these specific cell-types. To this end, we compared the protein expression of the ciliated-cell marker DNAI1, the club-cell marker CC16 and the pulmonary ionocyte-marker FOXI1 in the three cell lines at ALI day 30. The results show that DNAI1 expression is almost absent in BCi-CF1.1 cells (Figure 2.6A, B), suggesting that F508del-CFTR impairs ciliated-cell differentiation. This effect is specific to the homozygous cells, as BCi-Hz1.1 cells did not show a significant decrease in DNAI1 expression (Figure 2.6A, B). Similarly, immunostaining with an antibody against βTubulin-IV (another ciliated-cell marker) showed that, indeed, BCi-CF1.1 cells have fewer ciliated cells compared to the other genotypes (Figure 2.6D). Given this robust difference, we asked whether BCi-CF1.1 cells have a delayed ciliated-cell differentiation. To this end, we extended the ALI culture period to 40 and 50 days and assessed DNAI1 protein expression in BCi-NS1.1, BCi-Hz1.1 and BCi-CF1.1 cells. Our results show that, while DNAI1 expression increased over time in BCi-NS1.1 and BCi-Hz1.1 cells (only until day 50), no differences were observed in BCi-CF1.1 cells (Figure 2.6E, F), further demonstrating that wt-CFTR is required for proper ciliated-cell differentiation.

Moreover, we also assessed differentiation into pulmonary ionocytes and club cells. Interestingly, no significant differences were observed in FOXI1 expression (Figure 2.6A, B), suggesting that differentiation towards pulmonary ionocytes is not CFTR-dependent. On the other hand, the expression of CC16 significantly decreased in both cell lines (Figure 2.6A, B), although the immunofluorescence differences were not as striking as in ciliated cells (Figure 2.6C, D). Nevertheless, BCi-CF1.1 cells appear to have a defect in CC16 secretion vs BCi-NS1.1 and BCi-Hz1.1 cells (Figure 2.6C). Overall, our data show that BCi-CF1.1 cells have impaired epithelial cell differentiation, namely towards ciliated cells.

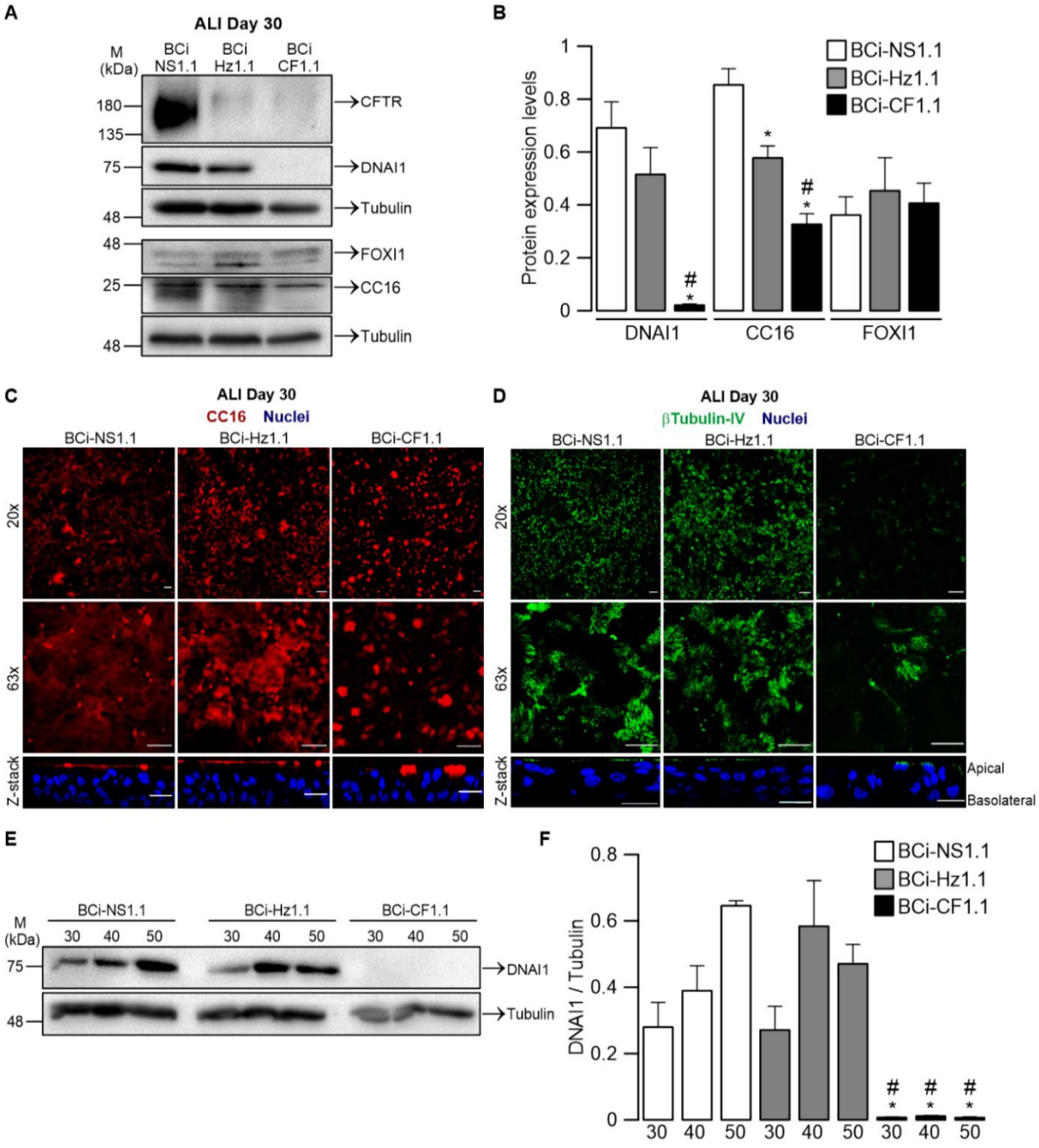

Figure 2.6 – BCi-CF1.1 cells display impaired ciliated-cell differentiation. (A) Western Blot (WB) showing the endogenous expression of the ciliated cell marker DNAI1 (~75 kDa), club cell marker CC16 (~25 kDa), and pulmonary ionocyte marker FOXI1 (~40 kDa) in BCi-NS1.1, BCi-Hz1.1 and BCi-CF1.1 cells following 30 days of ALI culture. α-Tubulin (~50 kDa) was used as the loading control. **(B)** Quantification by densitometry of the cell type-specific markers expression levels normalised to the loading control shown as mean ± SEM (n = 4). Asterisks and cardinals indicate significant differences compared to BCi-NS1.1 and BCi-Hz1.1, respectively (p-value < 0.05, unpaired t-test). **(C)** Confocal immunofluorescence microscopy images showing club cells stained with the CC16 antibody (red) in permeabilised BCi-NS1.1, BCi-Hz1.1 and BCi-CF1.1 cells at ALI day 30. Top: images acquired with the 20x objective (scale bar = 40 µm); middle: images acquired with the 63x objective (scale bar = 40 µm); bottom: Z-stack of a representative group of cells showing CC16 subcellular localisation in BCi-NS1.1, BCi-Hz1.1 cells and BCi-CF1.1 cells (scale bar = 20 µm). **(D)** Confocal immunofluorescence microscopy images showing ciliated cells stained with the βTubulin-IV antibody (green) in permeabilised BCi-NS1.1, BCi-Hz1.1 and BCi-CF1.1 cells, at ALI day 30. Top: images acquired with the 20x objective (scale bar = 40 µm); middle: images acquired with the 63x objective (scale bar = 40 µm); bottom: Z-stack of a

representative group of cells showing βTubulin-IV subcellular localisation in BCi-NS1.1, BCi-Hz1.1 cells and BCi-CF1.1 cells (scale bar = 20 µm. Images were acquired with a Leica TCS SP8 confocal microscope. **(E)** WB showing endogenous expression of the ciliated-cell marker DNAI1 (~75 kD) in BCi-NS1.1, BCi-Hz1.1 and BCi-CF1.1 cells cultured for 30, 40 and 50 days under ALI conditions. α-Tubulin (~50 kDa) was used as a loading control. **(F)** Quantification by densitometry of DNAI1 expression levels normalised to the loading control shown as mean ± SEM (n = 3). Asterisks and cardinals indicate significant differences vs the corresponding time point in BCi-NS1.1 and BCi-Hz1.1, respectively (p-value < 0.05, unpaired t-test).

5. Partial rescue of F508del-CFTR and ciliated-cell differentiation in BCi-CF1.1 cells by VX-661 and VX-445

Next, we evaluated whether F508del-CFTR introduced in BCi-NS1.1 cells is rescued by the combination of the two corrector drugs approved for CF individuals carrying this mutation, VX-661/Tezacaftor and VX-445/Elexacaftor (Hoy, 2019). To this end, cells were cultured for 28 days under ALI and treated for the next 48 h either with both correctors or the vehicle (DMSO).

WB analysis shows that incubation with VX-661 and VX-445 led to the upregulation of CFTR protein expression in both BCi-CF1.1 and BCi-Hz1.1 cells, albeit in the latter not significantly (Figure 2.7A, B). Ussing chamber measurements showed that the application of IBMX and Forskolin resulted in a typical lumen-negative response for both BCi-NS1.1 and BCi-Hz1.1 cells, which was absent in BCi-CF1.1 cells (Figure 2.7C, D). Notwithstanding, a significant increase in this response was observed when BCi-CF1.1 cells were treated with the CFTR correctors (Figure 2.7C, D). Interestingly, although not statistically significant, BCi-Hz1.1 cells also showed a reduced response to IBMX and Forskolin compared to BCi-NS1.1 cells (Figure 2.7C, D), which is likely due to the lower CFTR expression levels (Figure 2.4B, C). Accordingly, exposure to the CFTR correctors slightly increased this response (Figure 2.7D, E) and protein expression (Figure 2.7A, B).

We next asked if correction of F508del-CFTR would rescue the differentiation defect observed in BCi-CF1.1 cells (Figure 2.6). Indeed, with a higher exposure time, we observed a significant increase in the DNAI1 protein expression levels of BCi-CF1.1 cells treated with the correctors VX-661 and VX-445 for 48h (Figure 2.7E, F). Additionally, although not statistically significant, the CC16 protein expression levels of BCi-Hz1.1 and BCi-CF1.1 cells also increased with the correctors (Figure 2.7E, G). Altogether, these data show that F508del-CFTR expressed in BCi-CF1.1 cells is rescued at the protein and functional level by the FDA-approved correctors VX-661 and VX-445, which also slightly increase the expression of the ciliated-cell marker DNAI1.

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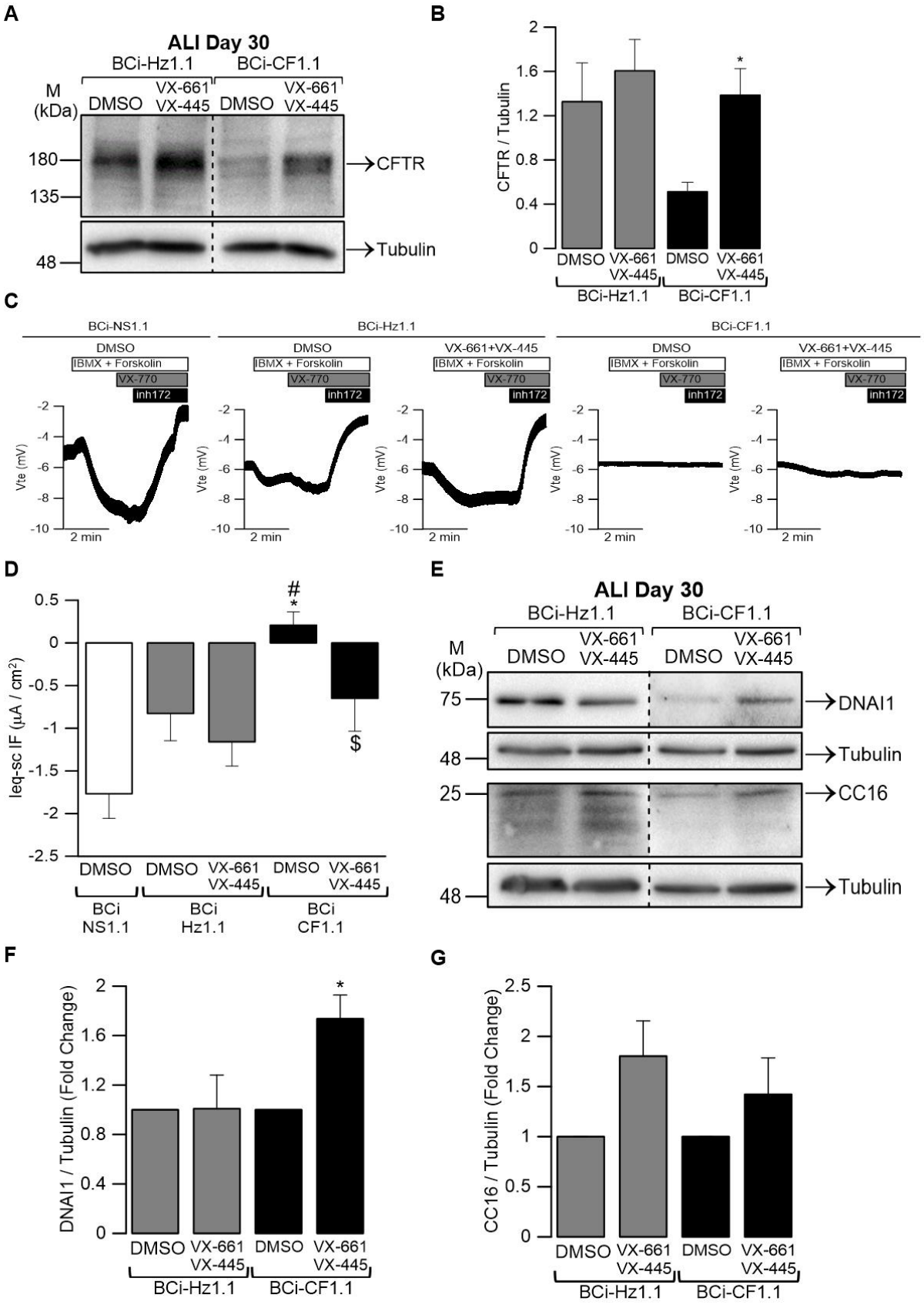

Figure 2.7 – VX-611 and VX-445 partially rescue CFTR expression and function, along with ciliated-cell differentiation in BCi-CF1.1 cells. (A) Western Blot (WB) showing the effect of VX-661 plus VX-445 on the endogenous expression of CFTR (~180 kDa) in BCi-Hz1.1 and BCi-CF1.1 cells. α-

Tubulin (~50 kDa) was used as a loading control. The dashed line indicates lanes run on the same gel but noncontiguous. Cells were treated either with the two correctors (3 µM each) or with the vehicle DMSO for 48h. **(B)** Quantification by densitometry of total CFTR protein levels from (A) normalised to the loading control shown as mean ± SEM (n = 3). The asterisk indicates a significant difference compared to BCi-CF1.1 cells treated with DMSO (p-value < 0.05, unpaired t-test). **(C)** Original Ussing chamber tracings showing transepithelial voltage measurements (V_{te}) for BCi-NS1.1, BCi-Hz1.1 and BCi-CF1.1 cells (at ALI day 30). A negative deflection is observed after apical stimulation with IBMX (100 µM) plus Forskolin (2 µM) and VX-770 (50 µM), in the presence of the epithelial Na^+ channel (ENaC) inhibitor, amiloride (30 µM). This effect was more pronounced when cells were pre-treated with VX-661 (3 µM) plus VX-445 (3 µM) for 48h and was reverted by the application of the CFTR inhibitor 172 (50 µM). [Ussing Chamber measurements were performed with Arthur Kmit and are included here with permission]. **(D)** Summary of the $I_{sc\text{-}eq}$ ($\mu A/cm^2$) calculated from the voltage deflection obtained for the responses to IBMX + Forskolin (IF). Values are mean ± SEM (n = 3 - 5). Asterisk, cardinal and dollar symbols indicate significant differences compared to the same cell line treated with DMSO, respectively (p-value < 0.05, unpaired t-test). **(E)** WB showing the effect of VX-661 plus VX-445 on the expression of the ciliated cell marker DNAI1 (~75 kDa) and the club cell marker CC16 (~25 kDa) in BCi-Hz1.1 and BCi-CF1.1 cells following 30 days of ALI culture. α-Tubulin (~50 kDa) was used as loading control. Cells were treated either with the two correctors (3 µM each) or with the vehicle DMSO for 48h. The dashed line indicates lanes run on the same gel but noncontiguous. The DNAI1 blot corresponding to BCi-CF1.1 cells was exposed for a longer time to detect faint bands. **(F)** Quantification by densitometry of the DNAI1 expression levels normalised to the loading control and the respective DMSO-treated cells shown as mean ± SEM (n = 3). Asterisk indicates significant differences compared to BCi-CF1.1 cells treated with DMSO. **(G)** Quantification by densitometry of the CC16 expression levels normalised to the loading control and the respective DMSO-treated cells shown as mean ± SEM (n = 3).

Discussion

To fully understand CF pathogenesis, it is essential to have physiologically relevant models that are easily accessible and mimic the airway epithelium by differentiating into the various airway epithelial cell types. Of note, Walter and colleagues produced a human airway basal cell line with multipotent differentiation capacity (BCi-NS1.1) that preserves the characteristics of the original primary cells and, importantly, with a much longer lifespan than primary cultures (Walters et al., 2013). BCi-NS1.1 cells thus emerge as a promising model to understand airway physiology and, in particular, the contribution of CFTR to epithelial differentiation. Ultimately, a deeper knowledge of the CFTR role in this process will help designing new therapeutic strategies aimed at improving epithelial regeneration, whose impairment is a major hallmark in CF, and thus prevent progression of the CF pathogenesis cascade (Amaral et al. 2020; Amaral and Kunzelmann 2007).

The main goal of this study was to determine the direct role of CFTR on epithelial cell differentiation in the absence of airway infection and inflammation. Beforehand, however, we had to characterise BCi-NS1.1 cells in terms of CFTR expression, localisation and function. Our results describe for the first time BCi-NS1.1 cells as a suitable *in vitro* model to study CFTR through different lines of evidence. Firstly, CFTR mRNA and protein expression increase during epithelial cell differentiation, being almost undetectable in basal cells and highly expressed in fully-differentiated cells (Figure 2.1). These findings are in agreement with previous studies in intestinal and nasal epithelial cells, where CFTR mRNA levels were shown to increase with cell differentiation and polarisation (Buchwald et al., 1991; Jun et al., 2001; Chahrzad Montrose-Rafizadeh et al., 1991; Sood et al., 1992). Secondly, CFTR localises at the apical membrane of differentiated BCi-NS1.1 cells (Figure 2.2A, B), recapitulating the staining pattern observed in primary human bronchial epithelial cells (Gorrieri et al., 2016; Scudieri et al., 2012). Ultimately, electrophysiological experiments show that CFTR is functional in differentiated BCi-NS1.1 cells, secreting Cl^- ions to the luminal space (Figure 2.2C, D). Altogether, these findings demonstrate that BCi-NS1.1 cells are a good model recapitulating the human airway epithelium in terms of CFTR mRNA/protein expression, cellular localisation and function.

Currently, there is growing controversy about CFTR distribution across the different cell types of the human airway epithelium (Hawkins & Kotton, 2018; Y. Tang et al., 2020). Ultimately, however, to better understand the CF pathophysiology, it is essential to define in which cell types CFTR is expressed in the airway epithelium so as to determine which cells should be targeted to rescue CFTR and fully restore epithelial functions. Here, we observed that upon full differentiation of BCi-NS1.1 cells into various respiratory cell types, CFTR is primarily expressed in ciliated (60%) and club cells (30%) and about 5% of CFTR-expressing

cells are ionocytes (Figure 2.3B, C). Nevertheless, these data do not invalidate that pulmonary ionocytes express high CFTR levels, and subsequent studies in BCi-NS1.1 cells should quantify CFTR expression levels in each cell-type. Moreover, it remains unknown if rescue of CFTR specifically in pulmonary ionocytes has an impact on CF phenotype improvement. Interestingly, a recent study demonstrated that CFTR function is maintained in primary human tracheal cells when the expression of FOXI1 – the transcription factor specific to pulmonary ionocytes (Montoro et al., 2018; Plasschaert et al., 2018) – is abolished (Goldfarbmuren et al., 2020). On the other hand, CFTR delivery to ciliated cells derived from CF individuals was shown to restore almost completely CFTR function, airway hydration and mucus transport (Zhang et al., 2009). Overall, it is very likely that CFTR has different functions according to the cell-type where it is expressed. For example, CFTR might support airway hydration in ciliated cells by secreting Cl^- ions into the luminal space (Jiang & Engelhardt, 1998), whereas in club cells, it might be essential to regulate the volume of secreted fluid and respective pH through bicarbonate secretion, particularly under inflammatory conditions (Rehman et al., 2020).

To pursue our main aim of determining the role of CFTR in epithelial differentiation, and given that BCi-NS1.1 cells differentiate into the various respiratory epithelial cell types expressing functional CFTR, we then generated a homozygous (BCi-CF1.1) and a heterozygous (BCi-Hz1.1) cell line of the most common CF-causing mutation F508del, corresponding to the CF and the carrier genotype, respectively (Figure S2.2). Analysis of CFTR mRNA expression demonstrated no significant differences among the three cell lines (Figure 2.4A), following what is described for CF individuals with the F508del/F508del genotype (Beck et al., 1999; Trapnell et al., 1991). On the other hand, BCi-CF1.1 cells showed a clear reduction in CFTR protein expression, a non-apical irregular sub-cellular distribution and significantly decreased function (Figure 2.4B-E, Figure **2.7**C-D). This CF cell model thus clearly recapitulates the well-described phenotype of CF primary cells (Awatade et al., 2015; Kreda et al., 2005), resulting from a processing and trafficking defect of CFTR to the cell surface, which is retained in the ER and prematurely degraded in the proteasome (Farinha & Amaral, 2005). Importantly, treatment with the combined correctors VX-661 and VX-445 partially rescued CFTR expression and function, indicating that BCi-CF1.1 cells respond to the drugs available in the clinic (Figure 2.7A-D). Moreover, BCi-Hz1.1 cells also showed decreased CFTR protein expression and function (Figure 2.4B-C, Figure **2.7**C-D), albeit to a lesser extent than that observed in BCi-CF1.1 cells, highlighting the contribution of each allele to these processes and mimicking what is described for CF carriers (Penque et al., 2000; Pranke et al., 2017). Overall, these results validate the BCi-Hz1.1 and BCi-CF1.1 cells as *bona fide* models that recapitulate the carrier and CF phenotypes in terms of CFTR expression, subcellular localisation, function and response to modulators. As novel basal cell

models that differentiate into several airway cell types, they also emerge as physiologically relevant to assess how mutant CFTR directly impacts airway differentiation in the absence of the CF infection and inflammation. Our results clearly show that BCi-CF1.1 cells evidence impaired epithelial polarisation as shown by their decreased TEER and irregular distribution of the tight junction protein ZO-1, which resulted in decreased tight junction organisation rate (Figure 2.5). Importantly, this phenotype was not observed in BCi-Hz1.1 cells (Figure 2.5). Indeed, previous studies report a similar ZO-1 staining pattern in native CF lung tissue (Quaresma et al., 2020) and describe an association between CFTR and ZO-1, with the presence of CFTR at the apical plasma membrane being required for proper cell polarisation (Ruan et al., 2014).

Furthermore, we also explored how mutant CFTR impacts airway epithelial differentiation into ciliated cells, club cells and pulmonary ionocytes, the three cell-types which we found positive for CFTR expression in fully-differentiated BCi-NS1.1 cells (Figure 2.3B, C). Interestingly, while BCi-CF1.1 cells presented a significant reduction in the expression levels of markers specific for ciliated and club cells, no effect was observed in the fraction of FOXI1 expression in pulmonary ionocytes (Figure 2.6A, B). These data are supported by another study where the number of pulmonary ionocytes in nasal epithelial cells was not significantly different between CF individuals and healthy controls (Scudieri et al., 2020).

In this study, we assessed club-cell differentiation by measuring the CC16 protein levels and determining its localisation by immunofluorescence. CC16 is abundantly secreted from club cells to the epithelial lining fluid, where it exerts anti-inflammatory effects by modulating the activity and production of phospholipase A2, interferon-γ and TNF-α (Dierynck et al., 1996). Previous studies describe a reduced concentration of CC16 in the bronchoalveolar lavage fluid of individuals with CF, particularly upon chronic inflammation and pulmonary exacerbations (Laguna et al., 2015; Starosta et al., 2006). Here, we demonstrate that BCi-CF1.1 cells have a defect in the production and secretion of CC16 (Figure 2.6A-C) in the absence of infection and inflammation, suggesting that it is a direct consequence of CFTR dysfunction. Indeed, exposure to the CFTR correctors slightly increased the CC16 protein expression levels (Figure 2.7E, G), even though this effect was not statistically significant, which may be explained by an insufficient rescue of CFTR expression at the plasma membrane or the need for functional CFTR to fully rescue this phenotype. Overall, our data suggest that CF airway epithelial cells have reduced anti-inflammatory functions, which will compromise the immune responses when exposed to infectious agents.

Besides reduced expression of ciliated cell markers, our results also demonstrate that ciliated cells are the main compromised cell-type in BCi-CF1.1 cells in numbers (Figure 2.6A, B, D). Consistently, previous studies demonstrated that CF nasal epithelial cell cultures have fewer ciliated cells vs non-CF cultures (Adam et al., 2015). These results are likely explained

by the downregulation of genes involved in ciliogenesis described occurring in CF cells (Clarke et al., 2013), including the ciliated-cell marker DNAI1 used in this study (Figure 2.6A, Figure S2.1). Interestingly, our results also suggest that even low CFTR expression is enough to drive proper ciliated-cell differentiation, as the differentiation defect was not observed in BCi-Hz1.1 cells (Figure 2.6A, B, D), despite the significant reduction in CFTR protein (Figure 2.4B, C). Moreover, while we could not observe the recovery of the differentiation defect of CF cells even at longer culture times (Figure 2.6E, F), a significant increase in the DNAI1 protein expression levels was detected in BCi-CF1.1 cells treated with VX-661 and VX-445 (Figure 2.7E, F), suggesting that rescue of F508del-CFTR to the plasma membrane partially restores this phenotype. Altogether, these results indicate that CF airways have a defect in ciliated-cell differentiation that is intrinsic to CFTR dysfunction, which, together with airway dehydration and mucus hypersecretion, will further compromise mucociliary clearance, worsening recovery from infection and inflammation.

In conclusion, here we developed novel basal CF and carrier cell lines complementing the previously reported BCi-NS1.1 model (Walters et al., 2013) as useful tools to assess the direct impact of dysfunctional CFTR on epithelial differentiation and on other aspects of human airway physiology that will thus enable a better understanding of CF pathogenesis. Importantly, the BCi-NS1.1, BCi-Hz1.1 and BCi-CF1.1 cells have endogenous CFTR expression controlled by the native CFTR promoter, contrasting other commonly used airway epithelial cell lines that overexpress CFTR variants from cDNA (Gruenert et al., 2004). Furthermore, as they maintain their multipotent differentiation capacity for over 40 passages, they will be particularly relevant to identify cellular and molecular targets involved in epithelial cell differentiation and regeneration, innate immunity of airway epithelial cells and response to therapeutic candidates.

Supplementary Data

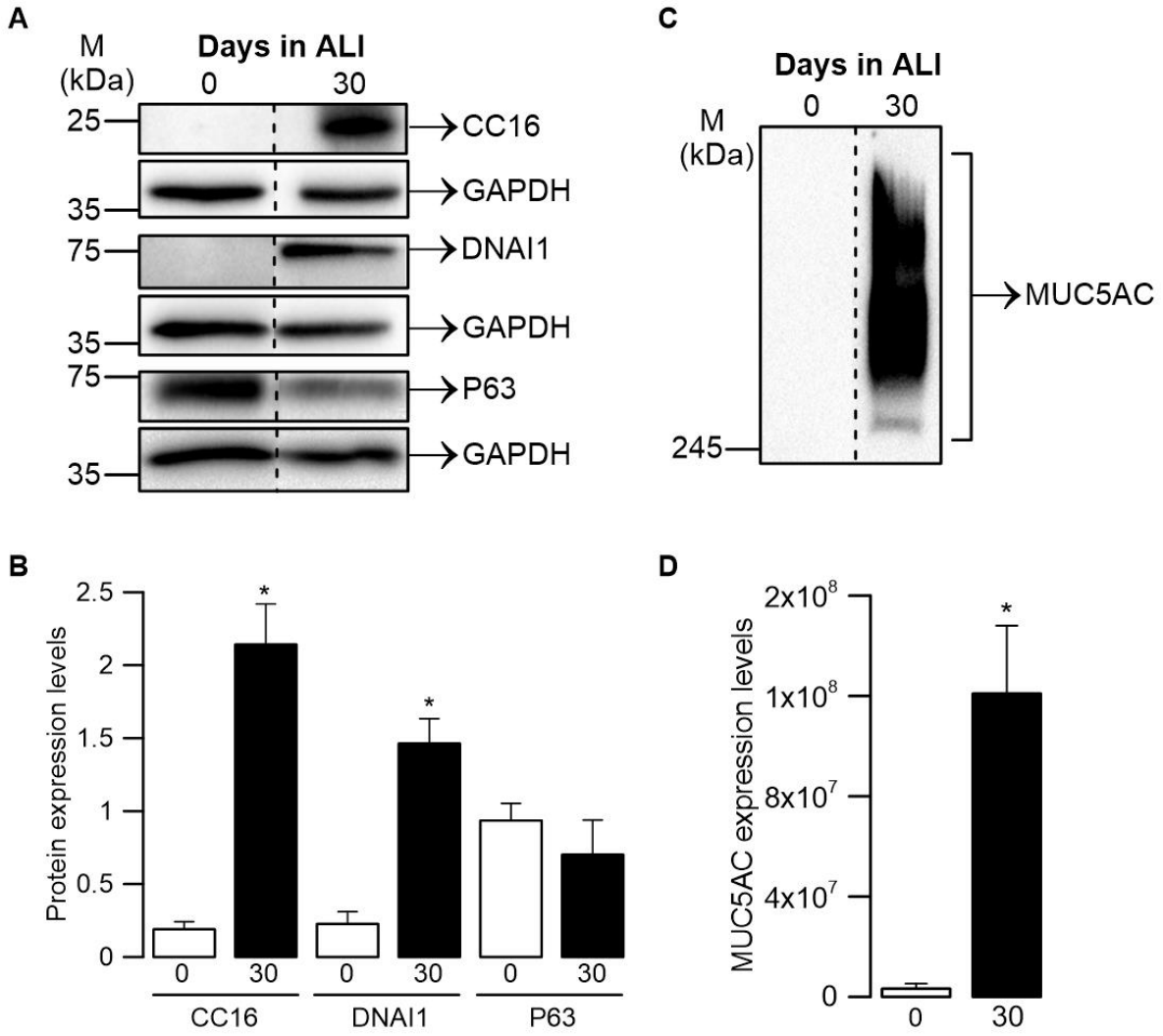

Figure S2.1 – Expression of cell-type-specific markers for ciliated, club, goblet and basal cells in undifferentiated and differentiated BCi-NS1.1 cells. (A) Western Blot (WB) showing an increase in endogenous expression of specific markers for differentiated cell types during differentiation of BCi-NS1.1 cells (days 0 and 30), namely club cell marker CC16 (~25 kD) and ciliated cell marker DNAI1 (~75 kD) in parallel with a decrease, albeit not significant, in basal cell marker P63 (~75 kD). GAPDH (~36 kD) was used as a loading control. **(B)** Quantification by densitometry of CC16, DNAI1 and P63 WB normalised to the loading control shown as mean ± SEM (n = 4). **(C)** Mucin agarose electrophoresis also showing an increase in the expression of the goblet cell marker MUC5AC (>300 kDa) during differentiation of BCi-NS1.1 cells (days 0 and 30). The dashed line indicates lanes run on the same gel but noncontiguous. **(D)** Quantification by densitometry of MUC5AC expression shown as mean ± SEM (n = 4). Asterisks indicate significant difference compared with day 0 (p-value < 0.05, unpaired t-test).

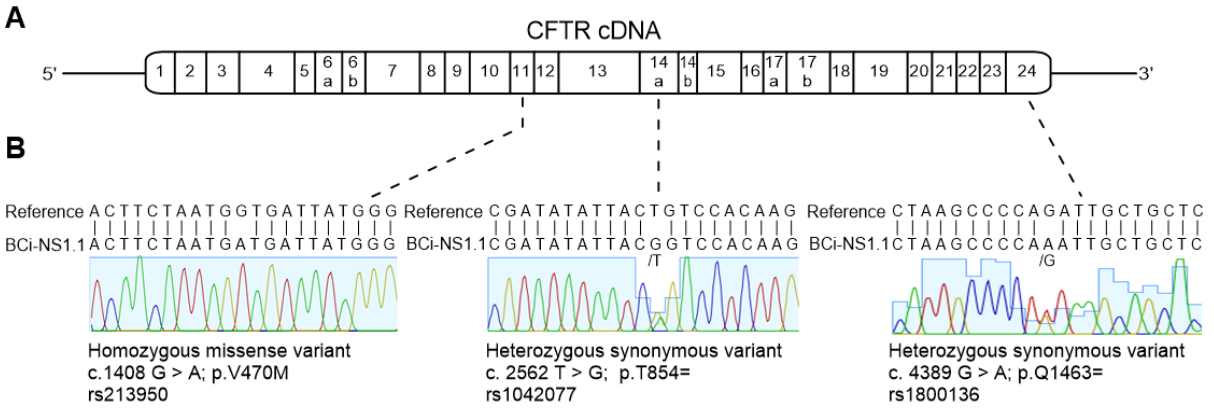

Figure S2.2 – Genotyping of BCi-NS1.1 cells. (A) Schematic representation of the CFTR cDNA. **(B)** Electropherograms displaying the non-CF causing variants identified in the CFTR transcripts of differentiated BCi-NS1.1 cells by Sanger sequencing, with the respective DNA sequence above each electropherogram: 1) a homozygous missense variant (rs213950) in exon 11, corresponding to a substitution of a G to an A at position 1408 (c. 1408 G>A) of the CFTR cDNA sequence, resulting in an alteration of valine to methionine at position 470 of the CFTR amino acid sequence (p. V470M); 2) one heterozygous synonymous variant in exon 14a (rs1042077) corresponding to a T to G substitution at cDNA position 2562 (c. 2562 T>G); and 3) another heterozygous synonymous variant in exon 24 (rs1800136), corresponding to a G to A substitution at cDNA position 4389 (c. 4389 G>A). [Data is from Margarida Quaresma and is included here with permission].

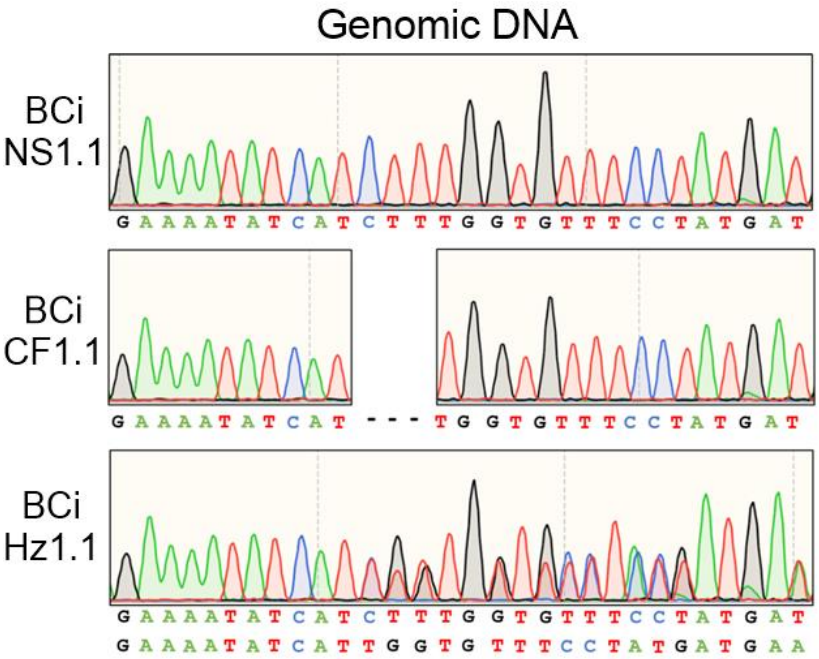

Figure S2.3 – CRISPR/Cas9 gene-editing of CFTR alleles. Sanger sequencing electropherograms of the endogenous CFTR gene at the genomic locus of the F508del mutation from BCi-NS1.1 cells (top panel), cells homozygous for the F508del-CFTR mutation (BCi-CF1.1, middle panel) and cells heterozygous for the F508del-CFTR mutation (BCi-Hz1.1, bottom panel). Three black dashes represent the CTT nucleotide deletion corresponding to F508del. [Data is from Lúcia Santos and is included here with permission].

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Chapter III – Cross-Talk between Inflammatory Mediators and Airway Epithelial Cells Reveals CFTR as a Major Target Modulated by Inflammation

Data presented in this chapter was included in the following work:

Simões, F. B., Kmit, A., & Amaral, M. D. (2021) "Cross-Talk of Inflammatory Mediators and Airway Epithelium Reveals CFTR as a Major Target" ERJ Open Res – accepted for publication

Abstract

Airway inflammation, mucus hyperproduction and epithelial remodelling are hallmarks of many chronic airway diseases, including asthma, chronic obstructive pulmonary disease and Cystic Fibrosis. Thus, to fully understand the pathogenesis of these diseases, it is essential to address how inflammation affects airway epithelium physiology. While several cytokines are dysregulated in chronic airway diseases, most studies focus on the response of airways to Th2 cytokines IL-4 and IL-13, which were shown to induce mucus hyperproduction and shift the airway epithelium towards a hypersecretory phenotype.

Here, we hypothesised that other cytokines might play similar roles in inducing the expression of chloride (Cl^-) channels/transporters, regulating epithelial differentiation and mucus production. To this end, fully-differentiated human airway basal cells (BCi-NS1.1) were treated with different cytokines identified as dysregulated in those diseases, namely interleukins-8, 1β, 4, 17A, 10, 22, and tumour necrosis factor-α (TNF-α).

Our results show that CFTR stands out as the main Cl^- channel modulated by inflammation, in contrast to TMEM16A, whose levels only changed upon IL-4 stimulation. Furthermore, our data establish novel roles for IL-10 and IL-22 by influencing epithelial differentiation towards ciliated cells and away from pulmonary ionocytes. Contrarily, IL-1β and IL-4 decreased the number of ciliated cells while increasing club cells. Interestingly, while IL-1β, IL-4 and IL-10 upregulated CFTR expression, IL-4 was the only cytokine that increased both its function and the number of CFTR-expressing club cells, suggesting that this cell-type may be the main contributor for CFTR function. Additionally, all cytokines assessed here increased mucus production through a differential upregulation of MUC5AC and MUC5B transcript levels.

Altogether, this study reveals a novel insight into differentiation resulting from the cross-talk of inflammatory mediators and airway epithelial cells, which is particularly relevant for chronic airway diseases.

Introduction

The human airway epithelium is a dynamic tissue that protects the lung from the deleterious effects of the inhaled pathogens and pollutants by providing a complex physical barrier, maintaining lung fluid homeostasis, mediating the clearance of invasive particles/pathogens and recruiting immune cells in response to infection (Knight & Holgate, 2003). These functions are carried out by a heterogeneous population of epithelial cells, namely ciliated cells, which drive clearance through the coordinated beating of motile cilia; goblet cells that secrete mucus to trap particles and pathogens; and club cells that produce surfactants and antiproteases with a protective function (Crystal et al., 2008). The continuous regeneration of the epithelium is guaranteed by basal progenitor cells that proliferate and differentiate into the other cell-types (Rock & Hogan, 2011a).

Efficient Mucociliary Clearance (MCC) depends on a regular airway surface liquid (ASL), which thickness and composition are tightly regulated by ion channels and transporters that are differentially distributed between the basolateral and apical membranes of epithelial cells (Hollenhorst et al., 2011). Proper airway hydration is mainly achieved by apical sodium (Na^+) absorption and chloride (Cl^-) secretion, with water passively flowing according to the osmotic gradient. Apical Cl^- secretion is driven by the Cystic Fibrosis Transmembrane conductance Regulator (CFTR), the calcium (Ca^{2+})-activated Cl^- channel (CaCC)/Transmembrane protein 16 A (TMEM16A, also Anoctamin 1 or ANO1) and exchangers of the solute carrier family 26 (SCL26A9 and SLC26A4, also Pendrin), while apical Na^+ absorption is mediated by the epithelial sodium channel (ENaC) (Hollenhorst et al., 2011; Webster & Tarran, 2018). Additionally, several Cl^- channels/transporters are also permeable to bicarbonate (HCO_3^-) ions (Jung et al., 2013; L. Tang et al., 2009) that are essential for mucus release and expansion (Gustafsson et al., 2012; Quinton, 2010)

Besides acting as a physical barrier and maintaining MCC, the airway epithelium directly contributes to host defence through a complex cross-talk with the immune system. Upon infection, airway epithelial cells are exposed to various inflammatory mediators (e.g. interleukins, chemokines, growth factors) that are recognised as signals to increase mucus and fluid secretion, thus promoting MCC and remove intrusive pathogens (Hiemstra et al., 2015; Sala-rabanal et al., 2015; Stanke, 2015). Indeed, previous studies established a link between inflammation and ion transport, with inflammatory mediators modulating the expression and function of ion channels. However, the primary focus has been on the response to T-helper (Th) 2 pro-inflammatory cytokines, namely interleukin (IL)-4 and IL-13, that shift the airway epithelium into a secretory phenotype through the upregulation of CFTR and TMEM16A channels while inhibiting ENaC activity (Dames et al. 2015; Danahay et al. 2002; Galietta et al. 2002; Skowron-Zwarg et al. 2007). Regarding other inflammatory

mediators, the results are often contradictory, depending on the *in vitro* model used. For example, IL-1β and Tumor Necrosis Factor-α (TNF-α) are described to both upregulate (Brouillard et al., 2001; Gray et al., 2004; Q. He et al., 2010) and downregulate (Baudouin-Legros et al., 2005; Ramachandran et al., 2013) CFTR expression or function, whereas no information is available about their effect on other Cl^- channels and transporters. Furthermore, it remains unknown how certain groups of cytokines modulate the airway epithelium properties, namely epithelium-secreted chemokines (e.g., IL-8), which are required to recruit immune cells to the site of infection (Jundi & Greene, 2015), and anti-inflammatory cytokines (e.g., IL-10), essential to resolve an inflammatory response (Opal & DePalo, 2000).

Apart from the modulation of ion transport, inflammatory mediators are also described to regulate the distribution of different airway epithelial cell-types, thus affecting tissue homeostasis. Airway remodelling is a key feature common to many airway diseases, namely Cystic Fibrosis (CF), Chronic Obstructive Pulmonary Disease (COPD) and asthma (Fahy & Dickey, 2010). Although these conditions are characterised by an imbalance in the production of several inflammatory mediators (Chung, 2001; Courtney et al., 2004), most reports only focus on IL-4 and IL-13, which were shown to increase the number of mucus-secreting goblet cells at the expense of ciliated cells (Henry Danahay et al., 2015; Laoukili et al., 2001; Scudieri et al., 2012). Notably, there is no information about the potential effect of inflammation on pulmonary ionocytes, a recently identified airway epithelial cell-type that is described to be very rare but highly enriched in CFTR expression (Montoro et al., 2018; Plasschaert et al., 2018), even though their contribution for CFTR function is still controversial (Scudieri et al., 2020).

Overall, there are still gaps in our understanding of how inflammation impacts the different airway epithelial cell types, being this knowledge essential to define molecular mechanisms occurring in chronic airway diseases. Thus, the main goal of this study was to determine the direct effect of different inflammatory mediators on the airway epithelium in terms of expression of Cl^- channels/transporters, epithelial differentiation and mucus production. To this end, we assessed the effects of different groups of cytokines that are either generally secreted upon infection (IL-8, IL-1β, TNF-α, IL-17A) (Nembrini et al., 2009; Toews, 2001), terminate an inflammatory response (IL-10) (Opal & DePalo, 2000) or have a tissue-protective function (IL-22) (Dudakov et al., 2015). IL-4 was also included in this study as a proof of concept, as its effect on the regulation of mucus and epithelial Cl^- transport is well described (Dabbagh et al., 1999; Galietta et al., 2002).

Our data, using a human respiratory basal cell line (BCi-NS1.1) with the ability to differentiate into the various airway epithelial cell types (Walters et al., 2013), demonstrate that CFTR stands out as the main Cl^- channel modulated by inflammation, leading all cytokines tested to an increase in its expression, except for TNF-α, which has the opposite effect.

Remarkably, some of these cytokines also regulate epithelial cell differentiation, as evidenced by changes in both the expression of cell-type markers and in the number of ciliated cells (IL-1β, IL-4, IL-10, IL-22), secretory club cells (IL-1β, IL-4) or pulmonary ionocytes (IL-10, IL-22). Furthermore, when comparing the effect of IL-1β, IL-4 or IL-10 on CFTR function, we observed that IL-4 was the only cytokine that significantly upregulated the cAMP-activated currents. This result may be explained by the induced increase in the number of CFTR-expressing club cells and/or in TMEM16A expression, an enhancer of CFTR function (Benedetto et al., 2017; Benedetto, Ousingsawat, et al., 2019). Of note, all cytokines studied here upregulate mucus production by increasing the transcript levels of either MUC5AC (IL-4, IL-10, IL-22), MUC5B (IL-1β, TNF-α, IL-17A) or both (IL-8). Finally, by integrating the information obtained on mucus production, expression of Cl^- channels/transporters and epithelial differentiation, we grouped the different cytokines according to their overall effect on the airway epithelium.

Altogether, these findings contribute to elucidate the mechanisms by which cytokines modulate ion transport, mucus production and epithelial differentiation, which may help to design novel cytokine-specific therapeutic strategies aimed at solubilising mucus secretions for the treatment of diseases causing airway obstruction.

Results

1. Effect of different inflammatory mediators on the expression of airway epithelial Cl^- channels/transporters

Given the growing evidence supporting that inflammatory mediators modulate transepithelial Cl^- transport, we first examined the effect of various cytokines on the expression levels of the Cl^- channels/transporters present in airway epithelial cells CFTR, TMEM16A, SLC26A4 and SLC26A9. To this end, fully-differentiated BCi-NS1.1 cells (at ALI day 27) were chronically exposed to IL-8, IL-1β, TNF-α, IL-4, IL-17A, IL-10 or IL-22 for 72h. The levels of CFTR, TMEM16A and SLC26A4 proteins were analysed by Western blot (WB) following exposure to cytokines. However, as we did not detect any WB signal with two different antibodies specific for SLC26A9 (data not shown), instead we assessed mRNA levels of this transporter by quantitative reverse-transcriptase PCR (qRT-PCR).

Our results show that all cytokines tested upregulate endogenous CFTR expression levels, except for TNF-α, which had the opposite effect (Figure 3.1A, D). In contrast, IL-4 was the only cytokine to increase TMEM16A protein expression, which is undetectable in control fully-differentiated BCi-NS1.1 cells (Figure 3.1B, D), as previously described (Chapter I, Simões et al. 2019). Moreover, the expression levels of SLC26A4 were also only significantly upregulated by IL-17A and downregulated by IL-22 (Figure 3.1C, D). Contrarily, these cytokines did not show any effect on the SLC26A9 mRNA expression levels, which however, significantly increased upon exposure to IL-8, IL-1β and IL-4 (Figure 3.1E).

Furthermore, the contribution of CFTR, TMEM16A, SLC26A4 and SLC26A9 for total Cl^- channels/transporters expression was calculated for all cytokines by dividing the fold-change of each channel's protein/mRNA expression levels (Figure 3.1D, E) by the sum of all (Figure 3.1F). Among the cytokines assessed, IL-4 was the only inducing TMEM16A expression (Figure 3.1B, D) which accounts for approximately 36% of total Cl^- channels/transporters expression (Figure 3.1F). Interestingly, CFTR contribution was highest (~60%) in IL-10-treated cells (the cytokine that terminates an inflammatory response), while SLC26A4 and SLC26A9 have a maximal expression upon exposure to IL-17A and IL-1β, respectively, representing approximately 57% and 39% (Figure 3.1F).

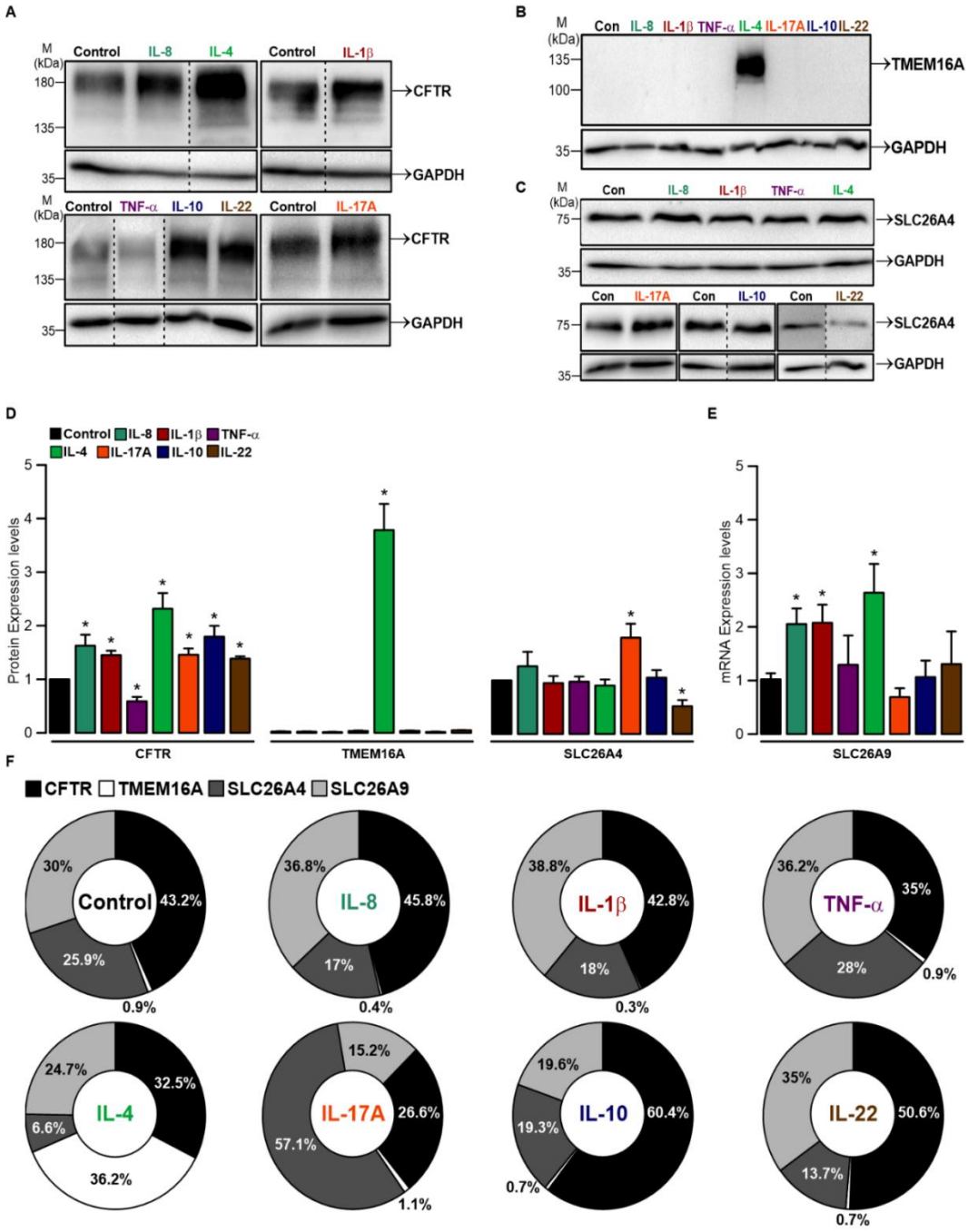

Figure 3.1 – Effect of inflammatory mediators on the expression of airway chloride channels and transporters in differentiated BCi-NS1.1 cells. Western blot (WB) of endogenous **(A)** CFTR (~180 kDa), **(B)** TMEM16A (~120 kDa) and **(C)** SLC26A4 (~85 kDa) expression following exposure to cytokines. GAPDH (~36 kDa) was used as a loading control. For each cytokine, representative blots are shown. The dashed line indicates lanes run on the same gel but noncontiguous. **(D)** CFTR, TMEM16A and SLC26A4 protein expression levels detected by WB, quantified by densitometry and normalised to the loading control and control untreated cells. Data are shown as mean ± SEM (n = 3 - 4). For each protein, asterisks indicate significant differences vs control (p-value < 0.05, unpaired t-test). **(E)** SLC26A9 mRNA expression levels following exposure to cytokines, quantitatively measured by qRT-PCR and normalised to the housekeeping gene GAPDH. Fold-change values are mean ± SEM, relative to control untreated cells (n = 3 - 4). Asterisks indicate significant differences vs control (p-value < 0.05, unpaired t-test). **(F)** Contribution of CFTR (black), TMEM16A (white), SLC26A4 (dark grey) and SLC26A9 (light grey) for total chloride channels/transporters expression following exposure to each cytokine. Values were calculated by dividing the fold change of each channel's protein/mRNA expression levels shown in (D) and (E) by the sum of all.

2. Effect of inflammatory mediators on epithelial cell differentiation towards ciliated, club cells and pulmonary ionocytes

Previous studies showed that proteins secreted from immune cells regulate the balance and distribution of the various respiratory epithelial cell-types (Danahay et al., 2015; Laoukili et al., 2001; Scudieri et al., 2012). Indeed, changes in the percentage of specific cell-types may also explain the observed variation in CFTR expression levels upon inflammation (Figure 3.1A, D). We have previously shown that differentiated BCi-NS1.1 cells express CFTR in ciliated, club cells and pulmonary ionocytes (Chapter II). Thus, we next asked whether the cytokines under study induce changes in the differentiation of BCi-NS1.1 cells into any of these cell types.

To answer this question, we compared protein expression levels of different markers that are specifically expressed in these cell types, namely: Club Cell secretory protein 16 (CC16) for club secretory cells; Dynein Axonemal Intermediate Chain 1 (DNAI1) for ciliated cells; and Forkhead box protein I1 (FOXI1) for pulmonary ionocytes. Following exposure to cytokines, levels of these three protein markers were analysed by WB (Figure 3.2). Results demonstrate that while IL-1β significantly downregulated CC16 protein expression, IL-4 significantly increased levels of this marker by ~3-fold (Figure 3.2A, D). Moreover, IL-1β and IL-4 decreased levels of ciliated-cell marker DNAI1, in contrast to IL-10 and IL-22, which increased its expression by ~2.5-fold (Figure 3.2B, D). Regarding pulmonary ionocytes, IL-10 and IL-22 were the only cytokines showing a significant downregulation of FOXI1 protein levels (Figure 3.2C, D).

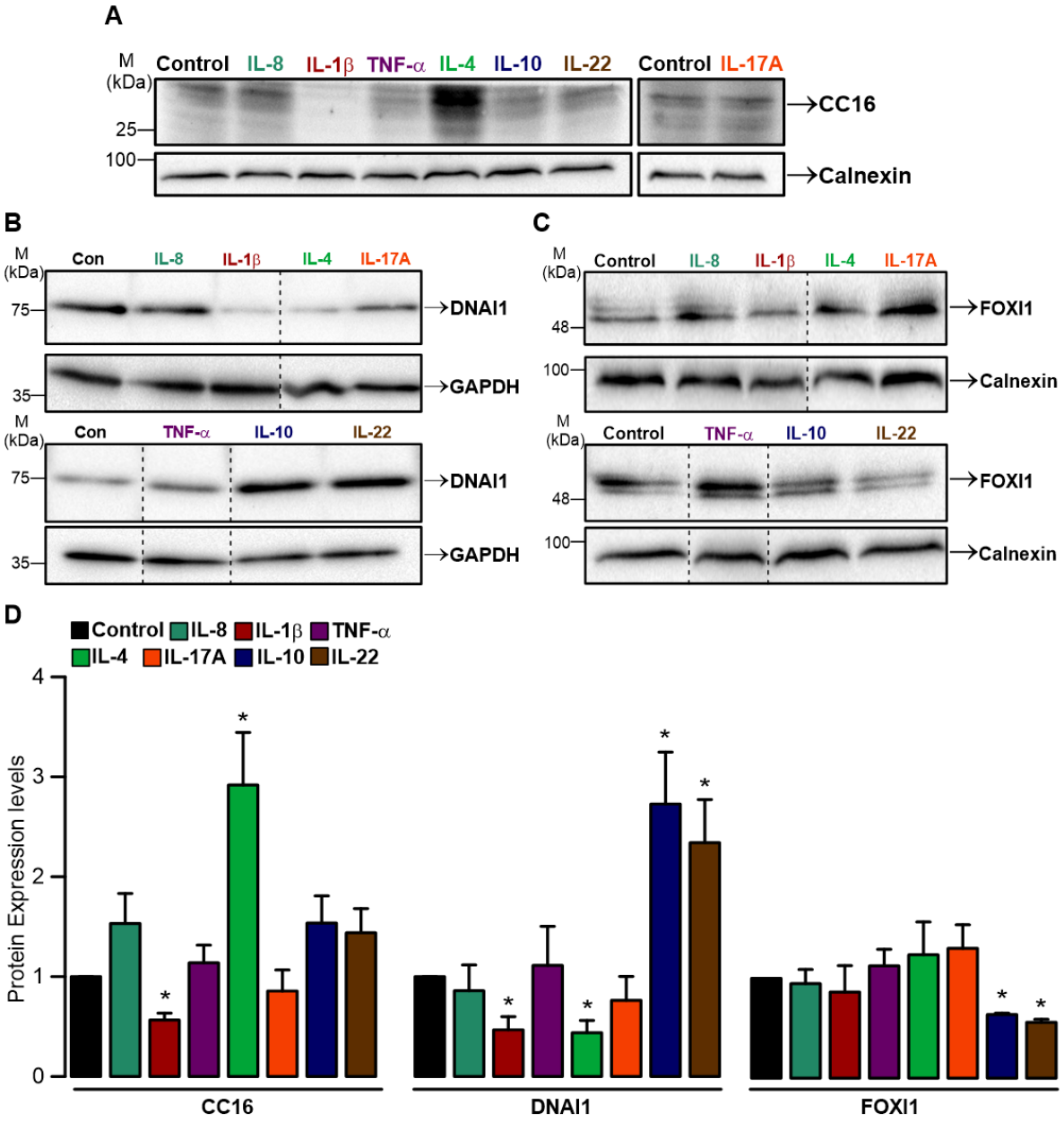

Figure 3.2 – Effect of inflammatory mediators on cell-type markers specific for ciliated, club cells and pulmonary ionocytes. Western blot (WB) comparing the endogenous expression of the **(A)** club-cell marker CC16 (~25 kDa), **(B)** ciliated-cell marker DNAI1 (~75 kDa) and **(C)** pulmonary ionocyte marker FOXI1 (~40 kDa) following exposure to cytokines. GAPDH (~36 kDa) and Calnexin (~90 kDa) were used as loading controls. For each cytokine, representative blots are shown. The dashed line indicates lanes run on the same gel but noncontiguous. **(D)** DNAI1, CC16 and FOXI1 protein expression levels detected by WB, quantified by densitometry and normalised to the loading control and control untreated cells. Data are shown as mean ± SEM (n = 3 - 4). For each protein, asterisks indicate significant differences vs control (p-value < 0.05, unpaired t-test).

To determine whether these changes in protein levels correspond to equivalent differences in the percentage of ciliated, club cells and pulmonary ionocytes, next we performed immunofluorescence experiments with antibodies against βTubulin-IV (another ciliated-cell marker), CC16 and FOXI1, and we co-stained each marker with CFTR to determine the distribution of this channel under the different inflammatory conditions.

Our immunofluorescence results corroborate the WB data as the observed differences matched those in the levels of cell-type-specific markers and also the percentage of ciliated, club cells and pulmonary ionocytes (Figure 3.2Figure 3.3). Moreover, co-staining of βTubulin-IV and CFTR demonstrated that, even though IL-10 and IL-22 increased the percentage of ciliated cells by approximately 1.5-fold (Figure 3.3A, D), the total number of CFTR-expressing βTubulin-IV positive cells did not change (Figure 3.3E). These data suggest that the contribution of ciliated cells for CFTR expression does not change upon exposure to IL-10 or IL-22. On the other hand, treatment with IL-1β and IL-4 significantly decreased the percentage of ciliated cells by half (Figure 3.3A, D) and reduced the contribution of ciliated cells to total CFTR expression (Figure 3.3E). Interestingly, co-labelling of CFTR and CC16 showed that IL-4 increased not only the percentage of club cells (Figure 3.2Figure **3.3**B, D) but also the number of CFTR-expressing club cells (Figure 3.3E), which seems to indicate that upregulation of CFTR by IL-4 (Figure 3.1A, D) occurs in club cells. Contrarily, this was not observed in IL-1β-treated cells, which did not significantly change the percentage of cells co-labelled with CFTR and CC16 (Figure 3.3E), despite the decrease in the total number of club cells (Figure 3.3B, D). Furthermore, the IL-10 and IL-22-induced downregulation of FOXI1 protein levels (Figure 3.2C, D) was also associated with a significant decrease in the percentage of pulmonary ionocytes (Figure 3.3C, D), although the number of CFTR-expressing $FOXI1^{+}$ cells did not change (Figure 3.3E).

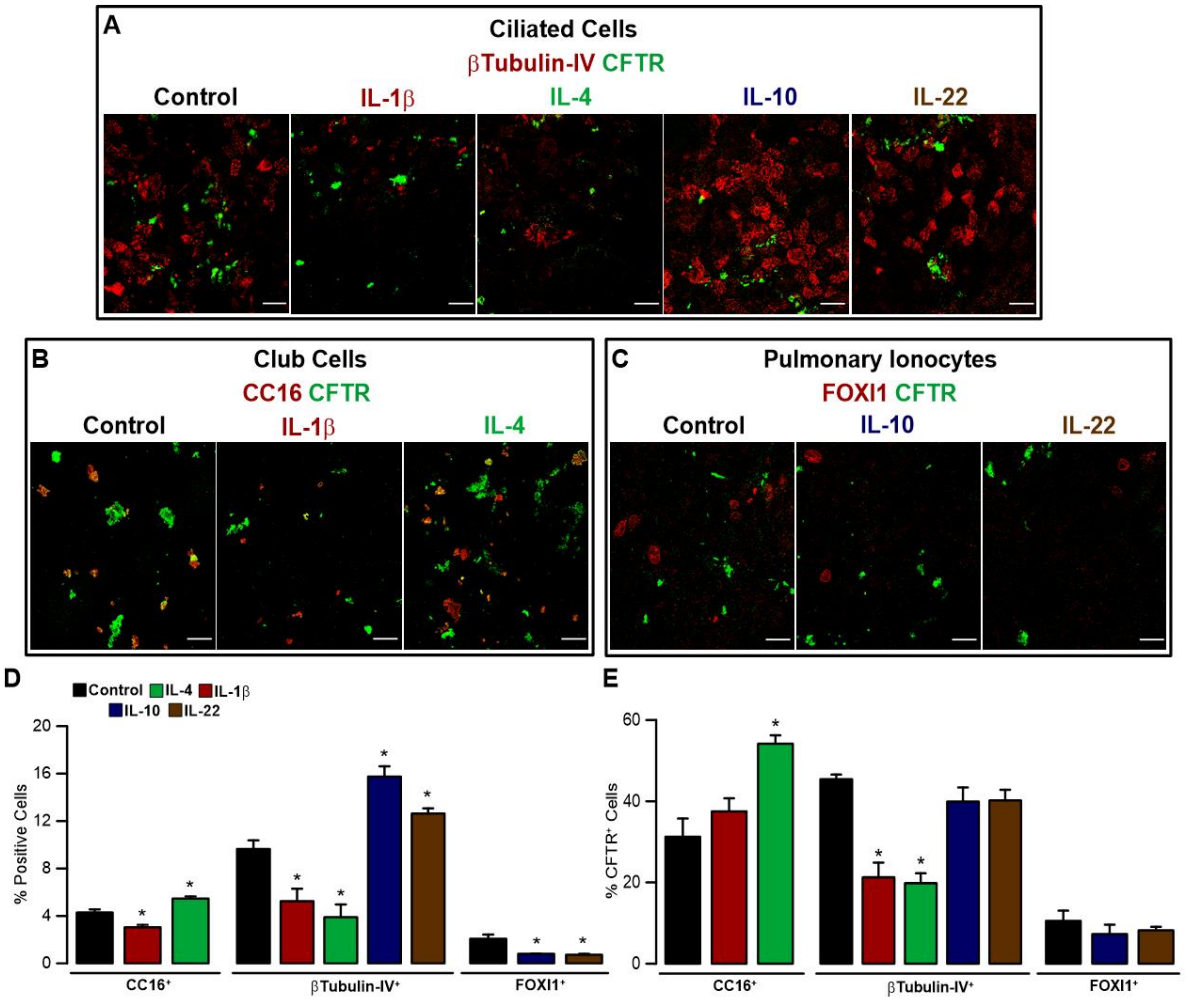

Figure 3.3 – Modulation of epithelial differentiation towards ciliated, club cells and pulmonary ionocytes by IL-1β, IL-4, IL-10 and/or IL-22 in BCi-NS1.1 cells. Confocal immunofluorescence microscopy images showing CFTR (green) and **(A)** ciliated cells (labelled with βTubulin-IV in red), **(B)** club cells (labelled with CC16 in red) or **(C)** pulmonary ionocytes (labelled with FOXI1 in red) in permeabilised fully-differentiated BCi-NS1.1 cells (ALI day 30) exposed to IL-1β, IL-4, IL-10 or IL-22. Scale bar = 20 µm. Images were acquired with a Leica TCS SP8 confocal microscope (objective 63x oil, NA 1.4). **(D)** Quantification of the % of ciliated, club cells and pulmonary ionocytes (stained with the βTubulin-IV, CC16 and FOXI1 antibodies shown in A-C, respectively). The number of each cell-type was normalised to the total number of nuclei. **(E)** Quantification of the % of cells co-stained with CFTR and CC16, βTubulin-IV or FOXI1 normalised to the total number of CFTR$^+$ cells. Quantifications were performed in 15 images randomly acquired in three independent experiments (5 images/experiment). Values are mean ± SEM (n = 3). For each cell-type, asterisks indicate significant differences vs control (p-value < 0.05, unpaired t-test).

3. Modulation of CFTR activity by IL-1β, IL-4 and IL-10

Next, we assessed CFTR function by Ussing chamber measurements in polarised BCi-NS1.1 cells treated with IL-1β, IL-4 and IL-10. Although these three cytokines upregulated the CFTR protein expression levels (Figure 3.1A, D), they showed opposite effects in the percentage of ciliated, club secretory and pulmonary ionocytes (Figure 3.2A-D). Thus, by comparing CFTR function in IL-1β, IL-4 and IL-10-treated cells, we can conclude which cell-

type contributes more to total CFTR function. Ussing chamber measurements demonstrated that IL-4 was the only cytokine that significantly increased the cAMP-activated currents (Figure 3.4A, C), elicited by the co-application of IBMX and Forskolin (IF) and sensitive to the CFTR inhibitor 172 (inh172). These data suggest an appreciable contribution of club cells to CFTR function, as this was the major cell type induced by IL-4 (Figure 3.2, Figure **3.3**). Additionally, we also observed that IL-1β and IL-4 significantly decreased the amiloride-sensitive Na^+ absorption currents (Figure 3.4A, B), indicating that ENaC function is downregulated upon exposure to these cytokines.

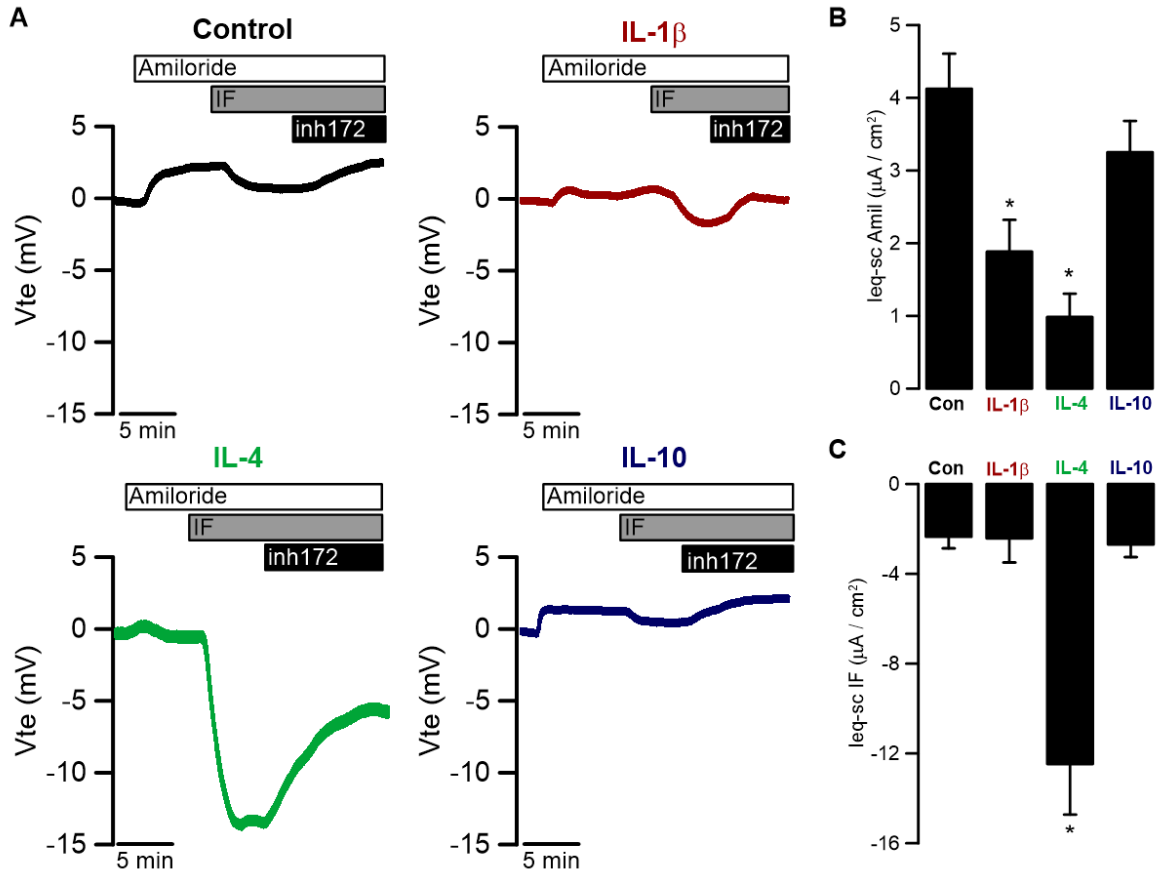

Figure 3.4 – Effect of IL-1β, IL-4 and IL-10 on the cAMP-activated and amiloride-sensitive currents in polarised BCi-NS1.1 cells. (A) Representative Ussing chamber tracings of transepithelial voltage measurements (V_{te}) for untreated BCi-NS1.1 cells (control, black) and treated with IL-1β (red), IL-4 (green) and IL-10 (blue). A positive deflection is observed after stimulation with the epithelial Na^+ channel (ENaC) inhibitor, amiloride (30 µM), which is followed by a negative deflection after co-stimulation with apical IBMX (100 µM) and Forskolin (2 µM). This effect was reversed upon the addition of the CFTR inhibitor-172 (30 µM). **(B)** Summary of the $I_{sc\text{-}eq}$ (µA/cm²) calculated for the response to amiloride (Amil). **(C)** Summary of the $I_{sc\text{-}eq}$ (µA/cm²) calculated for the response to IBMX + Forskolin (IF). Values are mean ± SEM (n = 3 - 5). Asterisks indicate significant differences vs control. (p-value < 0.05, unpaired t-test). [Ussing Chamber measurements were performed with Arthur Kmit and are included here with permission].

4. Regulation of mucin gene expression by IL-8, IL-1β, TNF-α, IL-4, IL-17A, IL-10 and IL-22

Next, we determined the effect of the cytokines under study on mucus production, particularly on the mRNA expression levels of MUC5AC and MUC5B, the two main gel-forming mucins present in human airway mucus (Ridley & Thornton, 2018). These two mucins are essential components of the MCC machinery, with MUC5B forming mucus strands upon secretion that are coated with MUC5AC plaques to ensure proper mucus transport (Ermund et al., 2017; Xie et al., 2018). Therefore, it is relevant to determine the ratio between these two mucins to better define how the mucus properties change according to the inflammatory stimuli.

To answer this question, we assessed levels of MUC5AC and MUC5B transcripts by qRT-PCR in fully-differentiated BCi-NS1.1 cells following chronic exposure to IL-8, IL-1β, TNF-α, IL-4, IL-17A, IL-10 or IL-22. Our results demonstrate that all cytokines induced an overall increase in MUC5AC and MUC5B transcripts, albeit at differential levels and thus resulting in different ratios. Indeed, IL-8, IL-4, IL-10 and IL-22 significantly increased MUC5AC transcripts (Figure 3.5A), being IL-4 the cytokine inducing the greatest unbalance as it had no significant effect on MUC5B transcript levels (approximately 12-fold in the MUC5B vs MUC5AC levels). On the other hand, MUC5B transcript levels were higher upon exposure to IL-1β (approximately 8-fold), followed by IL-8, TNF-α and IL-17A (Figure 3.5B).

Additionally, the relative levels of transcripts of both mucins were calculated for all cytokines by dividing the MUC5AC or MUC5B fold-change expression levels by the sum of the two (Figure 3.5C). While IL-8 significantly increased the MUC5AC and MUC5B mRNA expression levels (Figure 3.5A, B), the relative percentage of each mucin is similar to the control condition. In contrast, IL-4, IL-10 and IL-22 increased mucus production mainly by upregulating MUC5AC, whereas MUC5B represents over 60% of mucin mRNAs in cells treated with IL-1β, TNF-α or IL-17A (Figure 3.5C).

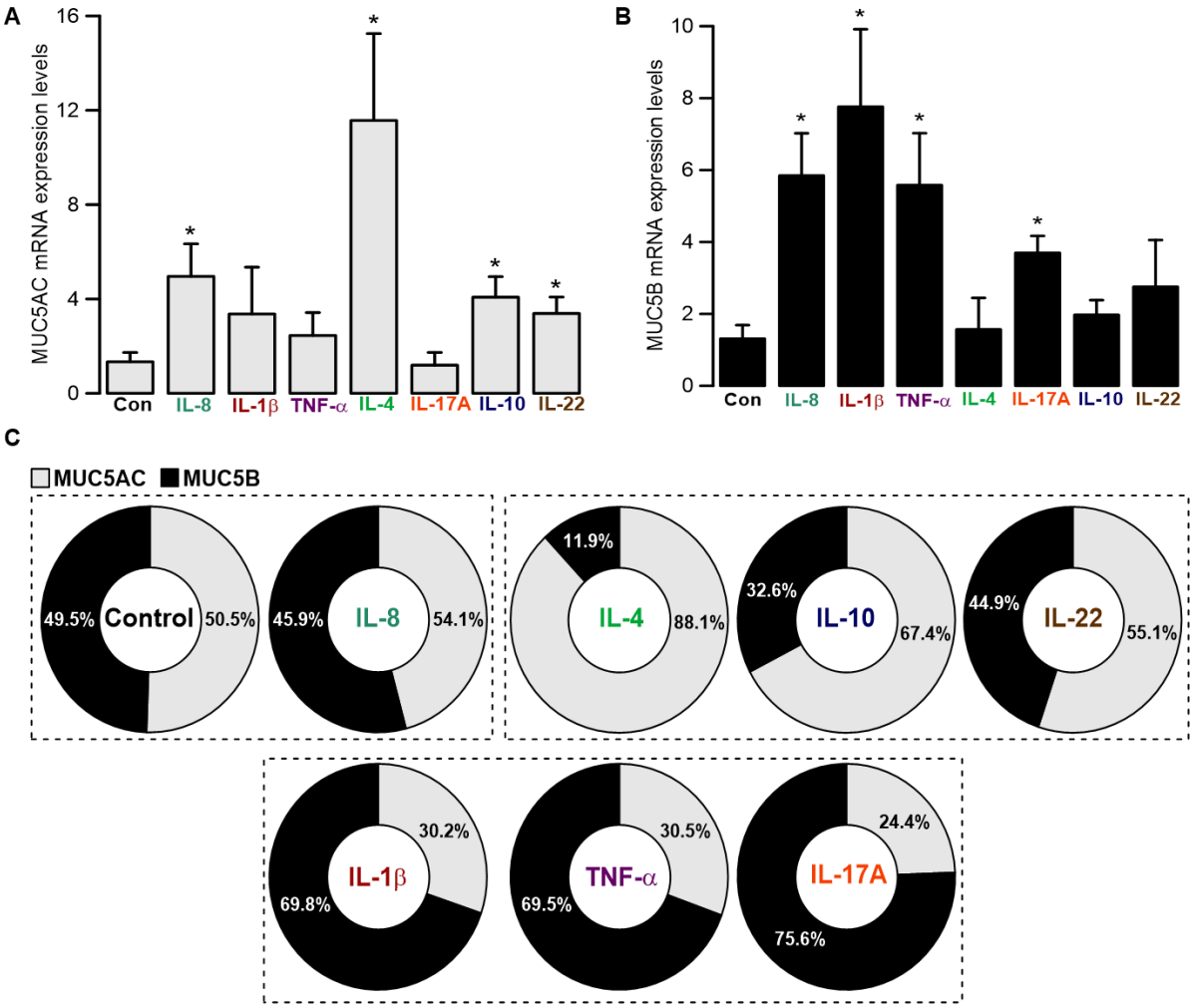

Figure 3.5 – MUC5AC and MUC5B mRNA expression in differentiated BCi-NS1.1 cells following exposure to cytokines. (A) MUC5AC and **(B)** MUC5B mRNA expression levels following exposure to cytokines, quantitatively measured by qRT-PCR and normalised to the housekeeping gene GAPDH. Fold-change values are mean ± SEM, relative to control untreated cells (n = 3 - 5). Asterisks indicate significant differences vs control (p-value < 0.05, unpaired t-test). **(C)** Contribution of MUC5AC (grey) and MUC5B (black) for mucin production. Values were calculated by dividing each mucin's fold-change expression levels by the sum of the MUC5AC and MUC5B expression levels shown in (A) and (B). Dashed lines indicate groups of treatments that have matching contributions of MUC5AC or MUC5B expression.

5. Summary of the effect of different cytokines on the expression of Cl^- channels/transporters, epithelial differentiation and mucus production

The observed effects on the expression of Cl^- channels/transporters, epithelial differentiation and mucus production (Figure 3.1, Figure **3.2**Figure 3.5) are summarised in a heatmap (Figure 3.6) where the various cytokines are grouped into clusters according to their overall effect on the airway epithelium properties. Altogether, we observed that IL-4 induced the most significant changes in all these parameters. Moreover, IL-10 and IL-22 showed similar effects on differentiated BCi-NS1.1 cells by upregulating CFTR, DNAI1 and MUC5AC, while decreasing FOXI1. IL-1β and IL-8 are grouped into the same cluster as both increased

the CFTR, SLC26A9 and MUC5B expression levels. Finally, IL-17A and TNF-α were the two cytokines that demonstrated fewer effects on differentiated BCi-NS1.1 cells (Figure 3.6).

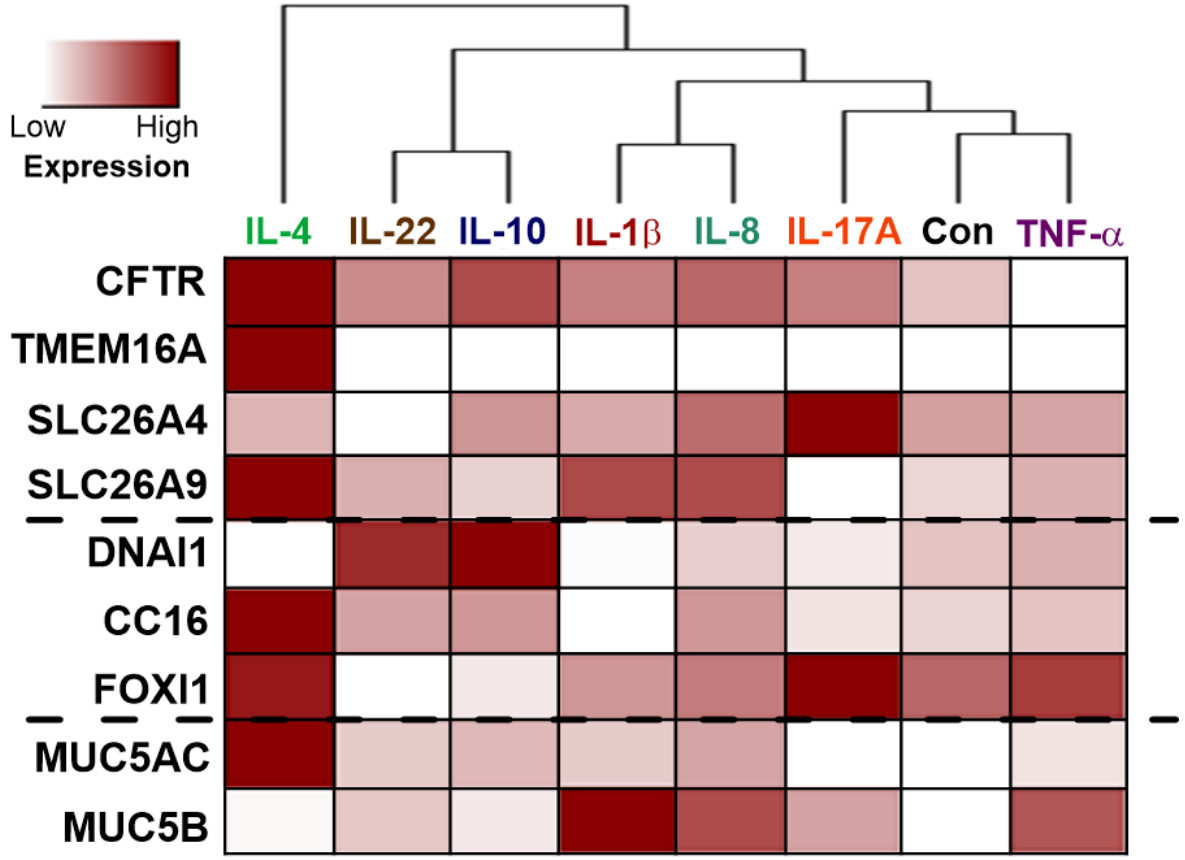

Figure 3.6 – Summary of the observed changes in different chloride channels/transporters expression, differentiation markers and mucus production upon exposure to cytokines. The heatmap was generated based on the results shown in Figs. 3.1, 3.2 and 3.5. Data was rescaled between 0 and 1, with lower and higher expression levels represented in white and red, respectively. Inflammatory mediators are grouped into clusters according to the relative expression levels of all the proteins analysed. Dashed lines separate the chloride channels/transporters, differentiation markers and mucins.

Discussion

Airway inflammation is a hallmark of nearly all airway obstructive pulmonary diseases, being associated with mucus hyperproduction and epithelial remodelling (Fahy & Dickey, 2010). Under these conditions, there is a complex cross-talk between airway epithelial cells and the immune system to ensure efficient MCC assisted by the secreted mucus. When efficient MCC fails, e.g. due to ASL dehydration, mucus plugging and airway clogging occur, followed by progressive lung failure (Hiemstra et al., 2015; Stanke, 2015). Therefore, to fully understand the pathogenesis caused by inflammation on obstructive airway diseases, it is essential to characterise how airway epithelial cells respond to inflammatory stimuli. Although several cytokines are dysregulated in chronic airway diseases (Chung, 2001; Courtney et al., 2004), most studies focus on the response of airway epithelial cells to Th2 cytokines, namely IL-4 and IL-13, due to their involvement in allergic airway conditions, such as asthma (Danahay et al., 2015; Laoukili et al., 2001; Scudieri et al., 2012). In this study, we screened a panel of seven cytokines that are dysregulated in CF, COPD and/or asthma, namely IL-8, IL-1β, TNF-α, IL-4, IL-17A, IL-10 and IL-22 (Bonfield et al., 1995; Chung, 2001; Paats et al., 2013). To determine the effect of these cytokines on airway epithelial cells, we used a human airway basal cell line (BCi-NS1.1) that differentiates into the different cell types of a pseudostratified mucociliated epithelium (Walters et al., 2013) and assessed the expression of Cl^- channels/transporters, the epithelial differentiation status and mucus production in terms of MUC5AC and MUC5B.

Firstly, we evaluated the impact of the various cytokines on the expression of airway epithelial Cl^- channels/transporters, namely CFTR, TMEM16A, SCL26A4 and SLC26A9. Increasing evidence supports the concept that inflammatory stimuli modulate the transepithelial ion transport, which is essential to regulate the ASL height and ion composition, thus maintaining the homeostasis of the epithelium barrier and ensuring host defence (Hiemstra et al. 2015; Stanke 2015). Overall, our results show that CFTR is the primary Cl^- channel modulated by inflammation, with all cytokines increasing its expression, except for TNF-α, which had the opposite effect (Figure 3.1A, D). Interestingly, while these observations agree with previous studies (reviewed in Sala-Rabanal et al. 2015; Stanke 2015), they also establish novel roles for IL-8, IL-10 and IL-22 as regulators of CFTR protein expression. IL-8 is a chemokine produced by airway epithelial cells to recruit immune cells to the site of infection (Jundi & Greene, 2015). Previous studies have demonstrated that expression of *wt*-CFTR suppresses IL-8 secretion by airway epithelial cells (Veit et al., 2012). Here, we show that IL-8 also has an autocrine effect on airway epithelial cells, leading to a significant upregulation of both CFTR and SLC26A9 (Figure 3.1A, D, E).

Furthermore, although all cytokines significantly increased mucus production (Figure 3.5), IL-4 was the only one that upregulated the TMEM16A protein expression levels (Figure 3.1B, D, F). This observation may shed some light on the current controversy on whether TMEM16A is required for mucus production (reviewed in Danahay and Gosling 2020). Indeed, we have previously shown that IL-4-mediated TMEM16A upregulation does not directly cause mucus hyperproduction, but instead, both events occur due to a concomitant increase in cell proliferation, namely of mucus-secreting cells (Chapter I, Simões et al., 2019).

Besides regulating the expression of Cl^- channels/transporters, we also show that inflammatory mediators modulate epithelial differentiation. Particularly, our results demonstrate that while IL-1β and IL-4 decrease the percentage of ciliated cells (Figure 3.2Figure 3.3A, D), they have the opposite effect on the number of club secretory cells (Figure 3.2Figure 3.3B, D). These effects should be considered when designing therapeutic strategies aimed at reducing mucus production in chronic airway diseases, as they might not be sufficient if the airway epithelium lacks ciliated cells to achieve proper MCC and thus propel the secreted mucus. This is particularly relevant for CF, a disease characterised by mucus plugging and stasis, in which IL-1β was identified as the main cytokine driving mucus hyperproduction (Chen et al., 2019). Contrarily, IL-10 and IL-22 biased the airway epithelium towards ciliated cells and away from pulmonary ionocytes (Figure 3.2Figure 3.3). These observations indicate that IL-10 and IL-22 might support mucosal defence by directly regulating MUC5AC production (Figure 3.5) and ciliated-cell differentiation (Figure 3.2Figure 3.3), thus ensuring efficient MCC of unwanted pathogens and particles. Moreover, the observed effect of IL-10 and IL-22 in reducing the number and percentage of pulmonary ionocytes (Figure 3.3C, D) may help to understand the function of this new cell-type on the airway epithelium.

Interestingly, our results showed that although IL-1β, IL-4 and IL-10 upregulated CFTR expression levels (Figure 3.1A, D), IL-4 was the only cytokine that significantly increased its function (Figure 3.4A, C). A possible explanation for this finding is that these cytokines induce opposite changes in the epithelial differentiation towards ciliated, club cells and pulmonary ionocytes (Figure 3.2Figure 3.3). Indeed, IL-4 increased the percentage of CFTR-expressing club cells (Figure 3.3E), suggesting that this cell-type is the main contributor to CFTR function in IL-4-treated cells. This conclusion is supported by other studies describing a significant contribution of secretory club cells to CFTR expression and function, particularly upon inflammation (Okuda et al., 2020; Rehman et al., 2020). Additionally, the effect of IL-4 on CFTR activity may also be explained by the induced upregulation of TMEM16A, as this channel has been reported to enhance CFTR function (Benedetto et al., 2017; Benedetto, Ousingsawat, et al., 2019). Furthermore, whereas IL-1β decreased the number of club cells, it did not affect the overall contribution of this cell-type for CFTR expression (Figure 3.3E) nor function (Figure 3.4A, C). Our results on IL-10 treated cells also show that pulmonary

ionocytes do not necessarily mediate CFTR activity, as this cytokine decreased their percentage (Figure 3.3C, D) while not showing any effect on cAMP-activated currents (Figure 3.4A, C), in agreement with other studies (Scudieri et al., 2020).

Inflammatory cytokines are among the main factors inducing mucin hyperproduction through transcriptional and post-transcriptional mechanisms (Thai et al., 2008). Indeed, our data show that all cytokines tested increase mucus production by upregulating mRNA expression of either MUC5AC (IL-4, IL-10, IL-22), MUC5B (IL-1β, TNF-α, IL-17A) or both mucins (IL-8) (Figure 3.5). These results corroborate previous reports describing a differential induction of MUC5AC and MUC5B due to activation of different transcription factors and signalling pathways, depending on the inflammatory stimulus (Bautista et al., 2009; Y. Chen et al., 2003; Enss et al., 2000). It is generally accepted that these mucins play distinct roles in maintaining lung homeostasis: while MUC5B is essential to regulate MCC and avoid infection, MUC5AC is considered an “acute response mucin”, with its levels rapidly increasing in response to an allergic inflammatory challenge (Ridley & Thornton, 2018). Furthermore, MUC5AC is a key controller of mucus transport by anchoring MUC5B-containing mucus strands that are usually secreted from submucosal glands (Ermund et al., 2017; Xie et al., 2018). Thus, the differential induction of MUC5AC and MUC5B mRNA expression will likely affect mucus transport and MCC. These effects are particularly relevant for CF, where MUC5B-mucus strands remain tethered to the submucosal gland ducts, leading to mucus stasis (Hoegger et al., 2014). Thus, by increasing MUC5B mRNA expression, cytokines IL-8, IL-1β, TNF-α and IL-17A may further compromise mucus transport and worsen mucus stasis in CF. Nonetheless, the overall cytokine-mediated mucus hyperproduction likely occurs as a protective response of airway epithelial cells to ensure proper clearance by trapping the unwanted inhaled pathogens and harmful agents in the secreted mucus. However, when CFTR – a key channel ensuring Cl^- secretion and ASL hydration – is deficient, the resulting effect of mucus hypersecretion is noxious.

While it is widely established that pro-inflammatory cytokines stimulate mucus production (Thai et al., 2008), less information is available about the effect of anti-inflammatory cytokines. In this study, MUC5AC transcript levels were also significantly upregulated by IL-10 (Figure 3.5), a potent anti-inflammatory cytokine essential to inhibit inflammatory responses and inactivate the production of other pro-inflammatory mediators (Opal et al., 2000). Interestingly, IL-10 was previously identified to control intestinal mucus production by regulating the expression of MUC2, the prominent intestinal mucin (Hasnain et al., 2013). This effect was mediated by the activation of the signal transducer and activator of transcription 1 and 3 (STAT1 and STAT3), the latter being also involved in the regulation of MUC5AC transcription (Hao et al., 2017). Moreover, our data identified a similar effect on MUC5AC mRNA expression by IL-22 (Figure 3.5), a cytokine that plays a crucial role in maintaining the

mucosal cell barrier by promoting epithelial cell regeneration and tissue repair (Dudakov et al., 2015). IL-22 belongs to the IL-10 family, and both cytokines activate similar signalling pathways, including STAT3 (Ouyang & O'Garra, 2019). Thus, further studies are required to determine how IL-10 and IL-22 regulate MUC5AC transcription levels.

In summary, by assessing the differential effects of these cytokines on the expression of 1) Cl^- channels/transporters; 2) epithelial differentiation status; and 3) mucus production, we grouped the different inflammatory stimuli according to their effect on the airway epithelium properties (Figure 3.6). Overall, we observed that the most extensive modifications on airway epithelial cells are induced by IL-4 by 1) upregulating CFTR, TMEM16A and SLC26A9 (Figure 3.1); 2) inducing club-cell differentiation (Figure 3.2Figure 3.3); 3) leading to MUC5AC hyperproduction (Figure 3.5A); and 4) significantly decreasing the number of ciliated cells (**Figure 3.3**A, D). Therefore, as IL-4 also increased CFTR function (Figure 3.4A, C), it would be interesting to elucidate the mechanisms behind this effect, which ultimately may lead to the identification of novel strategies to increase CFTR expression and function in CF cells.

Taking together all data in this study, we propose a novel model for the effect of each cytokine on airway epithelial cells (Figure 3.7). For simplicity, the cytokines grouped on the same cluster (Figure 3.6) are represented together. In summary, these models describe the effects of IL-1β, IL-4, IL-17A, and IL-10 on 1) the differential induction of MUC5AC and MUC5B; 2) the percentage of different airway epithelial cell types; and 3) the expression of Cl^- channels and transporters. Overall, these results will help to understand the impact of inflammatory mediators on airway epithelium properties. Further studies are required to fully characterise the signalling pathways activated by these cytokines that may improve MCC and lung defence so as to identify druggable molecular targets and design novel therapeutic strategies for the treatment of chronic pulmonary diseases while reducing the impact of inflammatory mediators. Additionally, this knowledge may also allow predicting a specific individual's pathophysiology according to the circulating levels of these inflammatory mediators.

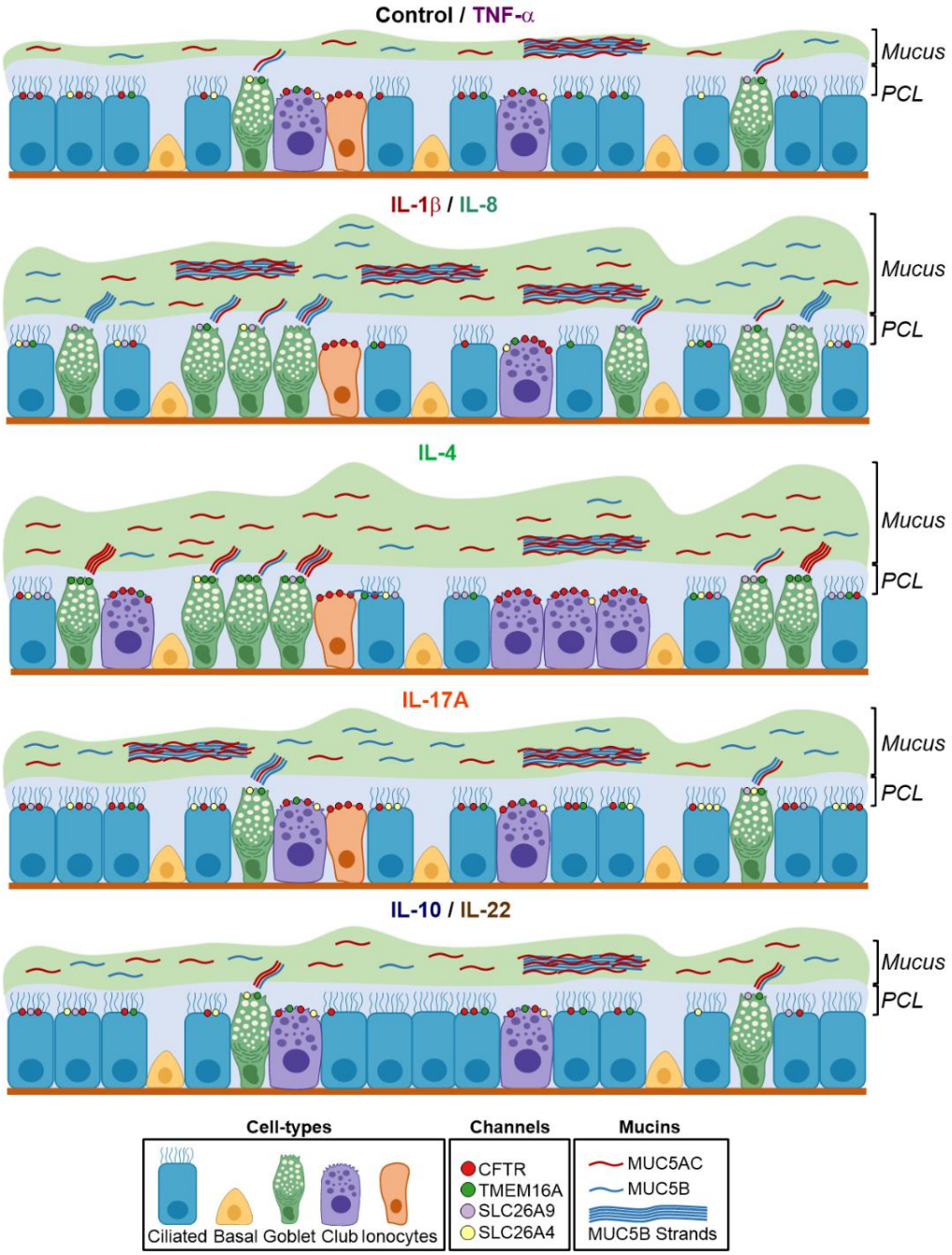

Figure 3.7 – Models for the impact of distinct cytokines on the airway epithelium in terms of differentiation status, mucus production and expression of chloride channels/transporters. Cytokines are represented based on the heatmap clusters (Fig.3.6) – TNF-α, IL-8 and IL-22 induced similar effects as control, IL-1β and IL-10, respectively. Mucus hyperproduction is represented by a higher thickness of the mucus gel layer (green) above the periciliary layer (PCL, blue), resulting from the upregulation of MUC5AC (red threads) or MUC5B (blue threads). MUC5B strands are also shown, which are usually secreted from submucosal glands and coated in the surface epithelium by MUC5AC. MUC5AC hyperproduction was prevalent in IL-4 and IL-10/IL-22-treated cells, while IL-1β and IL-17A increased MUC5B. The cytokine's effects on epithelial differentiation are displayed by changes in the number of ciliated cells (higher in IL-10/IL-22, lower in IL-1β and IL-4), club cells (higher in IL-4, lower in IL-1β) and pulmonary ionocytes (lower in IL-10/IL-22). The number of CFTR, TMEM16A, SLC26A4 and SLC26A9 channels/transporters vary according to the observed differences following exposure to cytokines (Fig.3.1) and have a cell-type distribution matching what is described for the human airway epithelium.

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IV. General Discussion and Future Perspectives

Since the identification of the CFTR gene in 1989 (Kerem et al., 1989; Riordan et al., 1989; Rommens et al., 1990), major advances have been made to assess the molecular basis of CF so as to treat it and thus increase the life expectancy of individuals affected by this disease. Nevertheless, despite the impressive progress in finding novel CFTR modulator drugs, a cure is not yet available to all individuals with CF, particularly to those with less common CFTR mutations. Indeed, CF is a complex disease with a wide range of clinical manifestations, which are not entirely explained by the basic defect in the CFTR-mediated Cl^-/HCO_3^- secretion. Increasing evidence supports the concept that CFTR has additional functions (reviewed in Amaral et al., 2020), which might explain the involvement of this protein in epithelial cell differentiation, ASL and mucus hydration and the increased susceptibility of CF airways to infection and inflammation. Apart from CFTR, other Cl^- channels and transporters have also been associated with mucus hyperproduction and airway inflammation, such as TMEM16A. Pharmacological activation of this Ca^{2+}-activated Cl^- channel has for long been considered a possible therapy for CF that may benefit all individuals, regardless of the mutations carried in the CFTR gene (Amaral, 2015). Notwithstanding, before embarking on a strategy to activate TMEM16A in CF airways, it is essential to define better the cellular and molecular mechanisms occurring in CF, as well as the role of this alternative channel.

The main goal of this doctoral work was to understand the role of Cl^- channels/transporters in mucus production, airway inflammation and epithelial cell differentiation, to ultimately identify therapeutic strategies for obstructive airway diseases, namely CF.

In the first part of this work, we aimed at determining the role of TMEM16A in mucus hyperproduction. This question emerged due to the growing number of controversial studies debating whether TMEM16A should be activated or inhibited in CF (reviewed in Danahay and Gosling 2020). Theoretically, as TMEM16A is a Cl^- channel expressed in airway epithelial cells, its activation should be beneficial for individuals with CF by compensating for the absence of the CFTR-mediated Cl^- secretion, thus improving airway hydration and MCC. Moreover, as TMEM16A was found to stimulate CFTR Cl^- channel activity (Benedetto et al., 2017; Benedetto, Ousingsawat, et al., 2019), its stimulation could also potentiate CFTR in individuals bearing residual CFTR function mutations. On the other hand, other studies proposed that inhibiting TMEM16A leads to decreased mucus production/secretion and, consequently, reduced inflammation (reviewed in Danahay and Gosling 2020). To help to solve this conflict, we used a multipotent human bronchial basal cell line (BCi-NS1.1) that differentiates into the various airway epithelial cell-types. This cell line mimics the responses of primary human bronchial epithelial cells, being physiologically relevant and robust. Overall, our results demonstrated that TMEM16A is not required for mucus production. Indeed, we

show that TMEM16A expression is regulated by cell proliferation and that MUC5AC production is independent of TMEM16A, both under homeostasis and upon exposure to the pro-inflammatory cytokine IL-4. Consistently, mucus hyperproduction can also occur without TMEM16A expression being upregulated and without cell proliferation, as evidenced by the activation of the notch signalling pathway with DLL4. On the other hand, we show that TMEM16A is essential for airway hydration, suggesting that activation of this channel *in vivo* can improve MCC without increasing mucus production. Therefore, this work led to the conclusion that activation of TMEM16A remains a *bona fide* therapeutic approach for CF.

Future studies should thus focus on identifying TMEM16A activators that stimulate the channel function specifically in non-proliferating airway epithelial differentiated cells and independently of Ca^{2+}. Remarkably, recent studies describe a promising TMEM16A potentiator that improves Cl^- secretion and MCC without affecting mucus production (Danahay et al., 2020). Additionally, it would be interesting to determine the effects of this potentiator on the proliferation of airway epithelial cells. Ultimately, the elucidation of the TMEM16A role in mucus hyperproduction is not only relevant for CF but also for other chronic obstructive pulmonary diseases. Furthermore, our findings relating TMEM16A upregulation to cell proliferation may also contribute to unravel the mechanisms regulating tumour growth and metastasis, as this protein is highly expressed in several cancers, being considered a cell proliferation and migration biomarker.

Besides mucus hyperproduction, CF airways are also characterised by epithelial remodelling and impaired differentiation (reviewed in Amaral et al., 2020), which are associated with the propensity to recurrent bacterial infections and chronic inflammation. Therefore, in the second part of this work, we explored the role of CFTR in airway epithelial cell differentiation. An initial assessment indicated that the expression of *wt* CFTR increases along with the differentiation of BCi-NS1.1 cells, being functional and mainly localised at the apical membrane of both ciliated and club secretory cells. These results are the first proof that differentiated BCi-NS1.1 cells express endogenous and functional CFTR. Given this observation, we then characterised how mutant CFTR affects the differentiation of basal cells into various airway epithelial cell-types. To this end, we produced two airway basal cell lines expressing the most common CF-causing mutation (F508del) in homozygosity (BCi-CF1.1) and heterozygosity (BCi-Hz1.1). Our data show that dysfunctional CFTR is sufficient to impair epithelial polarisation and differentiation, particularly towards ciliated cells, however with no effect on pulmonary ionocytes. Remarkably, albeit modest, we observed a significant increase in the expression of the ciliated-cell marker DNAI1 upon exposure to the CFTR modulators VX-661 and VX-445 for 48h, suggesting that CFTR modulators can reverse this differentiation defect.

Further studies should be carried out to determine the signalling pathways behind this defect, which can ultimately help to identify pharmacological targets that rescue the epithelial differentiation impairment in CF airways. Along these lines, our lab has already established the involvement of both the Akt signalling pathway (Sousa et al., 2020) and the transcriptional factor TWIST1 (Quaresma et al., 2020) in the CF differentiation defect. It will be interesting to determine whether chronic stimulation with the CFTR modulators for a longer period would further enhance the DNAI1 expression. However, it might also be that the levels of the CFTR correction are not sufficient to fully rescue this phenotype. An important open question is: What is the trigger signal for differentiation? Is it the presence of CFTR at the apical membrane? Or is it functional CFTR? If the latter is required, is it Cl^- or HCO_3^- (pH) that drives correct differentiation? In this regard, it will be very interesting to determine whether adding a potentiator (e.g. VX-770) will further correct the differentiation defect. Additionally, one could also compare the effect of different CFTR mutations on epithelial differentiation, such as the gating mutation G551D (a non-functional mutant that traffics to the apical membrane), which would allow further determining whether the defective differentiation phenotype is a consequence of the absence of CFTR at the plasma membrane or if the Cl^-/HCO_3^- channel function is also essential. Overall, it is essential to determine the role of mutant CFTR on epithelial differentiation, as therapies aimed at inhibiting mucus hyperproduction may not be sufficient to improve MCC if there are not enough ciliated cells to propel the secreted mucus.

Importantly, BCi-Hz1.1 and BCi-CF1.1 may constitute "intermediate" cell models between the usual cell lines that lack differentiation capacity (or CFTR-overexpressing cell lines) and primary cultures of airway epithelial cells which are limited resources and hard to manipulate. As the BCi cells have endogenous CFTR expression controlled by the native CFTR promoter and the potential of differentiating into all the respiratory epithelial cell-types, they constitute valuable tools to study CF pathogenesis under multiple aspects, such as 1) identification of cellular and molecular targets involved in epithelial differentiation and regeneration; 2) characterisation of innate immune responses of airway epithelial cells; or 3) evaluation of the potential effects of therapeutic targets on MCC and epithelial differentiation.

Although CF and other chronic obstructive airway diseases are characterised by an imbalance in the levels of several inflammatory mediators, most studies are focused on the responses of airway epithelial cells to IL-4 and IL-13 due to their involvement in asthma pathophysiology. The reason why in the first chapter, we also explored the effects of IL-4 on mucus hyperproduction, epithelial Cl^- transport and airway hydration.

However, in the last part of this doctoral work, our goal was to study the cross-talk between a panel of different disease-specific inflammatory mediators and the airway epithelium. Our results describe differential responses of the airway epithelium

transdifferentiating into distinct cell-type distributions in response to those inflammatory *stimuli*, with consequences at the level of mucus production and ion channels/transporters expression. Out of the seven cytokines analysed, IL-4 was the one that induced the most significant changes in the airway epithelium. Nonetheless, we also identified previously undescribed roles for IL-10 and IL-22 as enhancers of the MUC5AC production, CFTR expression and ciliated-cell differentiation while decreasing the percentage of pulmonary ionocytes. Whereas IL-10 is a potent anti-inflammatory cytokine (Opal & DePalo, 2000), IL-22 improves tissue regeneration and accelerates the wound-closure rate of CF airways (Guillon et al., 2019). Thus, further studies should assess the signalling pathways activated by these cytokines as they may have beneficial effects on CF.

In contrast, although IL-4 and IL-1β increased CFTR expression, they also impaired ciliated-cell differentiation. These two cytokines are described to be upregulated in CF airways, particularly upon infection by *Pseudomonas aeruginosa* (Bonfield et al., 1995; Brazova et al., 2005). Thus, IL-4 and IL-1β are likely to further impair the epithelial differentiation defect of CF airways. Additionally, our results indicate that in IL-4-treated cells, club secretory cells appear to be the main cell-type contributing to CFTR function. On the other hand, even though IL-10 decreased the percentage of pulmonary ionocytes, it did not affect the cAMP-activated currents, suggesting that CFTR in this cell type has additional functions that are not related to the CFTR-mediated ion transport.

In conclusion, we believe that this work contributed to a better understanding of how Cl^- channels, mucus production and epithelial differentiation are regulated in the airways, particularly under inflammatory modulators. Nonetheless, it also raised new questions that can be assessed in the future, namely:

1) Use BCi-CF1.1 cells to identify small molecules/drugs acting as novel TMEM16A potentiators that act specifically in non-proliferating differentiated airway epithelial cells.
2) Determine the signalling pathways responsible for epithelial differentiation in BCi-NS1.1 vs BCi-CF1.1 cells by identifying genes that are differentially expressed using single-cell RNA sequencing.
3) Assess the effect of disease-specific inflammatory mediators on BCi-CF1.1 cells, particularly to determine whether IL-10 and IL-22 can rescue the defect in ciliated-cell differentiation.
4) Determine the contribution of the different airway epithelial cell types to CFTR function by abolishing the expression of cell-type-specific transcription factors in BCi-NS1.1 cells, thereby modulating epithelial differentiation.

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