UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS

DEPARTAMENTO DE BIOLOGIA VEGETAL

Role of Ubiquitin Ligases in Pathophysiology of Cystic Fibrosis

Sara Inês de Ascensão Tavares Canato

Dissertação

Mestrado em Biologia Molecular e Genética

2012

UNIVERSIDADE DE LISBOA
FACULDADE DE CIÊNCIAS

DEPARTAMENTO DE BIOLOGIA VEGETAL

Role of Ubiquitin Ligases in Pathophysiology of Cystic Fibrosis

Sara Inês de Ascensão Tavares Canato

Dissertação orientada pelo Prof. Doutor Luka A. Clarke e pela

Prof. Doutora Margarida B. Telhada

Dissertação

Mestrado em Biologia Molecular e Genética

2012

Table of Contents

Acknowledgments/Agradecimentos

Ao concluir este trabalho, não posso deixar e agradecer a todos aqueles que de alguma forma contribuíram para a sua realização.

Em primeiro lugar gostaria de agradecer aos meus orientadores ao Professor Luka Clarke por toda a sua ajuda, disponibilidade e confiança depositada ao longo deste ano de trabalho. À Professora Margarida Telhada queria agradecer toda a preocupação, empenho, simpatia e ajuda prestada. Sem dúvida que a ajuda de ambos foi fundamental para o desenvolvimento deste trabalho.

Queria também agradecer à Professora Margarida Amaral por me ter dado a oportunidade de desenvolver o meu trabalho no seu laboratório, pela disponibilidade e confiança depositada.

Agradeço também à Rita Maldonado e ao grupo da Professora Isabel Sá-Correia por permitirem o desenvolvimento das experiências através da disponibilização do sobrenadante de bactéria.

Queria agradecer a todos os meus colegas de laboratório, com os quais partilhei um ano de muito crescimento quer a nível profissional quer a nível pessoal. À inna um muito obrigado, a primeira pessoa com quem contactei e meu deu a conhecer todo o fantástico mundo das células, à Marisa por ter sido incansável comigo, tendo prestado sempre a sua ajuda, à Marta (pessoa indispensável neste laboratório) pelos seus ensinamentos, paciência e ajuda, estando sempre disponível! Queria agradecer muito à Verónica e ao João Fernandes (Johnny), os meus fieis companheiros, com quem partilhei momentos de muita alegria fora e dentro do laboratório, sem os nossos momentos happy isto não teria sido a mesmo! Ao Simon, pela pessoa fantástica que é, sempre com uma palavra amiga a dar e uma novela romântica a fazer. Queria também agradecer ao João Coelho (Johnny Johnny), finalmente no 3º ano e à Georgina pela ajuda e companhia, ao Hugo, o meu colega de bancada que muito me apoio, à Ana Cachaça obrigado pelo apoio e disponibilidade. Agradeço também ao Professor Carlos e à Professora Anabela pela sua ajuda, ensinamentos e disponibilidade. Queria também agradecer ao Gabriel e ao Paulo Matos por disponibilizarem a sua ajuda no microscópio. Não posso deixar de agradecer ao grupo da Professora Margarida Gama Carvalho, à Ana Luísa, à Ana Margarida ao Jorge e à Sara pelos seus conselhos, ajuda e partilha.

Obviamente agradeço aos meus amigos de longa data, à Ana Cláudia Gonçalves um obrigado muito especial, com quem partilhei os 5 anos do meu percurso académico a sua companhia, ajuda e alegria contribuíram para todo o desenvolvimento desta tese. À filipa,

Ana, Marisa, Miguel e Mariana um obrigado por tudo, pelas pessoas fantásticas que são, que me acompanham desde há muitos anos, nesta tese está representado aquilo que vos fez esperar muitas horas.

Por último agradeço à minha família, à minha mãe ao meu pai e aos meus irmãos, sem eles nada disto tinha sido possível, graças a eles consegui chegar aqui. Muito obrigado!

Summary

Cystic Fibrosis (CF) is the most common lethal monogenic autosomal recessive disease in the Caucasian population and is caused by dysfunction of the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) protein which is located at the apical membrane of epithelial cells. F508del is the most predominant disease-causing mutation.

CF disease is characterized by an aggressive inflammatory response and chronic infection in the airway. E3 ubiquitin ligases are negative regulators of the TGF-β signaling pathway, which has important anti-inflammatory properties and is dysregulated in CF. Studies have led us to hypothesize that dysregulation of these negative regulators of TGF-β might be partly responsible for the pro-inflammatory phenotype of CF.

In an attempt to understand the role of E3 ubiquitin ligases in CF, we focused here on studying the expression of *SMURF1* and *SMURF2* and *NEDD4L* in CFBE41º- cells expressing wt-CFTR and F508del-CFTR.

Under basal conditions our results demonstrated that the F508del mutation is not sufficient to induce a significant differential expression of E3 ubiquitin ligases.

Application of TGF-β and TNF-α at the cell surface of unpolarized CFBE cells proved insufficient to mimic the inflammatory status observed in CF patients and to study the expression of E3 ubiquitin ligases under inflammatory stimuli.

We therefore performed further experiments in polarized CFBE cells. In this system, TGF-β and TNF-α increased the expression of *SMURF2* in cells expressing F508del-CFTR. TNF-α also increased the expression of *NEDD4L* in the F508del-CFTR cell line. These results may suggest an attenuation of the TGF-β signal that may be partly responsible for increasing pro-inflammatory signalling in CF.

Our results demonstrated that bacterial infection, cell polarization and CFTR genotype may each be partly responsible for alterations in E3 ubiquitin ligase expression that may contribute to inflammation in the CF airway.

Key words: Cystic Fibrosis, F508del mutation, E3 ubiquitin ligases, TGF-β, *B. cenocepacia*

Resumo

A Fibrose Quística (FQ) é a doença autossómica recessiva letal mais comum na população Caucasiana, causada por uma função anormal da proteína CFTR (do inglês, Cystic Fibrosis Transmembrane Conductance Regulator), localizada na membrana apical de células epiteliais. F508del consiste na mutação causadora da doença mais predominante.

A FQ é caracterizada por uma resposta inflamatória agressiva e infeção crónica das vias respiratórias. A via de sinalização TGF-β, envolvida na resposta anti-inflamatória é regulada negativamente por E3 ubiquitina ligases e parece estar desregulada na FQ. Desta forma, a nossa hipótese é de que uma desregulação destes reguladores negativos do TGF-β possa contribuir para o fenótipo inflamatório verificado na FQ.

Com o objetivo final de clarificar o papel das E3 ubiquitina ligases na FQ, estudamos a expressão de *SMURF1*, *SMURF2* e *NEDD4L* em células CFBE41º- que expressam wt-CFTR e F508del-CFTR.

Com base nesta análise, a mutação F508del não demonstrou ser suficiente para induzir um padrão diferencial da expressão das E3 ubiquitina ligases.

A exposição do TGF-β e TNF-α na superfície de células CFBE não polarizadas demonstrou ser insuficiente para mimetizar o perfil inflamatório observado em pacientes de FQ e para o estudo da expressão das E3 ubiquitina ligases sobre condições de inflamação.

Desta forma, começamos por realizar as experiências em células CFBE polarizadas. Neste sistema a linha celular F508del-CFTR quando tratada com TGF-β e TNF-α demonstrou aumentar os níveis de expressão de *SMURF2*. O mesmo foi verificado para *NEDD4L* em resposta ao TNF-α. Estes resultados sugerem uma atenuação da via de sinalização TGF-β que pode ser parcialmente responsável pelo aumento da sinalização pro-inflamatória na FQ.

Por último, os nossos resultados demonstram que a infeção bacteriana, a polarização celular e o genótipo CFTR podem ser parcialmente responsáveis pela alteração da expressão das E3 ubiquitina ligases, contribuindo para o fenótipo inflamatório da FQ.

Paravras-chave: Fibrose Quística, mutação F508del, E3 ubiquitina ligases, TGF-β, *B. cenocepacia*

Resumo Alargado

A Fibrose Quística (FQ) é numa doença autossómica recessiva letal causada por mutações ocorridas num gene, localizado no cromossoma 7 (7q31), que codifica para uma glicoproteína designada CFTR (do inglês, *Cystic Fibrosis Transmembrane Conductance Regulator*). Esta proteína consiste num canal de cloreto (Cl^-) presente na membrana apical de células epiteliais que regula o transporte transepitelial de iões e água. A população Caucasiana é a mais afetada com uma frequência de 1 em cada 2500-6000 nascimentos, sendo a principal consequência a doença pulmonar crónica, que é a principal causa de mortalidade e morbilidade em FQ. A doença pulmonar crónica é causada por um desequilíbrio iónico do líquido que reveste as vias respiratórias ASL (do inglês, airway surfasse liquid) levando ao aumento de espessura e desidratação do muco. Desta forma, pacientes com FQ apresentam elevada suscetibilidade a infeções bacterianas recorrentes, nomeadamente *Pseudomonas aeruginosa*, e a intensas respostas inflamatórias. A delecção de um único aminoácido, uma fenilalanina na posição 508 (F508del) está presente em aproximadamente 90% dos pacientes com FQ.

Estudos anteriores sugerem uma desregulação ao nível das cascatas de sinalização envolvidas na modulação da resposta inflamatória das vias respiratórias de pacientes FQ, que poderá estar envolvida no elevado número e proporção de neutrófilos e seus produtos, bem como elevados níveis de interleucinas pro-inflamatórias (IL1, IL6, IL8 e TNF-α), e seus mediadores (NF-kB e STAT) e baixo níveis de interleucinas anti-inflamatórias (IL-10).

TGF-β (do inglês, *transforming growth factor beta*) consiste numa citoquina maioritariamente envolvida em processos anti-inflamatórios, mediados pela via SMAD2/3. Esta cascata é iniciada aquando da ligação do TGF-β ao recetor tipo II, o qual forma um complexo com o recetor tipo I, promovendo a sua fosforilação. Posteriormente o recetor tipo I do TGF-β recruta e fosforila diretamente SMAD2/SMAD3, R-smads (do inglês, *receptor-regulated smad*), as quais vão formar complexos com SMAD4, uma Co-smad (do inglês, *common-partner smad*), sendo posteriormente translocados para o núcleo, onde se vão ligar a vários elementos de resposta de modo a regular a expressão dos genes envolvidos nos mais variados processos celulares (ex, sinalização celular, polarização e endocitose). Esta via de sinalização do TGF-β é regulada negativamente por E3 ubiquitina ligases, nomeadamente SMURF (do inglês, *Smad ubiquitination regulatory factor*) 1 e 2 e NEDD4L (do inglês, *Neural precursor cell expressed developmentally downregulated 4-like*), que promovem, juntamente com as enzimas E1 (do inglês, *E1 ubiquitin-activators*) e E2 (do inglês, *E2 ubiquitin-conjugation*) a ubiquitinação e subsequente degradação de proteínas

alvos pelo proteassoma 26S. SMURF1 e SMURF2 estão envolvidos, juntamente com SMAD7, uma I-smad (do inglês, *inhibitory smad*) na ubiquitinação do recetor tipo I do TGF-β. SMAD2 e SMAD3 também são promovidos para degradação por SMURF2 e NEDD4L, respetivamente.

Estudos têm demonstrado por parte do TGF-β um papel na gravidade da doença de FQ, tendo sido associado à inflamação mediada por neutrófilos e diminuição da função pulmonar, sugerindo uma possível desregulação na FQ. Além disso, dados recentes sugerem que proteínas do sistema de ubiquitinação, incluindo HECT E3 ubiquitina ligases, encontram-se desreguladas em estudos de expressão relacionados com F508del-CFTR. Tendo em conta esta informação, bem como estudos preliminares desenvolvidos no nosso grupo, a hipótese sugerida é de que uma possível desregulação das E3 ubiquitina ligases na FQ poderia estar implicada numa anormal modulação dos sinais inflamatórios por parte da via de sinalização TGF-β.

Com o objetivo final de esclarecer o papel das E3 ubiquitina ligases na FQ, centralizamos o nosso estudo na análise da expressão dos genes *SMURF1*, *SMURF2* e *NEDD4L* em células CFBE41º- (do inglês, *Cystic fibrosis bronchial epitelial cells*).

Numa primeira abordagem, experiências realizadas em células CFBE que expressam wt-CFTR e F508del-CFTR sobre condições basais, bem como em células epiteliais nasais, não verificámos uma diferença significativa na expressão dos genes em estudo.

Sendo a doença de FQ caracterizada por um perfil inflamatório, do qual as citoquinas TGF-β e TNF-α fazem parte, foi analisado o efeito destas citocinas na expressão de *SMURF1* e *NEDD4L* em CFBE não polarizadas. Não verificamos diferenças significativas no padrão de expressão de *SMURF1* e *NEDD4L* em células CFBE não polarizadas tratadas com TGF-β. No entanto, na linha celular F508del-CFTR quando tratada com TNF-α verificou-se uma diminuição na expressão de ambos os genes em estudo. Os resultados sugerem uma atenuação na regulação negativa por parte de SMURF1 e NEDD4L na via de sinalização TGF-β sob estímulos pró-inflamatórios (TNF-α) na FQ, o que poderia traduzir-se num balanço entre estímulos anti-inflamatórios e pro-inflamatórios, importantes na regulação das cascatas da resposta inflamatória. No entanto, o perfil inflamatório verificado em pacientes de FQ não demonstra haver esta regulação.

De seguida analisámos a distribuição dos recetores do TGF-β em células CFBE não polarizadas. Verificou-se que o recetor tipo II de TGF-β localiza-se essencialmente no citoplasma e nas regiões de adesão celular. Assim, este resultado sugeriu uma ineficiente resposta por parte das células não polarizadas ao TGF-β devido à localização dos seus recetores.

Desta forma foi estabelecido um modelo celular polarizado de células CFBE, no qual se verificou em ambas as linhas celular a aquisição de um fenótipo polarizado, caracterizado por uma elevada resistência transepitelial, com expressão de ZO-1, uma proteína das TJ (do inglês, *tight junction*) e *E-cadherin*, uma proteína de adesão celular. No entanto, células CFBE F508del-CFTR demonstraram um atraso na aquisição deste fenótipo.

Procedeu-se à análise da expressão dos genes *SMURF1*, *SMURF2* e *NEDD4L* em células CFBE polarizadas sob estímulos anti-inflamatórios (TGF-β) e pro-inflamatórios (TNF-α) aplicados nas membranas apical e basolateral de células epiteliais, de modo a mimetizar o perfil inflamatório ocorrido na FQ. Relativamente à expressão de *SMURF1* não se verificou diferenças significativas em ambas a linhas celulares. No entanto, TGF-β e TNF-α aumentaram significativamente a expressão de *SMURF2* em células CFBE polarizadas que expressam F508del-CFTR. O mesmo se verificou para a expressão de *NEDD4L* em células CFBE polarizadas tratadas com TNF-α que expressam F508del-CFTR. Estes resultados sugerem um aumento da expressão de *SMURF2* e *NEDD4L* na FQ em condições de inflamação. Dados sugerem um envolvimento de TGF-β na inibição de sinais pro-inflamatórios, incluindo IL6 pelo mediador SMAD2 e IL8, pela via SMAD3. Assim, devido ao aumento de expressão de *SMURF2* e *NEDD4L* e consequente possível diminuição de SMAD2 e SMAD3, respectivamente, estes resultados sugerem uma atenuação da via de sinalização TGF-β.

Sendo os recetores do TGF-β alvos da via proteolítica mediada pelas E3 ubiquitina ligases, os níveis de expressão do recetor tipo II do TGF-β foram também analisados. Os resultados demonstraram um aumentou na expressão do gene para o recetor tipo II do TGF-β em células CFBE polarizadas expressando F508del-CFTR quando tratadas com TNF-α, nas quais se verificou um aumento de *SMURF2*. Este resultado demonstra a necessidade de uma abordagem funcional complementar ao estudo da expressão génica.

A via de sinalização TGF-β é mediada também por uma via independente das smads, a qual tem demonstrado estar envolvida na polarização. Vários estudos têm sugerido uma implicação da via TGF-β independente das smads na dissolução das TJ, a qual é promovida por SMURF1. Os nossos resultados demonstraram uma diminuição da resistência transepitelial em células tratadas com TGF-β e TNF-α, bem como um padrão de organização de ZO-1 anormal, característico de uma eventual disrupção das TJ.

Sendo a FQ também caracterizada por uma intensa colonização bacteriana, também determinante do perfil inflamatório verificado em pacientes de FQ, a expressão dos genes *SMURF1*, *SMURF2* e *NEDD4L* foi analisada em células CFBE polarizadas e não

polarizadas sob estímulo de infeção com sobrenadante de *Burkholderia cenocepacia.* Os resultados demonstraram que tanto a infeção como o estado de diferenciação das células são ambos causadores de uma expressão diferencial por parte *SMURF1* e *SMURF2*. Com o intuito de averiguar este padrão de expressão, foram analisados marcadores inflamatórios e o estado de integridade epitelial. Os resultados não demonstraram uma significante afetação de componentes da inflamação e polarização/diferenciação por parte do sobrenadante da bactéria. No entanto, verificou-se uma redução do nível de expressão de CFTR em ambas as linhas celulares polarizadas tratadas com sobrenadante.

As observações incluídas neste estudo contribuem para uma melhor compreensão da expressão das E3 ubiquitina ligases na FQ e sua implicação nas vias de sinalização inflamatórias. Além disso, os resultados aqui incluídos demonstram a importância do modelo celular para estudos envolvidos na inflamação relacionada com FQ.

Abbreviations

Ω	Ohm
ABC	ATP-binding cassette
DAPI	49,6-diamidino-2-phenyl-indole, dihydrochoride
ASL	Airway surface liquid
ATP	Adenosine triphosphate
BAL	Brochoalveolar lavage
BSA	Bovine serum albumin
$CaCl_2$	Calcium Chloride
C-terminal	Carboxyl terminal
CF	Cystic fibrosis
CFBE	Cystic fibrosis bronchial epithelial cells
CFF	Cystic Fibrosis Foundation
CFTR	Cystic fibrosis transmembrane conductance regulator
Cif	CFTR inhibitor factor
Cl^-	Chloride
CO_2	Carbon dioxide
Co-smad	Common-parter Smad
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugation enzyme
E3	Ubiquitin-ligase enzyme
EMT	Epithelial to mesenchymal transition
ENaC	Epithelial sodium (Na^+) channel
ER	Endoplasmatic reticulum
FBS	Fetal bovine serum
HBSS	Hank's balanced salt solution
HCO_3^-	Bicarbonate
HECT	Homologous to E6-Ap C-terminal domain
I-smad	Inhibitory smad
IL	Interleukin
IFN-γ	Interferon-gama
IkB	Inhibitor of factor nuclear kappa B (NF-kB)
JAK	Janus Kinase
MAPK	MAP Kinase
MCC	Mucociliary clearance

Sara Inês Canto
Molecular Biology and Genetics

MDCK	Madin-Darby canine kidney
MEM	Minimal essential media
$MgCl_2$	Magnesium Chloride
MSD	Membrane spanning domain
N-terminal	Amino-terminal
NBD	Nucleotide binding domain
Nedd4l	Neural precursor cell expressed developmentally downregulated 4-2
NES	Nuclear exporter signal
NLS	Nuclear localization signal
PAGE	Polyacrylamide gel electrophorese
PBS	Phosphate buffered saline
PenStrep	Penicillin streptomycin solution
PIAS	Inhibitor of activated STAT
PI3K	Phosphatidynositol-3-kinase
PKA	Protein Kinase A
PCR	Polymerase chain reaction
PY	Proline rich region
qRT-PCR	Quantitative real time polymerase chain reaction
R-smad	Receptor-regulated Smads
RD	Regulatory domain
RING	Really interesting new gene
RT-PCR	Reverse transcription polymerase chain reaction
Smurf	Smad ubiquitin regulated factor
STAT	Signal transducer and activator of transcription
TER	Transepithelial resistance
TGF- β	Transforming growth factor β
TGF- βR	Transforming growth factor β receptor
TJ	Tight junction
TLR	Toll-like receptor
TM	Transmembrane segment
TNF-α	Tumor necrosis factor alpha
TNFR	Tumor necrosis factor receptor
Ub	Ubiquitin
UV	Ultraviolet
ZO-1	zona occludens

Sara Inês Canto
Molecular Biology and Genetics

I. General Introduction

1. Cystic Fibrosis

Cystic Fibrosis (CF) is the most common autosomic recessive lethal disorder in Caucasians, affecting about 1 in 2500-6000 live births and with a carrier frequency of 1 in 25 individuals[1]. CF is clinically characterized by pancreatic insufficiency and a progressive lung disease, caused by abnormal regulation of ion transport by the cystic fibrosis transmembrane conductance regulator (CFTR) channel in respiratory epithelium. However, current therapies have improved the quality of life and prognosis of CF patients, according to the Cystic Fibrosis Foundation (CFF), the life expectancy of individuals with CF is estimated at 37 years of age[2].

1.1 The CFTR Gene and Protein

The *CFTR* gene which spans 190kb is located on chromosome 7 at position 7q31.2, and is composed of 27 exons resulting in an mRNA of 6.5kb following transcription. The CFTR protein is composed of 1480 amino acids and has a molecular weight of 170 kDa[3]. It is a chloride (Cl^-) channel predominantly expressed at the apical membrane of epithelial cells. As a member of the ATP-binding cassette (ABC) transporter superfamily, the CFTR protein has two membrane spanning domains (MSD1 and MSD2), each composed of six transmembrane segments (TM1-TM12) which form the channel, two nucleotide binding domains (NBD1 and NBD2), where adenosine triphosphate (ATP) is hydrolysed, and finally a regulatory domain (RD) that contains phosphorylation sites (Fig. I. 1).

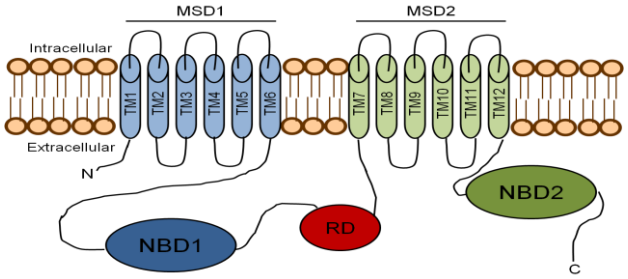


Figure I. 1 – Model of the CFTR protein at the plasma membrane

ATP hydrolysis by the NBD domains, together with R domain phosphorylation by cyclic adenosine monophosphate (cAMP)-regulated protein kinase A (PKA) are essential for activating the channel (Fig. I. 2)[4,5].

There are 1913 mutations described in the CFTR gene[6], and these mutations are grouped in five classes according to their specific effects upon CFTR biosynthesis and function (Table VIII. A1). F508del (the most common mutation) leads to CFTR retention in

the endoplasmic reticulum (ER), where it is prematurely degraded. Thus F508del-CFTR at the apical membrane of native epithelial cells from CF patients is reduced. Furthermore, the ion channel function of F508del CFTR protein is also reduced, even if it reaches the apical membrane[7].

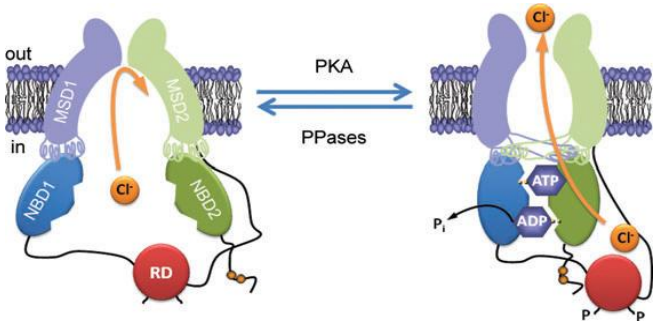


Figure I. 2 – Simplified model for Cl^- ion permeation through the plasma membrane via the CFTR channel [5]

1.2 Function of CFTR in Epithelia

CFTR functions as a Cl^- channel, and plays a fundamental role in fluid and electrolyte transport across the epithelial cells, and regulation of the airway surface liquid (ASL) composition. CFTR can also conduct bicarbonate (HCO_3^-), providing an important role as a regulator of transmembrane pH gradients[8]. This channel therefore ensures epithelial hydration and efficient mucociliary clearance (MCC), by regulating transepithelial salt and water movement. This is achieved through the regulation of several channels and transporters, including the epithelial sodium channel (ENaC), potassium (K^+) channels and aquaporin water channels[9,10]. Furthermore, it was reported that CFTR is implicated in the control of intracellular reactive oxidative species balance, via glutathione (GSH) transport[11].

1.3. Characteristic inflammatory status of CF lung disease

Defective CFTR function leads to a reduced ASL volume as well as thick and dehydrated airway mucus, which results in a self-perpetuating cycle of airway obstruction. This cycle is characterized by bacterial infections (*Pseudomonas aeruginosa*), and chronic inflammation, resulting in bronchiectasis and death[12,13,14]. Airways of CF patients are characterized by elevated concentrations of neutrophil chemoatractants, neutrophils and their products, as well as high levels of the pro-inflammatory cytokines tumor necrosis factor alpha (TNF-α), interleukins (eg, IL6, IL8 and IL1) and low concentrations of anti-inflammatory cytokines (eg, IL10)[15,16,17,18]. Although bacteria also contribute to the disease process by stimulating overproduction of pro-inflammatory cytokines[19,20], these inflammatory markers are found in CF, even in the absence of infection with characteristic CF pathogens[21,22,23]. Thus, inflammatory cascades could be deregulated in CF disease, which could promote an imbalance between pro- and anti-inflammatory responses, potentially causing an excessive

inflammation response, leading to epithelial damage and favouring bacterial colonization. However, other studies have suggested that infection happens early in the disease, anticipating the inflammatory response[24], and the exaggerated immune response in CF may result from the loss of CFTR apical membrane channel function, leading to concentration of pro-inflammatory mediators, reducing MCC of bacteria and leading to activation of cellular signalling[25].

2 E3 Ubiquitin Ligases: HECT family overview

The ubiquitin conjugation system is a major protein degradation pathway that regulates a wide variety of biological processes (eg, signal transduction and endocytosis)[26,27]. This cascade involves sequential enzymatic reactions catalyzed by three classes of enzymes, namely E1 ubiquitin-activators, E2 ubiquitin-conjugation enzymes and E3 ubiquitin ligases. Ubiquitin (Ub) is first activated by binding to an E1 through a thioester bond with a cysteine residue at the catalytic site and this is followed by subsequent transfer of the Ub to an E2 by transthiolation. Finally, an E3 ligase promotes the transfer of Ub from the E2 to the target protein for degradation by the 26S proteasome[28]. E3 ubiquitin ligases are responsible for the specific recognition of a large number of target proteins, a process that requires specificity and versatility, which are provided by the existence of more than 600 enzymes[29]. These proteins are grouped into three classes based on structure, namely HECT E3 ligases, RING-finger E3 ligases and U-box E3 ligases. HECT E3 ubiquitin ligases function as scaffold proteins that have binding sites for E2 conjugation enzymes, but unlike the other groups, they play a catalytic role by directly promoting the attachment of Ub to a substrate protein, through a conserved cysteine residue[30,31]. Thus, deregulation of HECT E3 ubiquitin ligase activity has been associated with the development of human diseases (eg, cancer) and a more general protein ubiquitination pathway including HECT E3 ubiquitin ligases has been found to be dysregulated in several studies of F508del-CFTR-related gene expression[32,33].

2.1 Structure of SMURF1, SMURF2 and NEDD4L

Nedd4-like proteins, such as SMURF (Smad ubiquitination regulatory factor) 1 and 2 and NEDD4L (Neural precursor cell expressed developmentally downregulated 4-like) are members of the HECT E3 ubiquitin ligase family[34]. This enzyme class is characterized by a calcium-dependent phospholipid binding C2 domain in the N-terminal region, that is involved in membrane binding[35], a central region with two to four WW domains, which interact with short proline-rich regions (PY motifs) of the substrate protein[36] and a HECT domain in the C-terminal region that is responsible for E2 ubiquitin–conjugating enzyme interaction, and is important for attachment of Ub to a substrate protein[37,38].

SMURF1 is characterized by an N-terminal C2, two WW domains and a C-terminal HECT domain, while SMURF2 and NEDD4L possess three and four WW domains, respectively[38]. Although the C2 domain promotes localization to the plasma membrane, it has also been found to be involved in an auto-inhibition mechanism of SMURF2, through a HECT domain interaction, which regulates the E3 ligase activity and protects the enzymes and their substrates from futile degradation[39]. In addition, a recent study demonstrated that the C2 domain also appears to be important for substrate selection[40]. Another important study has demonstrated that SMURF1 has a nuclear exporter signal (NES) in HECT domain, which is responsible for nuclear export via its interaction with a nuclear export receptor (CRM1). Thus, SMURF1 is predominantly localized in both nucleus and cytoplasm[41], while SMURF2, without the NES, is predominantly nuclear[42].

2.2 Role of E3 ubiquitin ligases in TGF-β Signaling Pathway

SMURF1, SMURF2 and NEDD4L are negative regulators of the transforming growth factor beta (TGF-β) signaling pathway via promotion the ubiquitination and subsequent degradation of its components[43,44,45]. TGF-β is a multifunctional cytokine, which has been implicated in important cellular activities (eg, immune response, cell adhesion, and polarization)[46,47,48]. Evidence suggests that alteration of TGF-β signaling is implicated in human diseases including inflammatory disorders[49], cancer[50] and Cystic Fibrosis[51].

2.2.1 TGF-β from receptor to smad

The classical TGF-β cascade (Fig. I. 3) involves the formation of the transmembrane heterotetrameric complex of type I and type II serine/threonine kinase receptor (TGF-βRI and TGF-βRII) at the cell membrane[52]. Following TGF-β isoform (TGF-β_1, TGF-β_2 or TGF-β_3) binding, the TGF-βRII phosphorylates the TGF-βRI, which in turn recruits and phosphorylates the TGF-β signaling mediators, such as SMAD2 and SMAD3, a receptor-regulated smad (R-smad), at the SS(V/M)S motif located in their C-terminal MH2 domain. The half-life of this phosphorylation kinetic is approximately 5 min[53] and following TGF-β stimulation of epithelial cells, receptors remain active for at least 3 to 4 hours[54]. Subsequently activated R-smads associate with SMAD4, a common-partner smad (Co-smad), and these complexes are translocated into the nucleus, via a lysine rich nuclear localization signal (NLS) within the N-terminal MH1 domain of which protein, that is also responsible for DNA-binding[55]. Once there, the smad complex binds to transcriptional factors, such as FoxH1, Mixer, Runx-related proteins and E2F, as well transcriptional co-activators (p300 and CBP) and co-repressors (cSki, and SnoN) to regulate transcriptional target genes[56]. Activated R-smad proteins undergo constant nucleocytoplasmic shuttling by interaction with nuclear pore complexes, via a nuclear export signal (NES) in the linker region, located between the MH1

and MH2 domains. The latter domain of smad is responsible for cytoplasmic retention by establishing contacts with anchor proteins and promoting R-smad/R-smad and R-smad/Smad4 complex formation through the phosphorylation of its last two serine residues[57,58].

However, some reports have demonstrated that there is smad-independent TGF-β signalling, which is activated by TGF-β receptors and leads to crosstalk between other pathways (eg, MAP kinase (MAPK), Rho GTPase, and phosphatidynositol-3-kinase (PI3K)/AKT)[59,60]. These pathways also regulate the interaction between smad and co-activators or co-repressors contributing to the specificity of TGF-β signal[61].

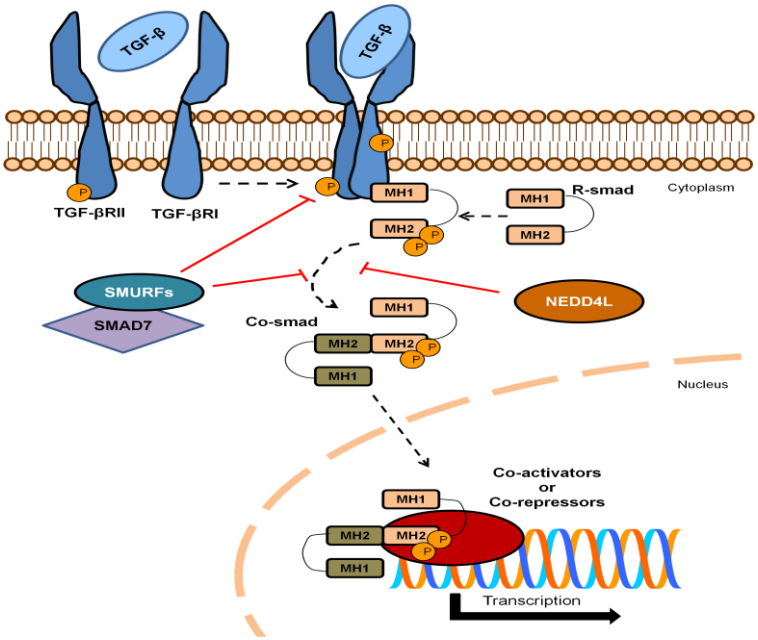


Figure I. 3 – Smad-dependent TGF-β signaling.

2.2.2 Regulation of TGF-β by inhibitory SMADs and E3 ubiquitin ligases

Smad-dependent TGF-β signals are regulated by SMAD7, an inhibitory smad (I-smad), which is induced by TGF-β signalling. This protein targets the R-smad and TGF-βRI for ubiquitin-mediated degradation, or competes with R-smads for binding to TGF-βRI, resulting in the inhibition of TGF-β signalling[38]. In addition, it was demonstrated that SMAD7 is predominantly located in the nucleus, where it can inhibit the TGF-β signal by binding the DNA and disrupting the smad-DNA complex[62]. I-smad and R-smad have PY motifs in their linker regions, allowing SMURF1, SMURF2 and NEDD4L to bind and thereby contribute to their negative feedback role. In response to TGF-β stimuli, SMURF1 and SMURF2 associate with SMAD7 in the nucleus, inducing a cytoplasmic accumulation of SMAD7/SMURF1 and SMAD7/SMURF2 complex, by NES and CRM1 interaction[41]. Consequently, SMAD7 recruits SMURF1 and SMURF2 to TGF-βRI, inducing its ubiquitination, followed by degradation

through the proteasome[42,43]. Consistent with the physical interaction between E3 ubiquitin ligases and smad proteins, other studies have reported that SMURF2 also targets SMAD2 for degradation[62,63], while NEDD4L promotes the degradation of both SMAD2 and SMAD3 by ubiquitination and limits the half-life of TGF-β activated smads[64]. Although SMAD4 lacks the PY motifs for E3 ubiquitin ligase interaction, it was demonstrated that SMURF2 and NEDD4L together with SMAD7 could promote SMAD4 degradation. This smad protein is predominantly localized in the cytoplasm, where it interacts with SMAD7, by MH2 domain, together with SMURF2 or NEDD4L, inducing its ubiquitination and degradation[65]. As demonstrated above, E3 ubiquitin ligases are important for localization, activity and stability of several components of TGF-β signaling pathways.

3 Relevance of the E3 ubiquitin ligases and TGF-β to CF disease

The major cause of morbidity and mortality in CF is lung disease, which is comprised of inflammation[66], bacterial infection[67], and epithelial repair and regeneration, each of which plays an important role in disease progression[68,69]. An important finding underpinning the present study is that TGF-β has been demonstrated to affect disease severity in CF[70,71,72]. In pulmonary tissue, TGF-β is produced essentially by bronchial epithelial cells[73]. TGF-β is elevated in CF bronchoalveolar lavage (BAL) and plasma and is associated with neutrophilic inflammation and diminished lung function[74]. It is not clear whether elevated TGF-β levels represent a cause or consequence of CF disease, but it may serve as a useful biomarker for CF lung disease, or as a modifier of CF lung disease severity[73].

3.1 TGF-β signal transduction and crosstalk with other cytokines

TGF-β modulates the inflammatory response through regulation of inflammatory cytokines. TGF-β signaling was shown to regulate IL-8 and IL-6 expression. Its regulation was mediated directly by changes in transcriptional activity of the cytokines at the level of promoter activity, or indirectly via interference of inflammatory mediators[74,75,76]. This provided evidence that TGF-β signaling exerts a crucial role in the anti-inflammatory response, by down-regulating the phosphorylation of STAT3 (signal transducer and activator of transcription 3) transcription factor activated through JAK (Janus kinase), which is induced by IL6 in intestinal epithelial cells[77,78]. Furthermore, a recent study also demonstrated that TGF-β - induced SMAD7 binds to PELLINO-1, a protein involved in toll-like receptor (TLR)-IL1R signaling, through its MH2 domain, and down-regulates the pro-inflammatory signal. SMAD7 blocks the ability of PELLINO-1 to form a complex with MyD88, IRAK1, IRAK4 and TRAF6. Thus, TRAF is unable to induce the degradation of the inhibitor of NF-kB (IkB), to promote the pro-inflammatory response[79,80]. SMURF1, in addition to TGF-β, has been found to function as an inhibitor of NF-kB signaling by inducing the ubiquitination of TRAF4, followed

by proteasome degradation[81]. In this context, it is interesting that in BAL fluid, sputum, serum, and respiratory epithelial cells and nasal epithelial cells from CF patients there are excessive concentrations of TNF-α, IL6, IL1 and IL8[15,16,17,18], whose synthesis is promoted by NF-ĸB[82,83]. Investigators have found that NF-ĸB signaling is deregulated, after finding exaggerated activation of NF-ĸB and altered IĸB processing in CF bronchial epithelial cells[84]. Another study reported that SMURF1 is a negative regulator of interferon-gamma (IFN-γ), a cytokine that plays essential roles in anti-viral, anti-proliferative and anti-tumoral activities. SMURF1 interacts with the PY motif of STAT1 (an IFN-γ signal mediator) promoting its degradation[85]. STAT1 and protein inhibitor of activated STAT1 (PIAS1) have been shown to be deregulated in CF[86].

3.2 Role of TGF-β in epithelial integrity and cell polarity

In epithelial cells, cell-cell adhesion is established with the assembly of tight junctions (TJ), which create a barrier to the diffusion of solutes across the cell, and also function as a boundary between the apical and basolateral membranes. This is an important process during cell differentiation and tissue homeostasis[87,88]. High levels of inflammation and infection may alter TJ barrier function, inducing severe modification of epithelial integrity[89,90]. After injury, airway epithelial cells modify their structure and function to adapt to the change or to repair the epithelium[91]. The CF airway epithelium was found to have a delay in differentiation[92], and the abnormal distribution of the CFTR protein in CF airway cells may also be caused by disruption of epithelial phenotype[93].

E3 ubiquitin ligases together with TGF-β play an important role in cell polarity. TGF-β induces the dissolution of TJ protein (zona occludens - ZO-1) and decreases the expression of epithelial markers (E-cadherin and occludin) during epithelial to mesenchymal transition (EMT) by triggering degradation of RhoA at cellular protrusions. TGF-β-induced RhoA degradation requires the phosphorylation of Par6 by TGF-βRII, that recruits SMURF1[95,96,97]. Interestingly, NEDD4L also appears to regulate TJ assembly by promoting occludin degradation[98]. In the case of SMURF2, it was demonstrated that this E3 ubiquitin ligase is required for the establishment of neuronal polarity by promoting interaction with mPar3 and degradation of Rap1B[99,100]. In addition, it was reported that NEDD4L interacts with ENaC and targets the channel for endocytosis and degradation. The increased number of ENaC channels at the membrane surface observed in CF also occurs in Liddle's syndrome, linked to mutations in the ENaC channel that alter or delete its PY motif, leading to disruption of the interaction between NEDD4L and this channel. In concordance with this study, the specific ablation of NEDDL4 in lung epithelia in mice demonstrated elevated ENaC levels and

function, leading to such CF-like symptoms as lung dehydration, hyper-inflammation and mucus accumulation in the airways[101].

Although the role of E3 ubiquitin ligases in the immune response is not clear, given the importance of these enzymes in anti- and pro-inflammatory cascades, and the abnormal levels of anti- and pro-inflammatory cytokines in CF, together with their function in cell polarity and paracellular pathways, a role in pathogenesis of CF lung disease can be envisaged.

II. Objectives of the present work

The present work aimed to study the role of E3 ubiquitin ligases such as SMURF1, SMURF2 and NEDD4L upon responses to inflammatory stimuli (TGF-β, TNF-α and Bacteria) using immortalized cystic fibrosis bronchial epithelial (CFBE) cell lines as a model. We thereby intended to understand whether dysregulation of E3 ubiquitin ligases associated with expression of mutant CFTR might influence TGF-β signaling, leading to a hyper-inflammatory phenotype.

We therefore proposed to evaluate:

- Differential expression of NEDD4L, SMURF1 and SMURF2 in CFBE cells;
- Patterns of E3 ubiquitin ligase expression during cell polarization;
- The hyper-inflammatory phenotype in cell models of CF;

Using this experimental approach we hoped to gain a better understanding of how altered expression of E3 ubiquitin ligases might contribute to molecular mechanisms of dysregulation in the TGF-β signalling pathway, thereby helping to explain the hyper-inflammatory phenotype and loss of epithelial integrity in CF disease.

III. Materials and Methods

1. Cell culture

1.1. Airway epithelial cell lines and Cellular growth conditions

Studies were performed using CFBE41º- cells stably expressing wild-type-CFTR or F508del-CFTR. These cells were grown in Minimum Essential Media (MEM - Glutamax) supplemented with 10% fetal bovine serum (FBS) (both from Invitrogen, USA), and 1% Penicillin:streptomycin solution (PenStrep) (Life technologies, UK). All cell lines were grown at 37ºC in 5% CO_2.

1.2. Epithelial cell polarization

When polarized cells were required, CFBE cells were seeded into collagen IV precoated transwell filter inserts (24mm diameter and 0,4µm pore size – 3450) at a density of $1x10^6$ cells per filter, or snapwell filter inserts (12mm diameter and 0,4 µm pore size - 3801) (both from Corning life sciences, USA) at density of $2x10^5$ cells per filter, and grown at the medium, but in this case with 5% FBS. Transepithelial resistance (TER) was used as a measure of cell polarization. Apical and basolateral compartments were washed with 1x Hank's balanced salt solution (HBSS) (invitrogen, USA), growth medium was replaced and the TER was measured with a Volt-Ohmmeter (MILLIPORE, USA, #MER500001). The TER was calculated according to the manufacturer´s instructions.

1.3. Epithelial cell stimulation with TGB-β, TNF-α and bacterial supernatant

CFBE cells were seeded at density of $1x10^6$ cells/well in 6 well plates, and when confluent were washed twice with 1x Phosphate buffered saline (PBS) and treated with 5ng/ml TGF-β or 80ng/ml TNF-α (both from Peprotech) in growth media for 24 h. The same experiment was performed on polarized cells on snapwell filters with TGF-β and TNF-α added to both apical and basolateral compartments.

Unpolarized CFBE cells were treated with supernatant resulting from the centrifugation of a culture of *B. cenocepacia* strain IST4113 which had been collected from the sputum of a CF patient, three years later, after a period of exacerbated pulmonary infection that compelled the patients to hospitalization and therapy[102]. The supernatant of *B. cenocepacia* grown between logarithmic and early-stationary growth phase was kindly provided by Rita Maldonado from Isabel Sá-Correia group at Technical University of Lisbon, Portugal. For this procedure, $1x10^6$ unpolarized CFBE cells seeded in 6 well plates were washed twice at confluence with 1XPBS (Invitrogen, USA) and exposed to the bacterial supernatant (0,3µg/ml), supplemented with a protease inhibitor cocktail in growth medium for 24h. This experiment was also performed on polarized cells grown at the medium on transwell filter inserts, adding the same bacterial supernatant mixture to the apical compartment only. The

experiments were performed in triplicate and were compared with untreated control cells in the same condition (basal condition). After 24h of treatment, cellular growth medium was removed and frozen at -80ºC for Enzyme-linked immunosorbent assay (ELISA). Cells were washed twice with 1xPBS (unpolarized cells) or 1xHBSS (polarized) cells and RNA and protein were isolated.

2. RNA and protein isolation

Unpolarized and polarized CFBE cells were lysed using 1ml TRIzol/Qiazol lysis reagent (Invitrogen, USA/Qiagen) per 3,5cm well, followed by total RNA and protein isolation, according to the manufacturer´s instructions. The concentration of resultant mRNA was analyzed by measurement of the A260nm on a NanoDrop ND-1000 spectrophotometer and the purity was confirmed by measuring the absorbance ratio at 260/280 nm and 260/230nm wavelengths. The resultant RNA was then treated with DNase to eliminate genomic DNA contamination and used as a template for reverse transcription-PCR (RT-PCR). In a total reaction volume of 11µl, 1µg RNA was treated with DNase I (1 U)(zymo research, USA) for 15 min at 37ºC and the reaction terminated by incubation for 15 min at 65ºC with 25mM EDTA.

3. Reverse Transcription-PCR

cDNA was synthesized using 20µl of reverse transcription reaction solution containing approximately 1µg of DNaseI treated total RNA, 0,2µg random hexamer, 0,5mM dNTPs (both from Fermentas), ribonuclease inhibitor, and 200 units of M-MuLV reverse transcriptase (both from Nzytech). The reverse transcription protocol was 37ºC for 50 min, 25ºC for 10 min and 70ºC for 15 min. Following that, cDNA was amplified by PCR in a 25µl reaction mixture containing 1µl of cDNA diluted 1:5, 1x PCR buffer supplemented with $MgCl_2$ (Invitrogen, USA), 12,5mM dNTPs, 2,5mM each forward and reverse primer for β-Actin (Table VII. B1) and 1 unit Biotaq DNA polymerase (Bioline, USA). The mixture was subjected to 30 cycles of amplification, 94°C for 2min, 60°C for 30sec and 72°C for 4min. PCR products were analyzed on 2% agarose gel containing safe red stain for ultraviolet (UV) light visualization (0,5µg/ml) (iNtRON Biotechnology, Korea).

The following techniques are in supplementary data (see section VII):

Quantitative real-time PCR
Western blotting
Immunofluorescence
Enzyme-linked immunosorbent assay method (ELISA)
Statistical analysis

IV. Results

1. Expression of E3 ubiquitin ligases in unpolarized CFBE cells under inflammatory stimuli

Previous results in our laboratory using microarray analysis have suggested that several E3 ubiquitin ligases such as SMURF1, SMURF2 and NEDD4L, show differential expression in nasal epithelial cells of patients with CF (Table VII. C1: Clarke et al, unpublished data, see supplementary data). Thus, we analyzed their expression in CFBE cells. For this purpose, *SMURF1*, *SMURF2* and *NEDD4L* transcript levels were analysed by qRT-PCR in unpolarized CFBE cells expressing wt and F508del-CFTR. As demonstrated in Figure IV. 1, the levels of transcripts of these enzymes did not show significant differences related to CFTR genotype.

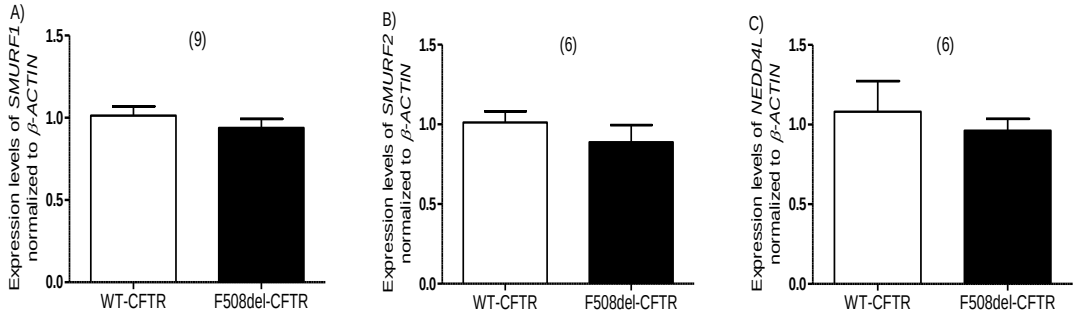


Figure IV. 1 - Expression of *SMURF1*, *SMURF2* and *NEDD4L* in unpolarized CFBE cells. A) *SMURF1*, B) *SMURF2* and C) *NEDD4L* expression analysed by qRT-PCR in unpolarized wt-CFTR or F508del-CFTR cell lines. Fold expression of mRNA levels was obtained by relative quantification (ddCt) method and was normalized to an internal control (β-Actin). Data plotted are means ± SE, (n) = number of experiments

SMURF1, SMURF2 and NEDD4L enzymes play important roles in the regulation of TGF-β signalling[43,44,45]. There is some evidence that suggest other factors regulating TGF-β including TGF-βRII (Table VII. C1, see supplementary data) and SMAD3 are altered in CF disease[51]. Subsequently, we examined the effect of TGF-β and TNF-α cytokines on E3 ubiquitin ligase expression related to CFTR genotype. Thus, we used the same experimental approach to measure *SMURF1* and *NEDD4L* expression in confluent CFBE cells expressing wt and F508del-CFTR and treated with TGF-β (5ng/ml) or TNF-α (80ng/ml) for 24h. qRT-PCR demonstrated that TGF-β decreases the transcript levels of both *SMURF1* (Fig. IV. 2A) and *NEDD4L* (Fig. IV 2B) in both cell lines, although the difference was only significant for *NEDD4L* expression in F508del-CFTR CFBE cells. In contrast, in wt-CFTR CFBE cells TNF-α increased *SMURF1* and *NEDD4L* mRNA levels, but not significantly (Fig. IV. 2A and 2B). Interestingly, TNF-α in F508del-CFTR CFBE cells didn't show the same response, since it reduced *SMURF1* and *NEDD4L* expression levels (significantly in the case of *NEDD4L*) (Fig.

IV. 2A and 2B). Thus, the data suggest possible down-regulation of *SMURF1* and *NEDD4L* following pro-inflammatory stimuli in the presence of F508del-CFTR expressing cells.

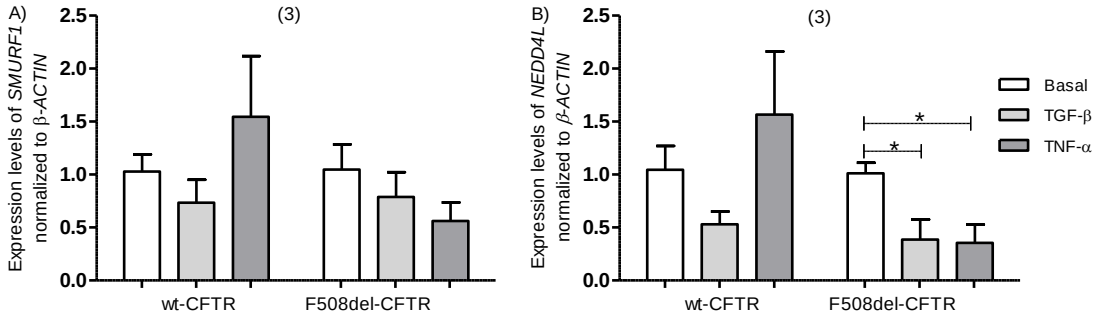
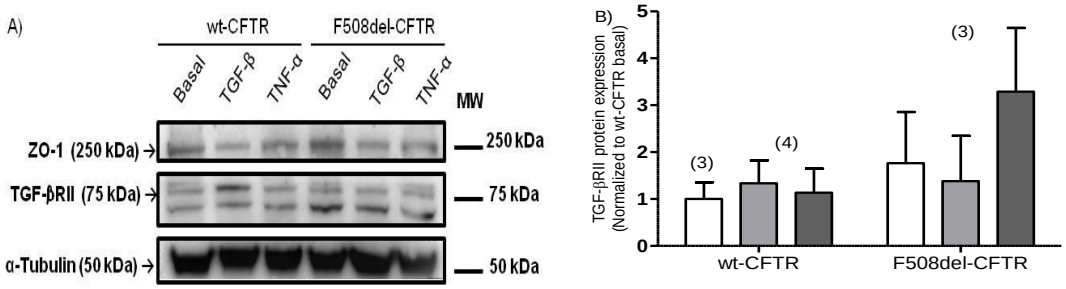


Figure IV. 2 – **Expression of *SMURF1* and *NEDD4L* in unpolarized CFBE cells under inflammatory conditions**. wt-CFTR and F508del-CFTR CFBE cells were treated with TGF-β (5ng/ml) or TNF-α (80ng/ml) for 24 h. Fold expression of (A) *SMURF1*, and (B) *NEDD4L* mRNA levels was obtained by relative quantification (ddCT) method normalized to an internal control (β-Actin). Data plotted are means ± SE, (n) = number of experiments. * indicates statistically significant difference (p<0,05).

SMURF1 promotes TGF-βR degradation[42,43] and together with smad-independent TGF-β signal also participates in Rho protein degradation, leading to TJ disruption[95,96,97].

To investigate whether TGF-β signalling changes TJ integrity and TGF-βRII levels in unpolarized CFBE cells, Western blot for detection of ZO-1 and TGF-βRII protein expression was performed following TGF-β (5ng/ml) and TNF-α (80ng/ml) treatment for 24h. Western blot analysis did not demonstrate significant changes in TGF-βRII protein levels in either cell line following treatment with TGF-β for 24h (Fig. IV. 3A and B). On the other hand, TGF-βRII protein levels increased in F508del-CFTR CFBE cells following TNF-α treatment (where *SMURF1* and *NEDD4L* appear to be down-regulated), which was in contrast to the lack of an effect in wt-CFTR CFBE cells (Fig. IV. 3A and B). Interestingly, we observed two bands of TGF-BRII in figure IV. 3A that may represent two different forms of TGF-βRII protein, probably two isoforms produced by alternative splicing (Uniprot data base - http://www.uniprot.org/)[105]. In addition, the intensity of these bands changed under TGF-β and TNF-α treatment in wt-CFTR cells, without apparent change in F508del CFTR cells. Figure IV. 3A also demonstrated that untreated wt-CFTR and F508del-CFTR CFBE cells expressed an intense band of ZO-1 protein, which decreased following both cytokines as confirmed by semiquantitative Western blot (only for wt-CFTR expressing cells) (Fig. IV. 3C), although the data were not statistically significant.



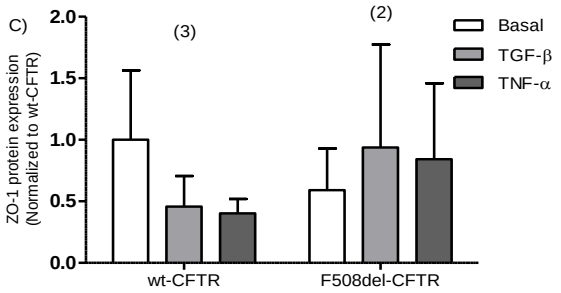
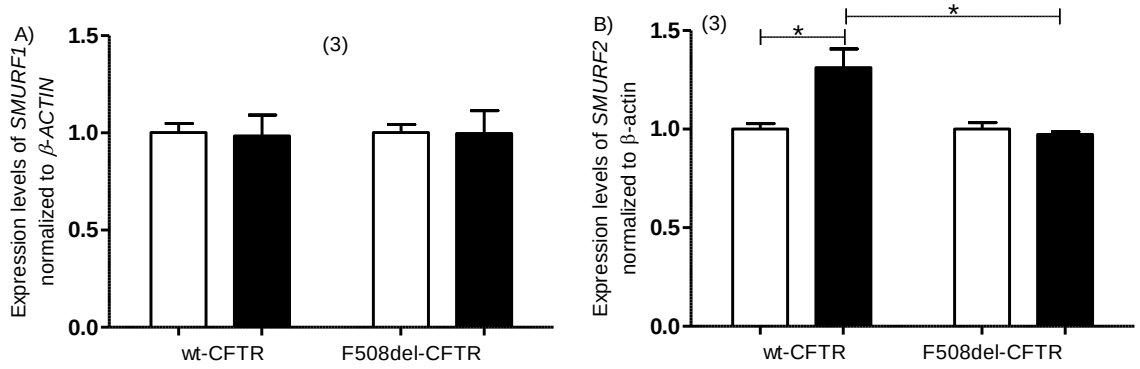


Figure IV. 3 – Alteration of tight junction organization and TGF-βRII protein expression in unpolarized cells following TGF-β and TNF-α treatment. A) Western blot for ZO-1 (top) and TGF-βRII (middle) protein expression. Equal amounts of proteins were loaded in each lane, as demonstrated by α-tubulin control (bottom). B) TGF-βRII and C) ZO-1 band intensities relative to wt-CFTR basal were quantified in the experiments in A and are shown as the mean ± SE, (n) = number of experiments.

B.cenocepacia bacteria is known to evoke a host immune response, contributing to a severe inflammatory response which plays an important role in CF pathogenesis. E3 ubiquitin ligases appear to have a role in inflammatory signalling pathways. In order to investigate E3 ubiquitin ligase expression under infection conditions, we measured *SMURF1*, *SMURF2* and *NEDD4L* mRNA levels in unpolarized CFBE cells treated with *B. cenocepacia* strain IST4113 supernatant for 24h by qRT-PCR analysis. At the time of writing, the composition of this bacterial supernatant is unknown, but it is known that this bacterial strain exhibits increased levels of resistance to different classes of antimicrobials[102] and releases secretory proteins that change innate responses and lead to disruption or alteration of pathways[106] No obvious alteration in *SMURF1* expression was observed (Fig. IV. 4A). On the contrary, *SMURF2* expression was increased following exposure to the supernatant in wt-CFTR expressing cells. In F508del-CFTR expressing cells there was no significant effect (Fig. IV. 4B). These data suggest that in unpolarized cells, *SMURF2* expression changes under bacterial infection conditions only in cells expressing wild type CFTR, with a similar trend observed for *NEDD4L* (Fig. IV. 4C). Overall this suggests a potential disruption of E3 ubiquitin ligase regulation associated with F508del CFTR expression.



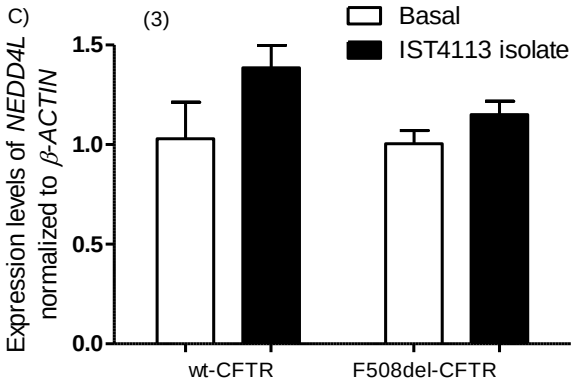
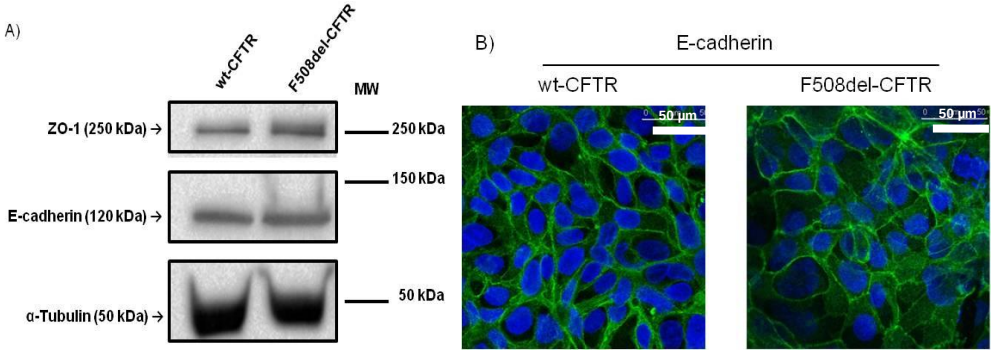


Figure IV. 4 – Expression of *SMURF1*, *SMURF2* and *NEDD4L* in unpolarized CFBE cells under infection condition. CFBE cells expressing wt-CFTR or F508del-CFTR were treated 24h with *B. cenocepacia* IST4113 strain supernatant (0,3µg/ml). Fold expression of (A*) SMURF1*, (B) *SMURF2* and (D) *NEDD4L* mRNA levels was obtained by relative quantification ddCT method and was normalized to an internal control (β-Actin). Data plotted are mean ± SE, number of experiments (n=3). * indicates statistically significant difference (p<0,05).

2. Characterization of the cell-cell contact and TGF-βR localization in unpolarized CFBE cells

An efficient biological response to cytokines depends on the specific localization of their receptors in the surface membrane. TGF-β signalling requires an intrinsic association of the TGF-βR to regions of cell-cell contact[107] and TNF-α receptors (TNFR) localize in both apical and basolateral surfaces of the membrane but their localization leads to different response intensities[108]. Therefore, we evaluated cell adhesion in unpolarized CFBE cells. Firstly we examined the TJ–associated cytoplasmic protein ZO-1 and intercellular adhesive protein E-cadherin. Western blot analysis showed that both of these proteins were expressed in confluent unpolarized CFBE cells with ZO-1 migrating at around 250 kDa and E-cadherin at 120 kDa (Fig. IV. 5A). Then, in accordance with Western blot, immunofluorescence staining using confocal microscopy demonstrated that these proteins had a characteristic location for epithelial cells. E-cadherin was located at the cell surface and appeared as a continuous line at the boundaries between neighboring cells (Fig. IV. 5B), and ZO-1 was very similar to E-cadherin (Fig. IV. 5C), although more boundary-specific. Our data demonstrated that unpolarized CFBE cells, when confluent, develop a partial epithelial status, which is predicted to confer specific distribution upon TGF-βR and TNFR.



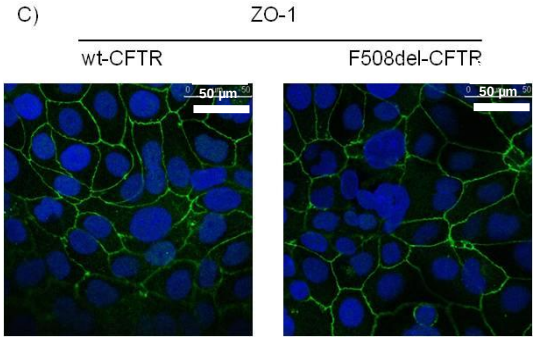


Figure IV. 5 – Tight Junction and intercellular adhesive protein expression and organization in unpolarized wt-CFTR and F508del-CFTR CFBE cells. A) Western blot for ZO-1 (top) and E-cadherin (middle) protein expression. Equal amounts of proteins were loaded in each lane, as demonstrated by α-tubulin control (bottom). Immunofluorescent staining for B) E-cadherin localized in the lateral membrane and C) for ZO-1 in intercellular TJ and detected by confocal microscopy with 63x oil-immersion objective. Green staining is the target protein under study (Alexa-488) and blue staining the nucleus (DAPI).

To determine the TGF-βR localization in unpolarized CFBE cells, immunofluorescence staining of endogenous TGF-βR was performed. Wt-CFTR CFBE cultures plated at high density to establish sites of cell contact were stained for both TGF-βRII and an additional staining for cell adhesion marker E-cadherin. TGF-βRII was found to be predominantly cytoplasmic (Fig. IV. 6A) and in some cases localized with E-cadherin to the lateral membrane (Fig. IV. 6A, vertical (XZ) sections seen in the lower image). When cells were treated with TGF-β (5ng/ml), for 24h confocal imaging demonstrated an identical localization, but higher intensity pattern of TGF-βRII expression (Fig. IV. 6B). Thus, TGF-βR is essentially cytoplasmic with some localization to the lateral membranes and consequent co-localizations with E-cadherin.

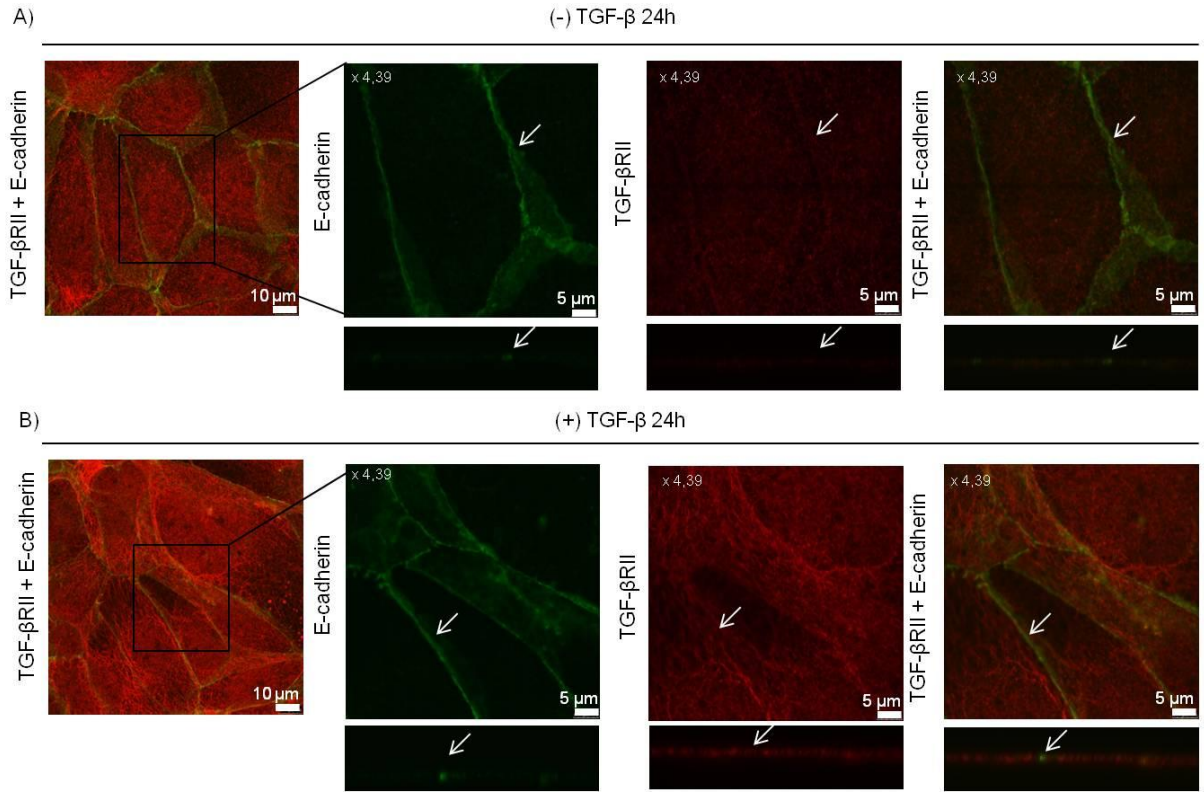


Figure IV. 6 – Localization of TGF-βRII in unpolarized CFBE cells. Immunofluorescent staining for TGF-BRII and E-cadherin in wt-CFTR CFBE cells untreated (A) and treated with TGF-β (5ng/ml) for 24h (B). The lower images represent vertical XZ flat section. Green staining (Alexa-488) is the E-cadherin and red staining (Alexa-568) the TGF-βRII, nuclear staining (blue) was not provided, since no DAPI filter was available. Open squares represent the magnified area (x 4.39) with 63x oil-immersion objective and arrow represents the localization of target protein at lateral membrane.

We performed an additional experiment to examine the effect of TGF-β and TNF-α on inflammatory cytokines stimulation in CFBE cells by ELISA analysis using medium collected after TGF-β and TNF-α exposure (Figure VII. C2, see supplementary data). However, the low sample number did not allow firm conclusions concerning unpolarized cells response to surface membrane application of TGF-β and TNF-α.

3. Characterization of cell polarity in CFBE cells

Because our data demonstrated that TGF-βII locates to the lateral surface of the membrane of CFBE cells, we suspected that TGF-β exposure to the surface membrane of unpolarized CFBE cells might lead to a minimal effect on TGF-β activity, which may not be prone to modulation of E3 ubiquitin ligases expression. Thus we used CFBE cells grown on filter at the medium to achieve a differentiated phenotype for the study of E3 ubiquitin ligase expression and for comparison with data from unpolarized cells. The differentiated phenotype of CFBE cells grown on filters was determined. Following 1 week of culture the transepithelial resistance (TER) of the cells was measured as demonstrated in Figure IV. 7. After 3 days wt-CFTR cultures developed TER≥600 $\Omega.cm^2$, characteristic of polarized cells. The TER peak was in the range of 1100-2000$\Omega.cm^2$ for wt-CFTR cultures after 7 days and 630-920 $\Omega.cm^2$ for F508del-CFTR cells, in which the development of a differentiated phenotype was retarded by comparison. After 5 days, F508del-CFTR expressing cells displayed a pronounced loss of TER compared with wt-CFTR expressing cells whose TER continued to increase, although at a lower rate. The maintenance of TER of polarized epithelial cells requires TJ complex assemblage. To investigate if there were differences in this process between wt-CFTR and F508del-CFTR expressing cells, we examined the epithelial markers ZO-1 and E-cadherin, in CFBE cells when polarized on filters. Western blot and immunofluorescence analysis showed normal protein expression (Fig. IV. 7B) and localization of E-cadherin (Fig. IV. 7C) and ZO-1 (Fig. IV. 7D) at membrane surface in both cell lines. However in CFBE cells expressing F508del-CFTR, associated with lower TER, we observed a smaller fraction of ZO-1 and E-cadherin at basolateral membrane than in wt-CFTR (Fig. IV. 7C and 7D). In summary, our results showed that acquisition of epithelial phenotype by F508del-CFTR CFBE cells is delayed compared with wt-CFTR expressing cells and this is reflected in the intensity pattern of expression of TJ markers.

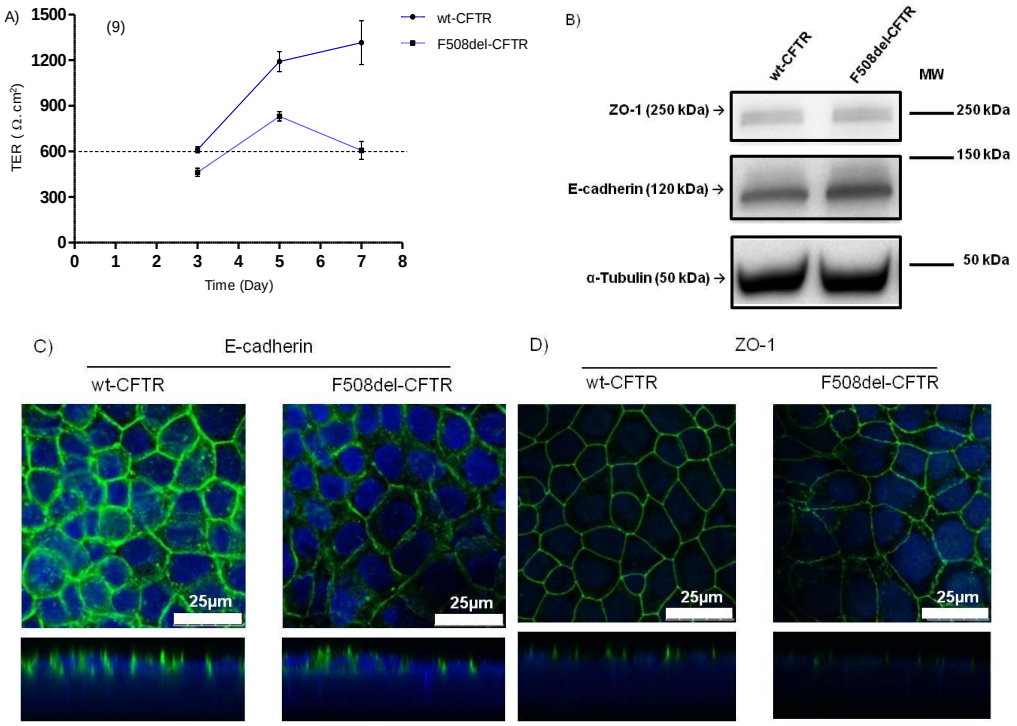


Figure IV. 7 – Development of TER and epithelial phenotype of polarized monolayer CFBE cells. A) TER of wt-CFTR (circle) and F508del-CFTR (square) CFBE cells grown on snapwell filters as a function of culture time. Values expressed as means TER ± SE, (n) = number of experiments. B) Western blot for ZO-1 (top) and E-cadherin (middle) protein expression. Equal amounts of proteins were loaded in each lane, as demonstrated by α-tubulin control (bottom) C) Immunofluorescent staining for E-cadherin localization in the lateral membrane and D) ZO-1 in intercellular tight junction detected by confocal microscopy with 63x oil-immersion objective. Green staining is the target protein under study (Alexa-488) and blue staining the nucleus (DAPI).

4. Expression of *SMURF1*, *SMURF2* and *NEDD4L* in polarized CFBE cells under inflammatory stimuli

SMURF1, *SMURF2* and *NEDD4L* expression was measured by qRT-PCR in polarized CFBE cells expressing wt and F508del-CFTR. The levels of transcripts of these enzymes did not show significant differences between both cell lines in study (Fig. IV 8). However, in CFBE cells expressing F508del-CFTR we observed a very slight increase on *SMURF1* expression in contrast with *NEDD4L* that decreased. These results demonstrated the same trend that was observed in previous experiments performed in our group (Data not shown). Thus, as observed for unpolarized cells (see Fig. IV. 1A-C) development of a polarized phenotype under basal conditions does not seem to be enough to produce CFTR-related differential expression of E3 ubiquitin ligases suggested by other studies, although the trends agree with previous data.

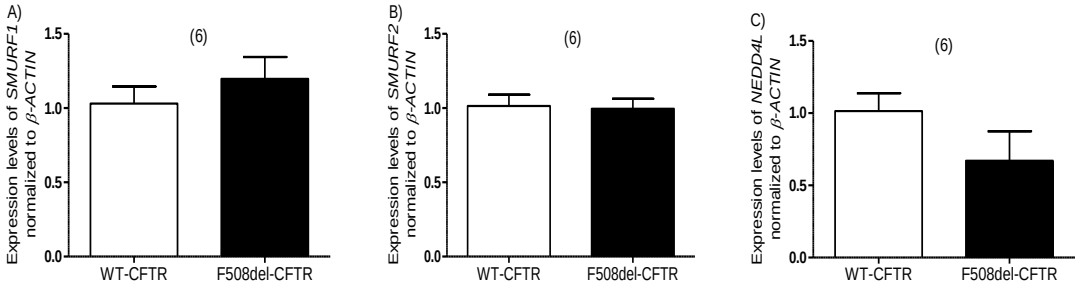
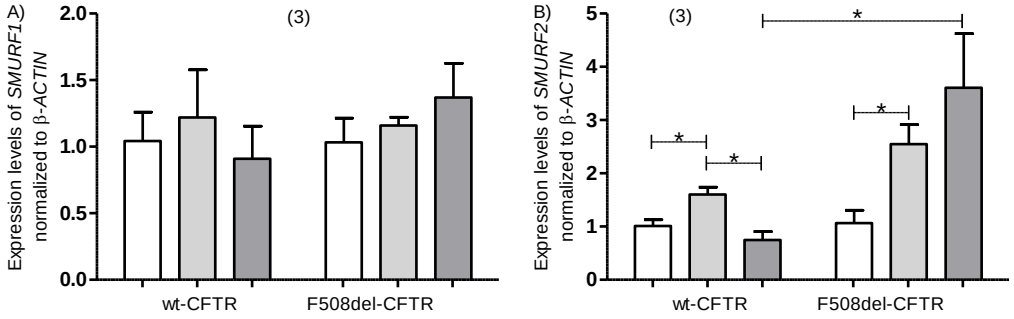


Figure IV. 8 - Expression of *SMURF1*, *SMURF2* and *NEDD4L* in polarized CFBE epithelial cells. (A) *SMURF1*, (B) *SMURF2* and (C) *NEDD4L* expression analysed by qRT-PCR in polarized wt-CFTR or F508del-CFTR CFBE cells. Fold expression of mRNA levels was obtained by relative quantification (ddCt) method and was normalized to an internal control (β-Actin). Data plotted as the mean ± SE, (n) = number of experiments.

Next, we measured the expression levels of E3 ubiquitin ligases following inflammatory stimuli (characteristic of CF disease) in polarized CFBE cells by qRT-PCR analysis. Pretreatment of wt-CFTR and F508del-CFTR expressing CFBE cells with TGF-β (5ng/ml) and TNF-α (80ng/ml) for 24h did not affect significantly the expression of *SMURF1* (Fig. IV. 9A). TGF-β up-regulated the mRNA levels of *SMURF2* in both cell lines, with a greater effect in F508del-CFTR expressing cells (Fig. IV. 9B). In contrast, TNF-α slightly decreased *SMURF2* expression in wt-CFTR, when compared with TGF-β. In addition, TNF-α significantly increased the expression of *SMURF2* in F508del-CFTR expressing cells. TGF-β markedly decreased the *NEDD4L* levels of transcripts in F508del-CFTR expressing cells when compared with the effect of TNF-α (Fig. IV. 9C). To determine whether changes in TGF-β–induced expression of E3 ubiquitin ligases are correlated with mRNA levels of *TGF-βRII*, mRNA was analysed by qRT-PCR. In wt-CFTR expressing CFBE cells, in which *SMURF1* and *NEDD4L* mRNA expression was relatively unaffected (see Fig. IV 9A and C), TGF-β also had little effect on the expression of *TGF-βRII* mRNA level (Fig. IV. 9D). However, in F508del-CFTR expressing cells, *TGF-βRII* mRNA level decreased and increased significantly upon treatment with TGF-β and TNF-α, respectively, with the differences parallel to those in *NEDD4L* expression (Fig. IV. 9C and D). These results suggest up-regulation of *SMURF2* in F508del-CFTR CFBE cells under inflammatory stimuli in polarized cells.



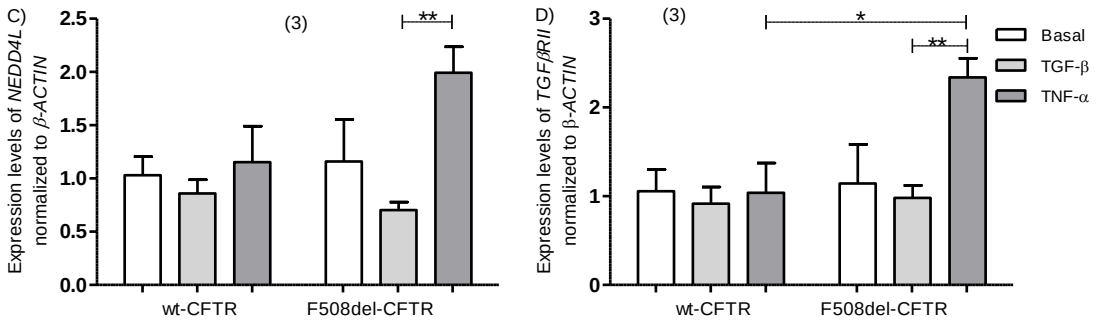


Figure IV. 9 – Expression of E3 ubiquitin ligases and TGF-βRII in polarized CFBE cells under inflammatory stimuli. wt-CFTR and F508del-CFTR CFBE cells were treated with TGF-β (5ng/ml) or TNF-α (80ng/ml) for 24 h. Fold expression of (A) *SMURF1*, (B) *SMURF2*, (C) *NEDD4L* and (D) *TGF-βRII* mRNA levels was obtained by relative quantification ddCT method and was normalized to an internal control (β-Actin). Data plotted as the mean ± SE, number of experiments (n=3). * indicates statistically significant difference (p<0,05) and ** p=0,001.

To understand the smad-dependent TGF-β response we performed Western blot to detect the TGF-β mediators (SMAD2) and SMAD7 in polarized CFBE cells treated with TGF-β and TNF-α. However, we failed to detect any smad protein (Data not shown).

The effect of TGF-β and TNF-α on the time course of development of TER in CFBE cells was determined. Cells were seeded on snapwell filter inserts and when polarized were treated with TGF-β (5ng/ml) and TNF-α (80ng/ml) followed by TER measurement during 24h. In approximately 30min the TER decreased in wt-CFTR CFBE cells treated with TGF-β and TNF-α. In addition, we also observed a decreasing of TER in untreated cells (Fig. IV. 10A). A possible explanation is that when we replaced the medium in apical chamber, some cells were detached from the filter. However using light microscope we didn't observe significant alterations. By contrast, in F508del-CFTR expressing cells, TER increased during the 6h following exposure to TGF-β and TNF-α. Untreated cells showed a slight decrease (Fig. IV. 10B). After that the TER of F508del-CFTR CFBE cells decreased to the borderline of TER characteristic of the unpolarized phenotype (~600). The exposure to TGF-β and TNF-α appears to be determinant for TER loss after 24h, but also the presence of F508del-CFTR. TGF-β seems to show an important role in epithelial injury of CF, which F508del-CFTR expressing cells appear not to resist.

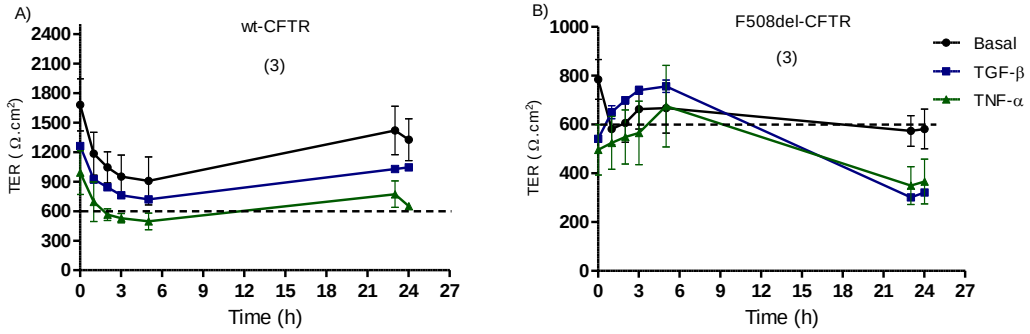


Figure IV. 10 – Effect of TGF-β and TNF-α on TER in polarized CFBE cells. wt-CFTR and F508del-CFTR CFBE cells were treated with TGF-β (5ng/ml) or TNF-α (80ng/ml) and TER was monitored over 24h. Data plotted as the mean ± SE, (n) = number of experiments.

Observing the effect of TGF-β and TNF-α on E3 ubiquitin ligases expression and TER in polarized CFBE cells (Fig. IV. 9A-C and 10A-B), which play a role in epithelial integrity, as above mentioned, next we studied whether TGF-β (5ng/ml) and TNF-α (80ng/ml) induced TJ dissolution in CFBE cells following 24h of exposure by immunofluorescence analysis. At the first examination, polarized F508del-CFTR CFBE cells exposed to TGF-β appeared to have punctate ZO-1 immunoreactivity that was localized away from the TJ (Fig. IV. 11 orange arrow) and the fluorescent intensity at TJ at this site decreased (Fig. IV. 11 orange arrow seen in XZ sections in the lower image). Cell exposure to TNF-α also demonstrated ZO-1 localization away from the TJ (Fig. IV. 11 XZ sections in the lower image). In both cells lines, TGF-β and TNF-α changed the TJ organization, but with more impact in CFBE cells expressing F508del-CFTR, in which these cytokines had significant effects on E3 ubiquitin ligase expression (Fig. IV. 9A-C).

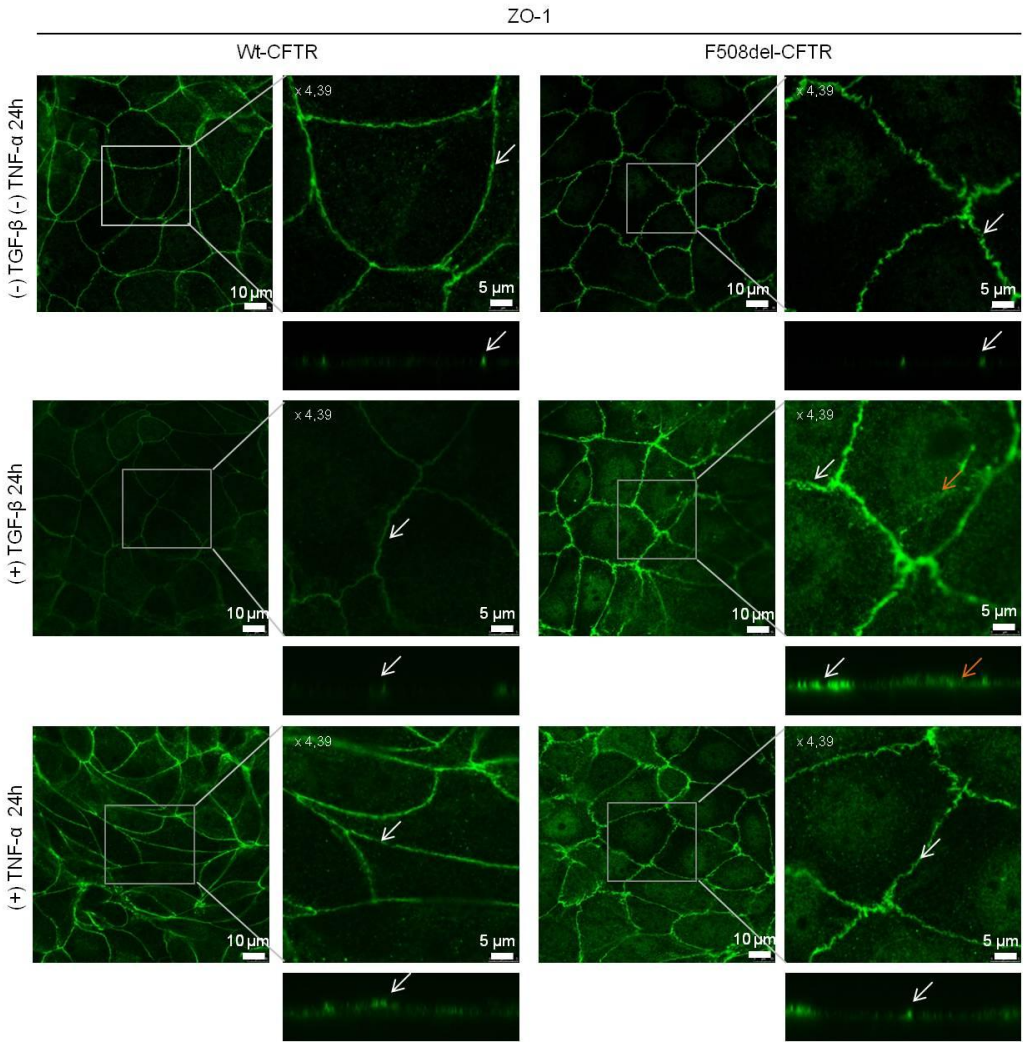


Figure IV. 11 – TGF-β and TNF-α exposure disrupt the tight-junction organization in CFBE cells. Immunofluorescent staining for ZO-1 in wt-CFTR and F508del-CFTR CFBE cells untreated and treated with TGF-β (5ng/ml) and TNF-α (80ng/ml) for 24h. The lower images represent vertical XZ flat section. Green staining is the target protein under study (Alexa-488) and nuclear staining (blue) was not provided, since no DAPI filter was available. Open square represents the magnified area (x 4.39) with 63x oil-immersion objective and arrow represents the localization of target protein at lateral membrane.

To address whether infection status, considered responsible for the potent pro-inflammatory response elicited by bacterial products changes the E3 ubiquitin ligase mRNA expression levels in CFBE cells when polarized, we examined *SMURF1*, *SMURF2* and *NEDD4L* expression in these cultures treated with *B. cenocepacia* strain IST4113 supernatant for 24h by qRT-PCR analysis. Figure IV. 12 demonstrates a very significant decrease of *SMURF1* transcripts levels in F508del-CFTR CFBE cells exposed to bacteria free-supernatant when compared with wt-CFTR expressing cells (Fig. IV. 12A). The expression of *SMURF2* also decreased in both wt- and F508del-CFTR CFBE cells following exposure to bacterial supernatant, but not significantly (Fig. IV. 12B), showing that the two smurfs have a differential response to infection conditions that is dependent upon CFTR genotype. The supernatant also significantly decreased *NEDD4L* expression in both cell lines (Fig. IV. 12C). The expression of *SMURF1* and *NEDD4L* appear to be down-regulated in polarized F508del-CFTR expressing cells under infection stimulus with *B. cenocepacia* supernatants. Therefore the polarized phenotype appears to enhance the responsiveness of E3 Ubiquitin ligase expression to stimuli that might be expected in vivo in the CF airway, in marked contrast to unpolarized cells (see Fig. IV. 6A-C), designating polarized CFBE cells as a much more valid model for infection and inflammation studies in CF disease.

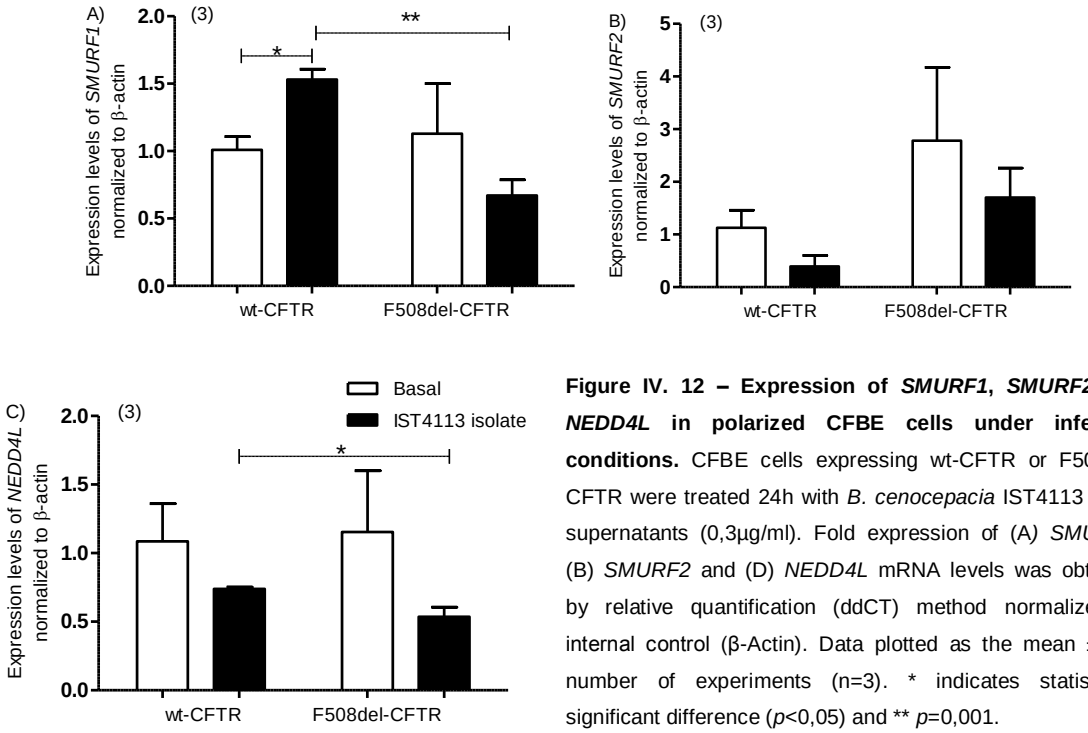


Figure IV. 12 – Expression of *SMURF1*, *SMURF2* and *NEDD4L* in polarized CFBE cells under infection conditions. CFBE cells expressing wt-CFTR or F508del-CFTR were treated 24h with *B. cenocepacia* IST4113 strain supernatants (0,3µg/ml). Fold expression of (A*) SMURF1*, (B) *SMURF2* and (D) *NEDD4L* mRNA levels was obtained by relative quantification (ddCT) method normalized to internal control (β-Actin). Data plotted as the mean ± SE, number of experiments (n=3). * indicates statistically significant difference (p<0,05) and ** p=0,001.

We then studied the potential effect of bacteria-free supernatant from *B.cenocepacia* IST411 isolate on epithelial and differentiation markers by Western blot. As demonstrated in Figure IV. 13A and B, E-cadherin, unlike ZO-1 (Fig. IV. 13A and C) had a non significant protein decrease in F508del-CFTR CFBE cells exposured to bacterial supernatant. Interestingly, in wt-CFTR cell line, in which *SMURF1* expression increased (Fig. IV. 12A), ZO-1 slight decreased (Fig. IV. 13C) and the contrary was observed in F508del-CFTR cell line, in which *SMURF1* levels decreased. CK14 and CK18 (differentiation markers) did not show any significant differences (Fig. IV. 13D). Although with a slight effect on E3 ubiquitin expression, the data indicated no significant effect of *B. cenocepacia* supernatant in epithelial and differentiated status of polarized CFBE cells.

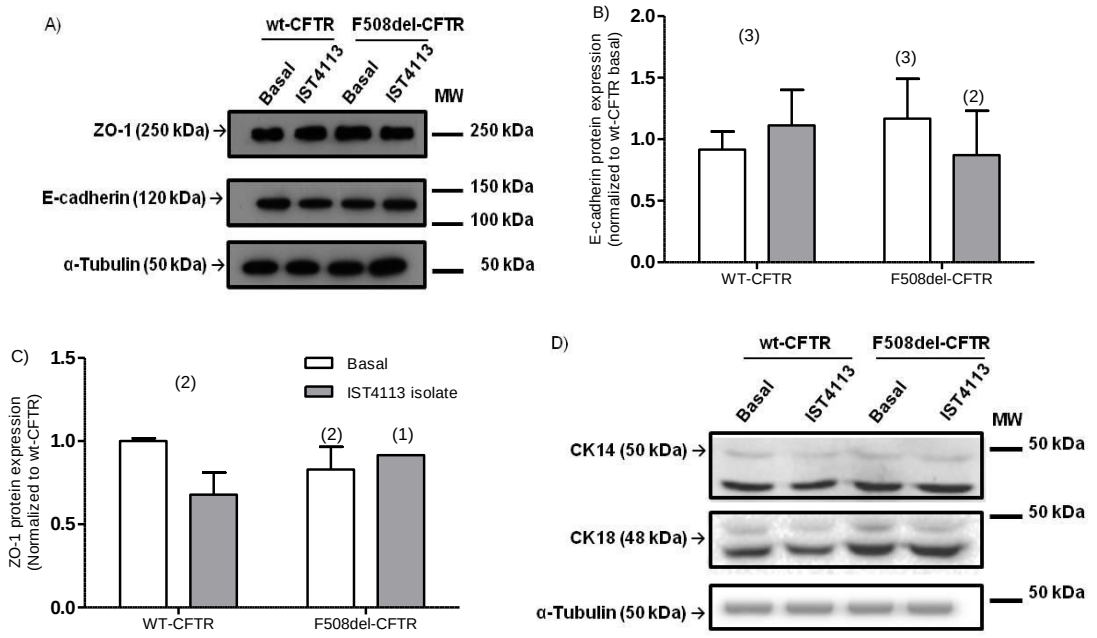


Figure IV. 13 – Effect of *B. cenocepacia* supernatant in epithelial and differentiated markers in CFBE cells A) Western blot for ZO-1 (top) and E-cadherin (middle) protein expression. Equal amounts of proteins were loaded in each lane, as demonstrated by α-tubulin control (bottom). B) E-cadherin and C) ZO-1 Band intensities relative to wt-CFTR basal were quantified in the experiments in A and are shown as the mean ± SE, (n) = number of experiments. D) Western blot for CK14 (top) and CK18 (middle) protein expression. Equal amounts of proteins were loaded in each lane, as demonstrated by α-tubulin control (bottom).

5. Effects of *B. cenocepacia* supernatant on CFTR protein, TGF-β and inflammation components'

To further define the effect of *B. cenocepacia* supernatant in CFTR protein and anti-inflammatory status Western blot was performed with total protein from polarized CFBE cells exposed to bacteria-free supernatant for 24h. Polarized CFBE cells expressing wt- and F508del-CFTR and treated with supernatant showed an apparent decline of CFTR protein expression (Fig. IV. 14A and B). TGF-βRII (Fig. IV. 14A and C) and NEDD4L protein levels (Fig. IV. 14A and D) did not show significant differences in cell lines under study following

supernatant exposure. Inflammatory response mediators, such as PELLINO1, and IL-8Rβ also did not show differences in cells exposed to bacteria supernatant. Only STAT1 protein, another inflammatory mediator appeared to increase in F508del-CFTR CFBE cells in the presence of bacterial supernatant (Fig. IV. 14E). These data suggested that the bacterial supernatant used contained a secreted toxin capable of reducing the expression of CFTR protein in both wt- and F508del-CFTR cell lines, by an unknown mechanism.

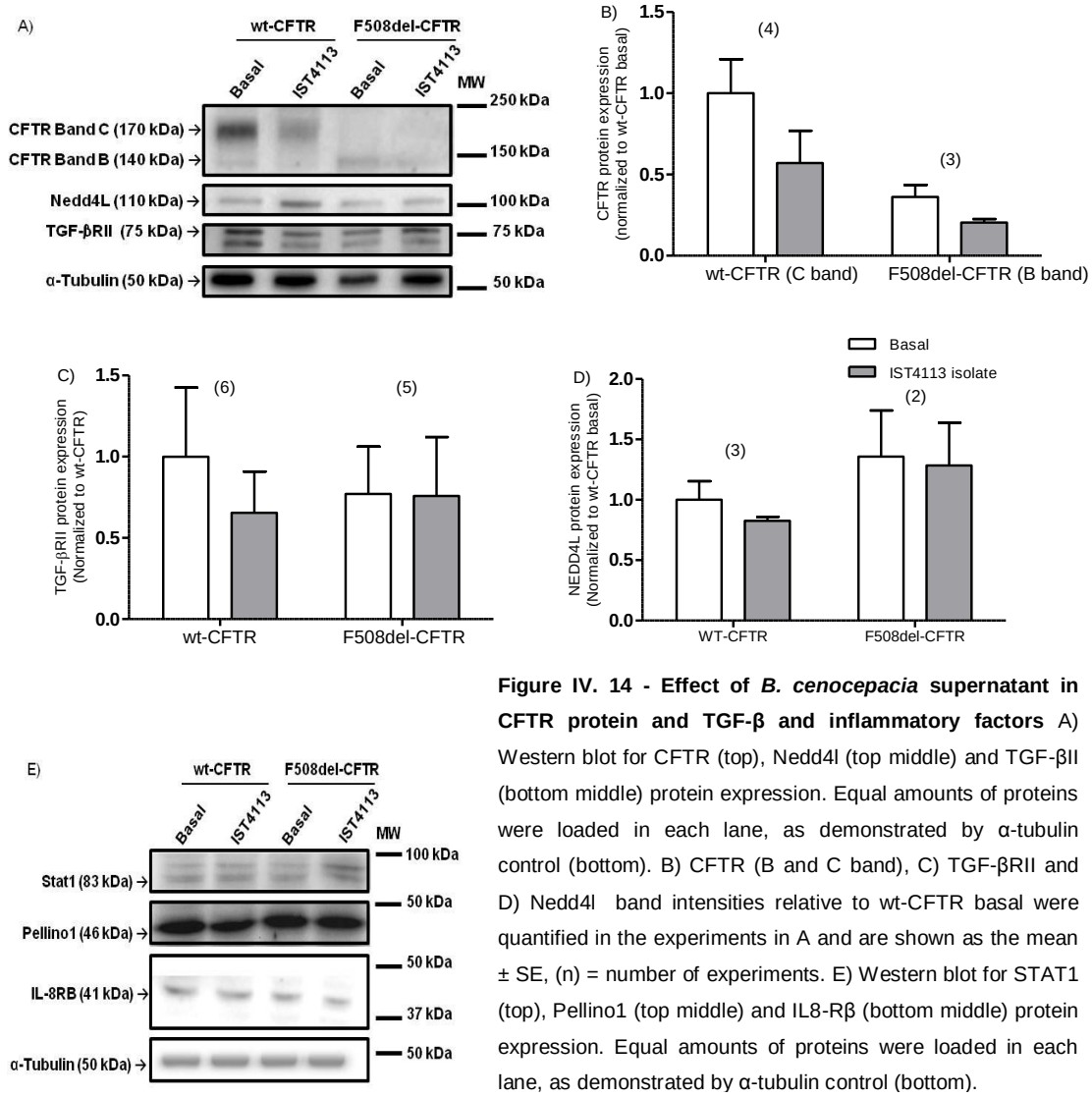


Figure IV. 14 - Effect of *B. cenocepacia* supernatant in CFTR protein and TGF-β and inflammatory factors A) Western blot for CFTR (top), Nedd4l (top middle) and TGF-βII (bottom middle) protein expression. Equal amounts of proteins were loaded in each lane, as demonstrated by α-tubulin control (bottom). B) CFTR (B and C band), C) TGF-βRII and D) Nedd4l band intensities relative to wt-CFTR basal were quantified in the experiments in A and are shown as the mean ± SE, (n) = number of experiments. E) Western blot for STAT1 (top), Pellino1 (top middle) and IL8-Rβ (bottom middle) protein expression. Equal amounts of proteins were loaded in each lane, as demonstrated by α-tubulin control (bottom).

6. Expression of E3 ubiquitin ligases in nasal epithelium from CF patients

Although the CFBE cells are a useful model in the CF studies, they are transfected cultured cells which do not express endogenous CFTR. Thus, we verified our findings of E3 ubiquitin ligase expression levels in freshly isolated nasal epithelial cells from CF homozygous patients for F508del-CFTR and non-CF healthy individuals. For this purpose, *SMURF1*, *SMURF2* and *NEDD4L* levels of transcripts were analysed by qRT-PCR. As demonstrated in Figure IV. 15, the levels of transcripts of these enzymes did not show

significant differences between the two CFTR genotypes. Thus in nasal epithelial cells the E3 ubiquitin ligases mRNA levels change as previous demonstrated, but not significantly. We observed the same trend for polarized CFBE cells.

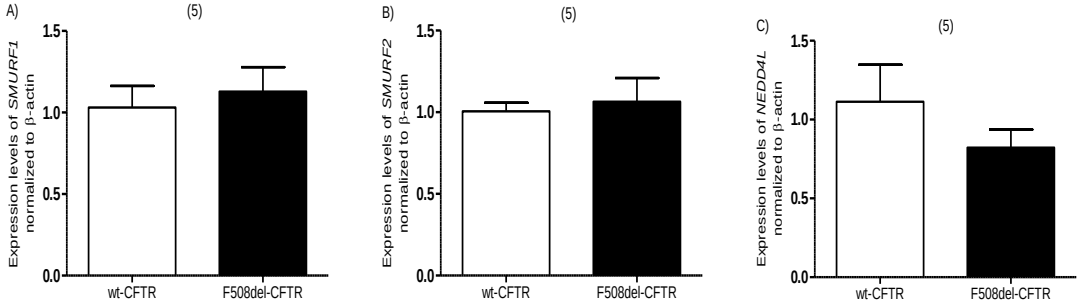


Figure IV. 15 - Expression of *SMURF1*, *SMURF2* and *NEDD4L* in nasal epithelial cells A) *SMURF1*, B) *SMURF2* and D) *NEDD4L* expression analysed by qRT-PCR in freshly nasal epithelial cells from non-CF and CF volunteers and patients homozygous for F508del-CFTR. Fold expression of mRNA levels was obtained by relative quantification ddCt method and was normalized to an internal control (β-Actin). Data plotted as the mean ± SE, (n) = number of experiments.

V. Discussion

The present course of study was based in part on previous reports suggesting that the TGF-β signalling pathway is dysregulated in CF lung disease[51], and partly on previous studies undertaken in our laboratory suggesting that E3 ubiquitin ligases including *SMURF1, SMURF2* and *NEDDL4* were up-regulated in the Cystic Fibrosis airway epithelium. We hypothesized that dysregulation of TGF-β signalling in CF epithelium, partly caused by differential expression of E3 ubiquitin ligases, could be partly responsible for the hyper-inflammatory phenotype that has long been known to characterize CF lung disease. In an attempt to understand the role of E3 ubiquitin ligases in CF, we focused here on studying the expression of *SMURF1*, *SMURF2* and *NEDD4L* that are activated downstream of the TGF-β signalling pathway[43,44,45].

In the current thesis, analysis of E3 ubiquitin ligase gene expression in CFBE cells and native nasal epithelial cells expressing wt-CFTR and F508del-CFTR under basal conditions demonstrated that the F508del mutation may not be sufficient to induce a significant differential expression of E3 ubiquitin ligases. Our result was not consistent with previous studies that suggested significant differential expression of ubiquitination-enzymes in CF. However, this effect on the gene expression is not yet in accordance[33,109,110,111].

We further hypothesized that altered expression of E3 ubiquitin ligases may be developed under certain inflammatory and infection conditions that characterize CF disease. Preliminary experiments were undertaken with unpolarized CFBE cells expressing wt-CFTR and F508del-CFTR and treated with TGF-β and TNF-α, which function predominantly as anti-inflammatory and pro-inflammatory cytokines, respectively. Previous studies have reported that both cytokines induce the expression of E3 ubiquitin ligases[112,123]. No significant differences in *SMURF1* and *NEDD4L* expression were detected in cells treated with TGF-β. This result was not consistent either with previous results in our laboratory or with previous research reporting that TGF-β exposure increases expression of E3 ubiquitin ligases in human alveolar epithelial adenocarcinoma (A549) cells and liver hepatocellular carcinoma (HepG2) cells[112,113].

Intriguingly, TNF-α administration resulted in down-regulation of both *SMURF1* and *NEDD4L* in CFBE cells expressing F508del-CFTR. This result suggests a potential decrease of negative regulation by these E3 ubiquitin ligases on anti-inflammatory pathways in the F508del-CFTR CFBE cell line. It is known that pro-inflammatory cytokines can induce anti-inflammatory mediators to regulate a destructive inflammatory status by negative

feedback[114]. Such regulatory networks governing inflammation cascades may also be present in CF lung disease, despite its inflammatory profile.

Because TGF-βR and ZO-1 are targeted by E3 ubiquitin ligases for degradation in a TGF-β-dependent manner[38,95,96,97], it was also pertinent to study their expression. We found that TGF-β and TNF-α decreased the ZO-1 levels in unpolarized CFBE cells. TNF-α, despite its effect on the expression of *SMURF1* and *NEDD4L,* increased levels of TGF-βRII in the F508del-CFTR cell line, suggesting, as mentioned above, a decrease of E3 unbiquitin ligase activity on the TGF-β signalling pathway in the F508del-CFTR cell line. This issue is, however, controversial in the context of CF disease. Moreover, the lack of statistical significance of these results, likely due to the low number of experimental replicates, does not allow firm conclusions to be drawn concerning the epithelial response to TGF-β and TNF-α in CF disease.

Nevertheless, we reasoned that our preliminary results might be explained by the lack of an effective cytokine response in unpolarized and undifferentiated cells. Epithelial cells have a distinct membrane organization, with specific membrane localization of their cell-surface receptors[115]. This is crucial for appropriate cytokine-receptor binding and an efficient cell signalling response. Our data therefore led us to speculate that the unpolarized cell system analyzed could not provide an appropriate response to TGF-β and TNF-α, with respect to E3 ubiquitin ligase expression, because TGF-β and TNF-α receptors are localized at the basolateral membrane in polarized airway epithelia.

We therefore investigated the epithelial status and localization of the TGF-βRII in unpolarized CFBE cells. We observed that these cells, when confluent, develop a partial epithelial status, with respect to the expression and localization of tight junction (TJ) proteins (eg, ZO-1) and cell adhesion markers (eg, E-cadherin). We also observed that as in intestinal epithelial cell line Caco2-BB3 and Madin-Darby canine kidney (MDCK) cells, TGF-βR in CFBE cells resides at the lateral membrane[77,107]. It was no surprise that we also found TGF-βR to be localized in the cytoplasm, since after TGF-β exposure TGF-βR are internalized via clathrin-dependent vesicles, to promote TGF-β signalling, or via lipid raft-caveolar internalization, together with SMAD7/SMURF2, for rapid receptor turnover[116].

Thus, taking into account other studies reporting that TNF-α exposure to the basolateral membrane surface of human airway epithelial cells resulted in increased IL8 release and NF-kB activation, while minimal effects were observed following exposure to the apical surface[115], our findings suggested that unpolarized CFBE cells have a restricted range of responses to TGF-β and TNF-α.

Taking into account the polarized localization of TGF-βR and TNFR in a fully differentiated airway, this issue would seem to be resolved by using a polarized cell system.

Thus, CFBE cells were grown on filters to enable receptor-cytokine binding and establish an efficient gradient of chemoattractants, which are cell signaling pathways activators. Polarized CFBE cells expressing wt-CFTR developed higher transepithelial resistance (TER) than cells expressing F508-del CFTR, with a slight differential pattern of expression of TJ markers. This result was in agreement with other studies suggesting that CFTR trafficking is essential for epithelial tightness and that therefore CFTR dysfunction can compromise regulation of paracellular transport[118].

Next, we measured expression of the E3 ubiquitin ligases under study, in polarized CFBE cells, following exposure to TGF-β and TNF-α. Although TGF-β has been reported to increase *SMURF2* mRNA and protein levels[112], interestingly, in our experiment TGF-β and TNF-α induced higher expression levels of *SMURF2* in polarized F508del-CFTR expressing cells than in those expressing wt-CFTR. The same study reported that this induction is mediated through smad- independent pathways, suggesting a tight regulation of the TGF-β-smad signaling pathway by SMURF2. In addition, SMURF2 was shown to interact with TNF receptor associated factor 2 (TRAF2), which is indispensable for TNF-induced activation of c-jun N-terminal kinase (JNK) and NF-kB, to promoting the ubiquitination of TNF receptor 2 (TNFR2), inducing its subcellular relocalization and consequently regulation of pro-inflammatory cytokine synthesis[118]. We also found in polarized F508del-CFTR expressing cells treated with TNF-α a significant increase in expression of *NEDD4L*, which regulates the TGF-β signal promoting SMAD3 degradation. It has been reported that in CF SMAD3 is reduced[51]. These data may therefore suggest an abnormal regulation of the TGF-β-smad signaling pathway by SMURF2 and NEDD4L in polarized CFBE cells expressing dysfunctional CFTR and a consequent increase of pro-inflammatory signals. There are some reports that suggest an involvement of the TGF-β signalling pathway in inhibition of pro-inflammatory signals, such as IL6, via SMAD2[77] and also IL8 via SMAD3[51]. These results suggest that, if E3 ubiquitin ligases are up-regulated in CF under inflammation, they could therefore be partially responsible for the increased pro-inflammatory mediators that characterize CF disease (Fig. V. 1).

Curiously, in the polarized F508del-CFTR cell line treated with TNF-α, *TGF-βRII* expression was higher than in wt-CFTR expressing cells, which may suggest, contrary to the data above, an increased TGF-β signal. However, previous studies have demonstrated an inhibitory effect by TNF-α on TGF-β signalling through down-regulation of TGF-βRII protein in human dermal fibroblasts, without decreasing the *TGF-βRII* mRNA[119]. This reinforces the idea that we cannot exclude the possibility that enhanced TGF-βRII mRNA abundance may not be associated with increased protein expression or activity. Thus, in the future it will be important to study not only transcriptomic, but also proteomic effects.

Several reports have demonstrated that TGF-β is a key regulator of epithelial-mesenchymal transition (EMT), and promotes TJ dissolution together with SMURF1[48]. Our present data demonstrated that, although without a differential expression of *SMURF1* mRNA levels, TGF-β decreased TER in both polarized cell lines and altered the TJ protein expression, suggesting TJ disruption after 24h. In addition, with TNF-α we also observed decreasing TER with a similar effect on epithelial integrity. This result requires further clarification, since we observed the same decrease of TER in wt-CFTR expressing CFBE cells without treatment, although no apparent effect on TJ integrity. However, if TGF-β promotes TJ disruption in polarized CFBE cells, this may suggest that smad-independent TGF-β signalling is also dysregulation.

In polarized CFBE cells and under conditions mimicking infection with *B. cenocepacia* IST4113 supernatant, we found that the bacterial products increased expression of *SMURF1* in wt-CFTR cell lines and increased TGF-βRII protein levels, while in F508del-CFTR cell lines they decreased the expression of *SMURF1* and increased TGF-βRII protein levels. The inverse was observed in the case of *SMURF2* expression. These results may suggest a presumably functionally specific differential response of both ubiquitin ligases under bacterial infection. The expression pattern of E3 ubiquitin ligases observed in polarized CFBE cells was not verified in unpolarized cells, which may also suggest the involvement of differentiated cell status in differential expression of E3 ubiquitin ligases. In addition, following exposure to bacterial supernatant, no significant differences were observed in protein levels of inflammatory mediators or epithelial markers. Kim and Sajjan (2005) using the *B. cenocepacia* AU0355 isolate supernatant, also demonstrated an undisturbed TJ organization[90]. Interestingly, it was reported that the *B. cenocepacia* pathogen crosses the epithelial barrier to access the basolateral epithelial receptor and that this invasion leads to TJ disruption[120]. In our experiment the cells only made contact with the bacterial supernatant at the apical surface. It might therefore be instructive to expose the basolateral surface of the cells to bacterial supernatant, because, as mentioned at the outset, modulation of the airway inflammatory response changes with the differential exposure of the epithelium surface. Bacterial invasion at the apical surface that does not alter airway epithelial barrier function results in minimal epithelial cell activation by cytokines. However when elements of the basolateral immune system are activated, a strong response is rapidly generated to defend against assault[115].

Finally, this experiment also demonstrated an interference of *B. cenocepacia* supernatant with CFTR protein expression, as has also been demonstrated by other groups with *P. aeruginosa*. This latter bacteria secretes Cif (CFTR inhibitory factor) protein, which

inhibits endocytic recycling of CFTR, capable of reducing apical membrane expression of both wt-CFTR and F508del-CFTR expressing cell lines. Interestingly, a potential homolog sharing approximately 38% amino acid sequence identity with Cif was identified in *B. cepacia*[121,122]. In the future it will be important to understand what is the key factor in this process, and how it affects CFTR expression.

In summary the present work has demonstrated that the expression of E3 ubiquitin ligases may be dysregulated under conditions of bacterial infection and inflammation relevant to Cystic Fibrosis in CF epithelial cells. These alterations may have implications involving the modulation of inflammatory responses, including TGF-β and TNF-α signaling pathways. However, in the future it will be important to deepen our understanding of the impact of this dysregulation on inflammatory pathways via the ablation of these E3 ubiquitin ligases, for example using siRNAs. A better understanding of the roles of altered E3 ubiquitin ligase regulation in CF will hopefully lead to discovery of novel targets and to more effective anti-inflammatory therapies. Finally, the current work constitutes an important characterization of two cellular models of cystic fibrosis which can be used as the basis for future studies on CF related inflammation.

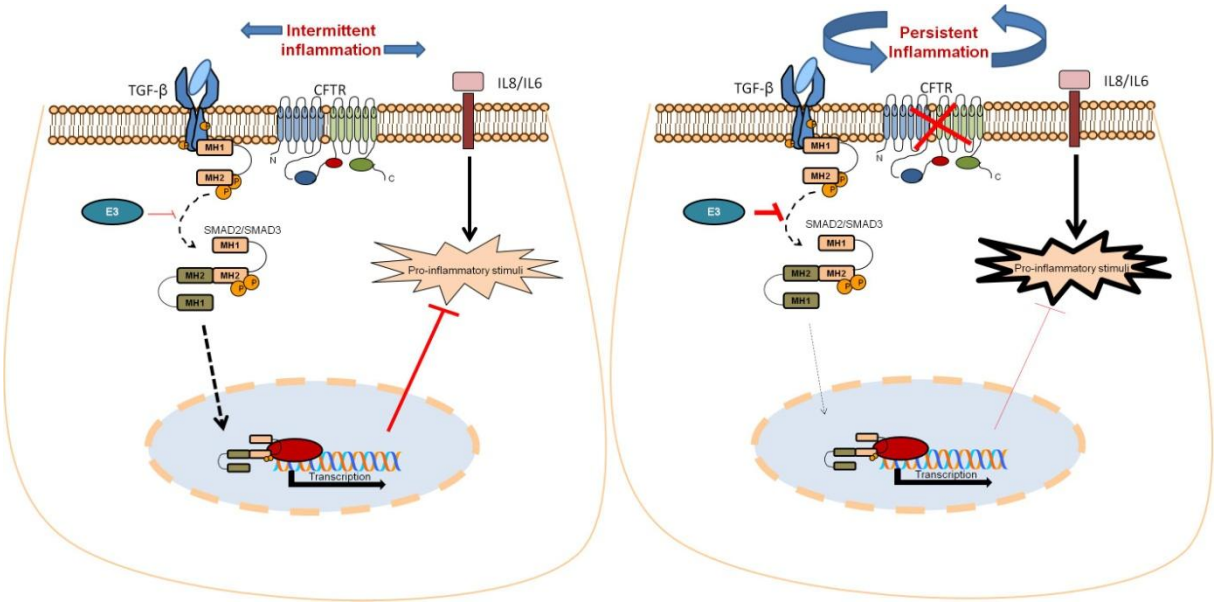


Figure V. 1 – Hypothetic model of role of E3 ubiquitin ligases in CF disease. In cells expressing wt-CFTR (left image) under inflammatory conditions (with TGF-β and TNF-α), the TGF-β binds to TGF-β receptors. TGF-β receptor type II phosphorylates TGF-β receptor I that recruits SMAD2/SMAD3. The latter is phosphorylated and translocated to the nucleus together with SMAD4 and binds to transcription factors implicated in inhibition of pro-inflammatory cytokines (IL8 and IL6). In F508del-CFTR cell lines (right image) the expression of E3 ubiquitin ligases (SMURF2 and NEDD4L) is increased, leading to TGF-β signal attenuation, promoting pro-inflammatory stimuli.

VI. References

1. Collins FS (1992). Cystic fibrosis: molecular biology and therapeutic implications**.** Science **256**, 774-779.
2. Cystic Fibrosis Foundation (2012) at http://www.cff.org/
3. Riordan JR, Rommens JM, Kerem B, Alon N, Rozmahel R, Grzelczak Z, Zielenski J, Lok S, Plavsic N, Chou JL, Drumm ML, Iannuzzi MC, Collins FS, Tsui L (1989). Identification of the cystic fibrosis gene: cloning and characterization of complementary DNA. Science. **245**, 1066 1073.
4. Gadsby DC, Vergani P, Csanády L (2006). The ABC protein turned chloride channel whose failure causes cystic fibrosis. Nature **440**, 477-483.
5. Hwang TC, Sheppard DN (2009). Gating of the CFTR Cl- channel by ATP-driven nucleotide-binding domain dimerisation. J Physiol **587**, 2151-2161.
6. Cystic Fibrosis Mutation Database (2012) at http://www.genet.sickkids.on.ca
7. Penque D, Mendes F, Beck S, Farinha C, Pacheco P, Nogueira P, Lavinha J, Malho R,Amaral MD (2000). Cystic fibrosis F508del patients have apically localized CFTR in a reduced number of airway cells**.** *Lab Invest* **80**, 857-868.
8. Hug MJ, Tamada T, Bridges RJ (2003). CFTR and bicarbonate secretion by [correction of to] epithelial cells. News Physiol Sci 18, 38-42.
9. Schreiber R, Nitschke R, Greger R, Kunzelmann K (1999). The cystic fibrosis transmembrane conductance regulator activates aquaporin 3 in airway epithelial cells. J Biol Chem 274, 11811-1186.
10. Reddy MM, Light MJ, Quinton PM (1999). Activation of the epithelial Na+ channel (ENaC) requires CFTR Cl- channel function. Nature 402, 301-304.
11. Kogan I, Ramjeesingh M, Li C, Kidd JF, Wang Y, Leslie EM, Cole SP, Bear CE (2003). CFTR directly mediates nucleotide-regulated glutathione flux. CFTR directly mediates nucleotide-regulated glutathione flux. EMBO J 22, 1981-1989.
12. Chemiel JF, Konstan MW (2010). Inflammation in the Cystic fibrosis Lung. In *Cystic Fibrosis*, eds. Allen JL, Panitch HB, Rubenstein RC, pp. 57-72. Informa, healthcare
13. Zhang L, Button B, Gabriel SE, Burkett S, Yan Y, Skiadopoulos MH, Dang YL, Vogel LN, McKay T, Mengos A, Boucher RC, Collins PL, Pickles RJ (2009). CFTR delivery to 25% of surface epithelial cells restores normal rates of mucus transport to human cystic fibrosis airway epithelium. PLoS Biol **7**, (7):e1000155
14. Ernst RK, Yi EC, Guo L, Lim KB, Burns JL, Hackett M, Miller SI (1999). Specific lipopolysaccharide found in cystic fibrosis airway *Pseudomonas aeruginosa*. Science 286, 1561-1565
15. Bonfield TL, Konstan MW, Berger M (1999). Altered respiratory epithelial cell cytokine production in cystic fibrosis. J Allergy Clin Immunol **104**, 72-78.
16. Carrabino S, Carpani D, Livraghi A, Di Cicco M, Costantini D, Copreni E, Colombo C, Conese M (2006). Dysregulated interleukin-8 secretion and NF-kappaB activity in human cystic fibrosis nasal epithelial cells. J Cyst Fibros **5**, 113-119.
17. Osika E, Cavaillon JM, Chadelat K, Boule M, Fitting C, Tournier G, Clement A (1999). Distinct sputum cytokine profiles in cystic fibrosis and other chronic inflammatory airway disease. Eur Respir J 14, 339-346.
18. Dean TP, Dai Y, Shute JK, Church MK, Warner JO (1993). Interleukin-8 concentrations are elevated in bronchoalveolar lavage, sputum, and sera of children with cystic fibrosis. Pediatr Res **34**, 159-161.
19. Kaza SK, McClean S, Callaghan M (2011). IL-8 released from human lung epithelial cells induced by cystic fibrosis pathogens Burkholderia cepacia complex affects the growth and intracellular survival of bacteria. Int J Med Microbiol **301**, 26-33.
20. Kube D, Sontich U, Fletcher D, Davis PB (2001). Proinflammatory cytokine responses to P. aeruginosa infection in human airway epithelial cell lines. Am J Physiol Lung Cell Mol Physiol **280**, 493-502.
21. Konstan MW, Berger M (1997). Current understanding of the inflammatory process in cystic fibrosis: onset and etiology. Pediatr Pulmonol **24**, 137-142.
22. Balough K, McCubbin M, Weinberger M, Smits W, Ahrens R, Fick R (1995). The relationship between infection and inflammation in the early stages of lung disease from cystic fibrosis. Pediatr Pulmonol **20**, 63-70.

23. Rosenfeld M, Gibson RL, McNamara S, Emerson J, Burns JL, Castile R, Hiatt P, McCoy K, Wilson CB, Inglis A, Smith A, Martin TR, Ramsey BW (2001). Early pulmonary infection, inflammation, and clinical outcomes in infants with cystic fibrosis. Pediatr Pulmonol **32**, 356-366.
24. Scheid P, Kempster L, Griesenbach U, Davies JC, Dewar A, Weber PP, Colledge WH, Evans MJ, Geddes DM, Alton EW (2001). Inflammation in cystic fibrosis airways: relationship to increased bacterial adherence. Eur Respir J **17**, 27-35.
25. Machen TE (2006). Innate immune response in CF airway epithelia: hyperinflammatory? Am J Physiol Cell Physiol **291**, 218-230
26. Conaway RC, Brower CS, Conaway JW (2002). Emerging roles of ubiquitin in transcription regulation. Science **296**, 1254-1258.
27. Marmor MD, Yarden Y (2004) Role of protein ubiquitylation in regulating endocytosis of receptor tyrosine kinases. Role of protein ubiquitylation in regulating endocytosis of receptor tyrosine kinases. Oncogene **23**, 2057-2070.
28. Hershko A, Ciechanover A. (1998). The ubiquitin system. Annu Rev Biochem **67**, 425-479
29. Fulda S, Rajalingam K, Dikic I (2012). Ubiquitylation in immune disorders and cancer: from molecular mechanisms to therapeutic implications. EMBO Mol Med **4**, 545-556
30. Ogunjimi AA, Briant DJ, Pece-Barbara N, Le Roy C, Di Guglielmo GM, Kavsak P, Rasmussen RK, Seet BT, Sicheri F, Wrana JL (2005). Regulation of Smurf2 ubiquitin ligase activity by anchoring the E2 to the HECT domain. Mol Cell. **19**, 297-308.
31. Scheffener M, Staub O (2008). HECT ubiquitin-protein ligases in Humam disease. In Protein degration: The ubiquitin-proteasome system and disease volume 4, eds. Mayer R, Ciechanover A, Rechsteiner M, pp. 77-105. WILEY-VCH
32. Berrnassola F, Karin M, Ciechanover A, Melino G (2008). The HECT family of E3 ubiquitin ligases: multiple players in cancer development. Cancer Cell. **14**, 10-21.
33. Hampton TH, Stanton BA (2010). A novel approach to analyze gene expression data demonstrates that the DeltaF508 mutation in CFTR downregulates the antigen presentation pathway. Am J Physiol Lung Cell Mol Physiol **298**, 473-482.
34. Rotin D, Kumar S (2009). Physiological functions of the HECT family of ubiquitin ligases. Nat Rev Mol Cell Biol 10, 398-409.
35. Plant PJ, Yeger H, Staub O, Howard P, Rotin D. (1997). e C2 domain of the ubiquitin protein ligase Nedd4 mediates Ca2+-dependent plasma membrane localization. J Biol Chem. **272**, 32329-32336.
36. Snyder PM, Olson DR, McDonald FJ, Bucher DB (2001). Multiple WW domains, but not the C2 domain, are for inhibition of the epithelial Na+ channel by human Nedd4. J Biol Chem. **276**, 28321-28326.
37. Maspero E, Mari S, Valentini E, Musacchio A, Fish A, Pasqualato S, Polo S (2011). Structure of the HECT:ubiquitin complex and its role in ubiquitin chain elongation. EMBO Rep. **12**, 342-349.
38. Izzi L, Attisano L (2004) Regulation of the TGFbeta signalling pathway by ubiquitin-mediated degradation. Oncogene **23**, 2071-2078.
39. Wiesner S, Ogunjimi AA, Wang HR, Rotin D, Sicheri F, Wrana JL, Forman-Kay JD (2007). Autoinhibition of the HECT-type ubiquitin ligase Smurf2 through its C2 domain. Cell. **130**, 651-662
40. Lu K, Li P, Zhang M, Xing G, Li X, Zhou W, Bartlam M, Zhang L, Rao Z, He F (2011). Pivotal role of the C2 domain of the Smurf1 ubiquitin ligase in substrate selection. J Biol Chem. **286**, 16861-16870.
41. Tajima Y, Goto K, Yoshida M, Shinomiya K, Sekimoto T, Yoneda Y, Miyazono K, Imamura T (2003). Chromosomal region maintenance 1 (CRM1)-dependent nuclear export of Smad ubiquitin regulatory factor 1 (Smurf1) is essential for negative regulation of transforming growth factor-beta signaling by Smad7. J Biol Chem. **278**, 10716-10721
42. Kavsak P, Rasmussen RK, Causing CG, Bonni S, Zhu H, Thomsen GH, Wrana JL (2000). Smad7 binds to Smurf2 to form an E3 ubiquitin ligase that targets the TGF beta receptor for degradation. Mol Cell. **6**, 1365-1375.
43. Ebisawa T, Fukuchi M, Murakami G, Chiba T, Tanaka K, Imamura T, Miyazono K (2001). Smurf1 interacts with transforming growth factor-beta type I receptor through Smad7 and induces receptor degradation. J Biol Chem. **276**, 12477-12480.
44. Tang LY, Yamashita M, Coussens NP, Tang Y, Wang X, Li C, Deng CX, Cheng SY, Zhang YE (2011). Ablation of Smurf2 reveals an inhibition in TGF-β signalling through multiple mono-ubiquitination of Smad3. EMBO J. **30**, 4777-4789.
45. Kuratomi G, Komuro A, Goto K, Shinozaki M, Miyazawa K, Miyazono K, Imamura T (2005). NEDD4-2 (neural precursor cell expressed, developmentally down-regulated 4-2) negatively

regulates TGF-beta (transforming growth factor-beta) signalling by inducing ubiquitin-mediated degradation of Smad2 and TGF-beta type I receptor. Biochem J. **386**, 461-470.
46. Li MO, Wan YY, Sanjabi S, Robertson AK, Flavell RA (2006). Transforming growth factor-beta regulation of immune responses. Annu Rev Immunol. **24**, 99-146.
47. Heino J, Ignotz RA, Hemler ME, Crouse C, Massagué J (1989). Regulation of cell adhesion receptors by transforming growth factor-beta. Concomitant regulation of integrins that share a common beta 1 subunit. J Biol Chem. **264**, 380-388.
48. Ozdamar B, Bose R, Barrios-Rodiles M, Wang HR, Zhang Y, Wrana JL (2005). ulation of the polarity protein Par6 by TGFbeta receptors controls epithelial cell plasticity. Science. **307**, 1603-1609.
49. Shull MM, Ormsby I, Kier AB, Pawlowski S, Diebold RJ, Yin M, Allen R, Sidman C, Proetzel G, Calvin D, Annunziata N, Doetschman T (1992). Targeted disruption of the mouse transforming growth factor-beta 1 gene results in multifocal inflammatory disease. Nature 359, 693-699.
50. Derynck R, Akhurst RJ, Balmain A (2009).TGF-beta signaling in tumor suppression and cancer progression. Nat Genet **29**, 117-1129.
51. Kelley TJ, Elmer HL, Corey DA (2001). Reduced Smad3 protein expression and altered transforming growth factor-beta1-mediated signaling in cystic fibrosis epithelial cells. Am J Respir Cell Mol Biol **25**, 732-738.
52. Wrana JL, Attisano L, Wieser R, Ventura F, Massagué J (1994). Mechanism of activation of the TGF-beta receptor. Nature **370,** 341-347.
53. Massagué J (1998). TGF-beta signal transduction. Annu Rev Biochem. **67**, 753-791.
54. Inman GJ, Nicolás FJ, Hill CS (2002). Nucleocytoplasmic shuttling of Smads 2, 3, and 4 permits sensing of TGF-beta receptor activity. Mol Cell. **10**, 283-294.
55. Baburajendran N, Jauch R, Tan CY, Narasimhan K, Kolatkar PR (2011). uctural basis for the cooperative DNA recognition by Smad4 MH1 dimers. Nucleic Acids Res. **39**, 8213-8222.
56. Shi Y, Massagué J (2003). Mechanisms of TGF-beta signaling from cell membrane to the nucleus. Cell 113, 685-700.
57. Massagué J, Gomis RR (2006). The logic of TGFbeta signaling. FEBS Lett. **580**, 2811-2820.
58. Chacko BM, Qin BY, Tiwari A, Shi G, Lam S, Hayward LJ, De Caestecker M, Lin K (2004). Structural basis of heteromeric smad protein assembly in TGF-beta signaling. Mol Cell. **15**, 813-823.
59. Zhang YE (2009). Non-Smad pathways in TGF-β signaling. Cell Res. **19**, 128–139.
60. Moustakas A, Heldin CH (2005). Non-Smad TGF-beta signals. J Cell Sci. **118**, 3573-3584.
61. Derynck R, Zhang YE (2003). Smad-dependent and Smad-independent pathways in TGF-beta family signalling. Nature **425**, 577-584.
62. Zhang S, Fei T, Zhang L, Zhang R, Chen F, Ning Y, Han Y, Feng XH, Meng A, Chen YG (2007). Smad7 antagonizes transforming growth factor beta signaling in the nucleus by interfering with functional Smad-DNA complex formation. Mol Cell Biol. **27**, 4488-4499.
63. Lin X, Liang M, Feng XH (2000). Smurf2 is a ubiquitin E3 ligase mediating proteasome-dependent degradation of Smad2 in transforming growth factor-beta signaling. J Biol Chem. **275**, 36818-36822.
64. Gao S, Alarcón C, Sapkota G, Rahman S, Chen PY, Goerner N, Macias MJ, Erdjument-Bromage H, Tempst P, Massagué J (2009). Ubiquitin ligase Nedd4L targets activated Smad2/3 to limit TGF-beta signaling. Mol Cell. **36**, 457-468.
65. Morén A, Imamura T, Miyazono K, Heldin CH, Moustakas A (2005). Degradation of the tumor suppressor Smad4 by WW and HECT domain ubiquitin ligases. J Biol Chem. **280**, 22115-22123.
66. Nichols D, Chmiel J, Berger M (2008). Chronic inflammation in the cystic fibrosis lung: alterations in inter- and intracellular signaling. Clin Rev Allergy Immunol **34**, 146-162.
67. Smith JJ, Travis SM, Greenberg EP, Welsh MJ (1996). Cystic fibrosis airway epithelia fail to kill bacteria because of abnormal airway surface fluid. Cell **85**, 229-236
68. Hajj R, Lesimple P, Nawrocki-Raby B, Birembaut P, Puchelle E,Coraux C (2007). Human airway surface epithelial regeneration is delayed and abnormal in cystic fibrosis**.** *J Pathol* **211**, 340-350.
69. Puchelle E, Zahm JM, Tournier JM, Coraux C (2006). Airway epithelial repair, regeneration, and remodeling after injury in chronic obstructive pulmonary disease. Proc Am Thorac Soc **3**, 726-733.
70. Collaco JM, Cutting RG (2008). Update on gene modifiers in cystic fibrosis. Curr Opin Pulm Med **14**, 559-566.
71. Cutting GR (2010). Modifiers genes in mendelian disorders: the example of cystic fibrosis. Amn N Y Acad Sci **1214**, 57-69.
72. Arkwright PD, Laurie S, Super M, Pravics V, Schwarz M (2000). TGF-β_1 genotype and accelerated decline in ling function of patients with cystic fibrosis. Thorax **55**, 459-462.

73. Harris WT, Muhlebach MS, Oster RA, Knowles MR, Clancy JP, Noah TL (2011). sma TGF-β_1 in pediatric cystic fibrosis: potential biomarker of lung disease and response to therapy. Pediatr Pulmonol **46**, 688-695.
74. Harris WT, Muhlebach MS, Oster RA, Knowles MR, Noah TL (2009). Transforming growth factor-β1 in bronchoalveolar lavage fluid from children with cystic fibrosis. Pediatr Pulmonol **44**, 1057-1064.
75. Lu S, Dong Z (2006). Characterization of TGF-beta-regulated interleukin-8 expression in human prostate cancer cells. Prostate **66**, 996-1004.
76. Ge Q, Moir LM, Black JL, Oliver BG, Burgess JK (2010), TGFβ1 induces IL-6 and inhibits IL-8 release in human bronchial epithelial cells: the role of Smad2/3. J Cell Physiol. **225**, 846-854.
77. Walia B, Wang L, Merlin D, Sitaraman SV (2003). TGF-beta down-regulates IL-6 signaling in intestinal epithelial cells: critical role of SMAD-2. FASEB J. 17, 2130-2132.
78. Guschin D, Rogers N, Briscoe J, Witthuhn B, Watling D, Horn F, Pellegrini S, Yasukawa K, Heinrich P, Stark GR (1995). A major role for the protein tyrosine kinase JAK1 in the JAK/STAT signal transduction pathway in response to interleukin-6. EMBO J. **14**, 1421–1429.
79. Choi KC, Lee YS, Lim S, Choi HK, Lee CH, Lee EK, Hong S, Kim IH, Kim SJ, Park SH (2006). Smad6 negatively regulates interleukin 1-receptor-Toll-like receptor signaling through direct interaction with the adaptor Pellino-1. Nat Immunol. **10**, 1057-1065.
80. Lee YS, Kim JH, Kim ST, Kwon JY, Hong S, Kim SJ, Park SH (2006). Smad7 and Smad6 bind to discrete regions of Pellino-1 via their MH2 domains to mediate TGF-beta1-induced negative regulation of IL-1R/TLR signaling. Biochem Biophys Res Commun. **393**, 836-843.
81. Li S, Lu K, Wang J, An L, Yang G, Chen H, Cui Y, Yin X, Xie P, Xing G, He F, Zhang L (2010). Ubiquitin ligase Smurf1 targets TRAF family proteins for ubiquitination and degradation. Mol Cell Biochem. **338**, 11-17.
82. Muselet-Charlier C, Roque T, Boncoeur E, Chadelat K, Clement A, Jacquot J, Tabary O (2007). Enhanced IL-1beta-induced IL-8 production in cystic fibrosis lung epithelial cells is dependent of both mitogen-activated protein kinases and NF-kappaB signaling. Biochem Biophys Res Commun **357**, 402-407.
83. Bodas M, Vij N (2010). The NFκB Signaling in Cystic Fibrosis Lung Disease: Pathophysiology and Therapeutic Potential. Discov Med **9**, 346–356.
84. Venkatakrishnan A, Stecenko AA, King G, Blackwell TR, Brigham KL, Christman JW, Blackwell TS (2000). Exaggerated activation of nuclear factor-kappaB and altered IkappaB-beta processing in cystic fibrosis bronchial epithelial cells. Am J Respir Cell Mol Biol **23**, 396-403.
85. Yuan C, Qi J, Zhao X, Gao C (2012). Smurf1 protein negatively regulates interferon-γ signaling through promoting STAT1 protein ubiquitination and degradation. J Biol Chem. **287**, 17006-17015.
86. Kelley TJ, Elmer HL (2000). In vivo alterations of IFN regulatory factor-1 and PIAS1 protein levels in cystic fibrosis epithelium. J Clin Invest **106**, 403-410.
87. Matter K, Balda MS (2003). Signalling to and from tight junctions. Nat Rev Mol Cell Biol **4**, 225-236
88. Matter K, Aijaz S, Tsapara A, Balda MS (2005). Mammalian tight junctions in the regulation of epithelial differentiation and proliferation. Curr Opin Cell Biol **17**, 453-458.
89. Coyne CB, Vanhook MK, Gambling TM, Carson JL, Boucher RC, Johnson LG (2002). Regulation of airway tight junctions by proinflammatory cytokines. Mol Biol Cell **13**, 3218-3234.
90. Kim JY, Sajjan SU, Krasan GP, LiPuma JJ (2005). Disruption of Tight Junctions during Traversal of the Respiratory Epithelium by *Burkholderia cenocepacia.*Infect Immun **73**, 7107–7112.
91. Puchelle E, Zahm JM, Tournier JM, Coraux C (2006). Airway epithelial repair, regeneration, and remodeling after injury in chronic obstructive pulmonary disease. Proc Am Thorac Soc **3**, 726-733.
92. Hajj R, Lesimple P, Nawrocki-Raby B, Birembaut P, Puchelle E, Coraux C (2007). Human airway surface epithelial regeneration is delayed and abnormal in cystic fibrosis. J Pathol **211**, 340-350.
93. Dupuit F, Kälin N, Brézillon S, Hinnrasky J, Tümmler B, Puchelle E (1995). CFTR and differentiation markers expression in non-CF and deltaF508 homozygous CF nasal epithelium. J Clin Invest **96**, 1601-1611.
94. Wang HR, Ogunjimi AA, Zhang Y, Ozdamar B, Bose R, Wrana JL (2006). Degradation of RhoA by Smurf1 ubiquitin ligase. Methods Enzymol **406**, 437-447.
95. Wang HR, Zhang Y, Ozdamar B, Ogunjimi AA, Alexandrova E, Thomsen GH, Wrana JL (2003). Regulation of cell polarity and protrusion formation by targeting RhoA for degradation. Science **302**, 1775-1779.
96. Bryan B, Cai Y, Wrighton K, Wu G, Feng XH, Liu M (2005). Ubiquitination of RhoA by Smurf1 promotes neurite outgrowth. FEBS Lett **579**, 1015-1019.

97. Ozdamar B, Bose R, Barrios-Rodiles M, Wang HR, Zhang Y, Wrana JL (2005). Regulation of the polarity protein Par6 by TGFbeta receptors controls epithelial cell plasticity. Science **307**, 1603-1609.
98. Raikwar NS, Vandewalle A, Thomas CP (2010). Nedd4-2 interacts with occludin to inhibit tight junction formation and enhance paracellular conductance in collecting duct epithelia. Am J Physiol Renal Physiol **299**, 436-444.
99. Schwamborn JC, Khazaei MR, Püschel AW (2007). The interaction of mPar3 with the ubiquitin ligase Smurf2 is required for the establishment of neuronal polarity. J Biol Chem **282**, 35259-35268.
100. Schwamborn JC, Müller M, Becker AH, Püschel AW (2007). Ubiquitination of the GTPase Rap1B by the ubiquitin ligase Smurf2 is required for the establishment of neuronal polarity. EMBO J **26**, 1410-1422.
101. Kimura T, Kawabe H, Jiang C, Zhang W, Xiang YY, Lu C, Salter MW, Brose N, Lu WY, Rotin D (2011). Deletion of the ubiquitin ligase Nedd4L in lung epithelia causes cystic fibrosis-like disease. Proc Natl Acad Sci USA **108**, 3216-3221.
102. Mira MP, Madeira A, Moreira AS, Coutinho CP, Sá-Correia I (2011). Genomic Expression Analysis Reveals Strategies of *Burkholderia cenocepacia* to Adapt to Cystic Fibrosis Patients' Airways and Antimicrobial Therapy. PLoS One **6**, e28831.
103. PrimerBank Data Base (2012) at http://pga.mgh.harvard.edu/primerbank/
104. ExonMine Data Base (2012) at http://www.imm.fm.ul.pt/exonmine/
105. UniProt (2012) at http://www.uniprot.org/
106. Mahenthiralingam E, Urban TA, Goldberg JB (2005).The multifarious, multireplicon Burkholderia cepacia complex. Nat Rev Microbiol **3**, 144-156.
107. Murphy SJ, Doré JE, Edens M, Coffey RJ, Barnard JA, Mitchell H, Wilkes M, Leof EB (2004). Differential Trafficking of Transforming Growth Factor-β Receptors and Ligand in Polarized Epithelial Cells. Mol Biol Cell **15**, 2853–2862.
108. Humlicek AL, Manzel LJ, Chin CL, Shi L, Excoffon KJ, Winter MC, Shasby DM, Look DC (2007). Paracellular permeability restricts airway epithelial responses to selectively allow activation by mediators at the basolateral surface. J Immunol **178**, 6395-6403.
109. Wright JM, Merlo CA, Reynolds JB, Zeitlin PL, Garcia JG, Guggino WB, Boyle MP (2006). Respiratory epithelial gene expression in patients with mild and severe cystic fibrosis lung disease. Am J Respir Cell Mol Biol **35**, 327-336.
110. Virella-Lowell I, Herlihy JD, Liu B, Lopez C, Cruz P, Muller C, Baker HV, Flotte TR (2004). Effects of CFTR, interleukin-10, and Pseudomonas aeruginosa on gene expression profiles in a CF bronchial epithelial cell Line. Mol Ther **10**, 562-573.
111. Zabner J, Scheetz TE, Almabrazi HG, Casavant TL, Huang J, Keshavjee S, McCray PB Jr (2005). CFTR DeltaF508 mutation has minimal effect on the gene expression profile of differentiated human airway epithelia. Am J Physiol Lung Cell Mol Physiol **289**, 545-553.
112. Ohashi N, Yamamoto T, Uchida C, Togawa A, Fukasawa H, Fujigaki Y, Suzuki S, Kitagawa K, Hattori T, Oda T, Hayashi H, Hishida A, Kitagawa M (2005). Transcriptional induction of Smurf2 ubiquitin ligase by TGF-beta. FEBS Lett 579, 2557-2563.
113. Zhang Y, Handley D, Kaplan T, Yu H, Bais AS, Richards T, Pandit KV, Zeng Q, Benos PV, Friedman N, Eickelberg O, Kaminski N (2011). High throughput determination of TGFβ1/SMAD3 targets in A549 lung epithelial cells. PLoS One **6**, e20319.
114. Carterson AJ, Höner zu Bentrup K, Ott CM, Clarke MS, Pierson DL, Vanderburg CR, Buchanan KL, Nickerson CA, Schurr MJ (2005). A549 lung epithelial cells grown as three-dimensional aggregates: alternative tissue culture model for Pseudomonas aeruginosa pathogenesis. Infect Immun **73**, 1129-1140.
115. Humlicek AL, Manzel LJ, Chin CL, Shi L, Excoffon KJ, Winter MC, Shasby DM, Look DC (2007). Paracellular permeability restricts airway epithelial responses to selectively allow activation by mediators at the basolateral surface. J Immunol **178**, 6395-6403.
116. Di Guglielmo GM, Le Roy C, Goodfellow AF, Wrana JL (2003). Distinct endocytic pathways regulate TGF-beta receptor signalling and turnover. Nat Cell Biol **5**, 410-421.
117. LeSimple P, Liao J, Robert R, Gruenert DC, Hanrahan JW (2010). Cystic fibrosis transmembrane conductance regulator trafficking modulates the barrier function of airway epithelial cell monolayers. J Physiol **588**, 1195-209.
118. Carpentier I, Coornaert B, Beyaert R (2008). Smurf2 is a TRAF2 binding protein that triggers TNF-R2 ubiquitination and TNF-R2-induced JNK activation. Biochem Biophys Res Commun **374**, 752-757.

119. Yamane K, Ihn H, Asano Y, Jinnin M, Tamaki K. (2003). Antagonistic effects of TNF-alpha on TGF-beta signaling through down-regulation of TGF-beta receptor type II in human dermal fibroblasts. J Immunol **171**, 3855-3862.
120. Kim JY, Sajjan US, Krasan GP, LiPuma JJ (2005). Disruption of tight junctions during traversal of the respiratory epithelium by Burkholderia cenocepacia. Infect Immun **73**, 7107-7112.
121. Swiatecka-Urban A, Moreau-Marquis S, Maceachran DP, Connolly JP, Stanton CR, Su JR, Barnaby R, O'toole GA, Stanton BA (2005). Pseudomonas aeruginosa inhibits endocytic recycling of CFTR in polarized human airway epithelial cells. Am J Physiol Cell Physiol **290**, 862-872.
122. MacEachran DP, Ye S, Bomberger JM, Hogan DA, Swiatecka-Urban A, Stanton BA, O'Toole JA (2007). The *Pseudomonas aeruginosa* Secreted Protein PA2934 Decreases Apical Membrane Expression of the Cystic Fibrosis Transmembrane Conductance Regulator. Infect Immun **75**, 3902–3912.
123. Kaneki H, Guo R, Chen D, Yao Z, Schwarz EM, Zhang YE, Boyce BF, Xing L. (2005)Tumor necrosis factor promotes Runx2 degradation through up-regulation of Smurf1 and Smurf2 in osteoblasts. J Biol Chem **281**, 4326-4333.
124. Amaral MD, Kunzelmann K (2007). Molecular targeting of CFTR as a therapeutic approach to cystic fibrosis. Trends Pharmacol Sci **28**, 334-341.

VII. Supplementary Data

A. Introduction supplementary

Table VII. A1 Classes of CFTR mutations [124].

Classes	Mutation Defect	CFTR quantity at cell surface	Cl^- transport ability	Mutation	Mutation-specific therapies
Class I ***Defective Protein Synthesis***	Failure to synthesize full-length protein	None	None	G542X	Aminoglycoside antibiotics
Class II ***Defective Protein Processing***	Improper folding or trafficking to cell mebrane	Little to none	Little to none	F508del	Chemical, molecular or pharmacological chaperones
Class III ***Defective Protein Regulation And Altered Conductance***	Inability of open channel	Normal	None	G551D	CFTR activators alkylxanthines (CPX) and flavonoid
Class IV ***Defective Protein Regulation and Altered Conductance***	Defect in channel that impairs ion transport	Normal	Some	R334W	Potentiators
Class V ***Reduced CFTR Level***	Splicing error causes variable synthesis of protein	Normal	Some	3272-26A>G	Splicing factors

B. Material and Methods supplementary

1. Quantitative real-time PCR

For differential E3 ubiquitin ligase and TGF-βRII expression analysis, quantitative real time PCR (qRT-PCR) amplifications were performed in a Cx96 real time PCR machine using 96 well plates with Evagreen reaction mixture (all from Bio-Rad laboratories). The sequences of primer sets used are shown in table III. Using the Harvard PrimerBank database (http://pga.mgh.harvard.edu/primerbank/)[103] and the ExonMine database (http://www.imm.fm.ul.pt/exonmine/)[104], primers were chosen to amplify across exon boundaries to minimise potential background amplification of products from genomic DNA. The total qRT-PCR reaction mixture of 20µl per well contained 5µl of the template cDNA (diluted 1:5), 250 nM of each forward and reverse primer (Table VII. B1), and 1x Evagreen PCR reaction mixture. The cycling protocol was 40 cycles of 10 sec at 95ºC and 30 sec at 60ºC. Melting curve with a temperature gradient from 65 to 95ºC was performed to confirm amplification of specific products. Relative abundance of mRNA was measured using samples run in duplicate using the Bio-Rad CFX Manager 2.1 software, with test gene values normalized to β-actin levels by calculating the ddCT value which is the threshold cycle (CT) of the endogenous control (β-actin) subtracted from the CT of the target gene under study. The fold difference in gene expression was calculated by the relative quantification method using the mathematical equation 2^{-ddCT}, since the efficiency of target gene and endogenous control amplifications was found to be approximately equal (≈100%), using standard curve amplification. For standard curve elaboration, 20µl reactions containing a series of 1:5

dilutions of cDNA, starting with approximately 50ng/reaction, were performed under the same conditions. The efficiency of primers was calculated using the formula $[10^{(-1/slope)} -1] \times 100$. The cDNA obtained by Luka Clarke at the Faculty of Sciences of the University of Lisbon, Lisbon, Portugal, from nasal epithelium cells from non-CF and F508del-CFTR homozygous patients collected at Santa Maria Hospital, Lisbon, Portugal, was also used to assess the mRNA expression level of E3 ubiquitin ligases by qRT-PCR performed under the same conditions.

2. Western Blotting analysis

Total protein was measured by modified Lowry assay. Briefly, 10µl of total protein was diluted in water and incubated 10min at 25ºC with 0,15% (w/v) sodium deoxycholate for protein solubilisation. Then proteins were precipitated with 72% (w/v) trichloroacetic acid and centrifuged 14000 rpm for 5min at 25ºC. The supernatant was aspirated and pellet resuspended in water and incubated 10min at 25ºC with solution A. After this, solution B was added and samples incubated 30min at 25ºC in the dark before measurement of the A750 nm. The regression equation for protein concentration was determined using bovine serum albumin (BSA) as a standard protein. Protein was detected by Western blot analysis. Equal amounts of protein were loaded on each lane for SDS polyacrylamide gels electrophoresis (PAGE), and run at 80 V for 2.5h. Subsequently proteins were transferred to membranes in transfer buffer at 400 mA for 1,5h. After the transfer, membranes were blocked overnight in 5% skimmed milk in 1xPBS supplemented with 0,1% (v/v) tween (PBS-T). Membranes were incubated 1h with primary antibody (Table VII. B2). As a control, Alpha Tubulin (α-Tubulin) antibody was also used to confirm equal loading of protein in each lanes. After three washes with PBS-T, the membranes were incubated 1h with secondary antibodies (Table VII. B2) and washed another three times. All antibodies were diluted in blocking solution. Chemiluminescent detection was performed using Immobilon HRP chemiluminescent substrate peroxide solution and luminol reagent (Millipore, USA). The quantification of band intensity was performed using the Image Lab software (Bio-Rad laboratories) and normalized to alpha-Tubulin.

3. Immunofluorescence

CFBE cells grown on glass coverslips were fixed using a methanol-acetone method. Briefly, cells were washed twice with PBS supplemented with calcium chloride ($CaCl_2$) and magnesium chloride ($MgCl_2$) (PBS+/+) for 5min and incubated 5min with methanol at -20ºC. After this, cells were incubated with acetone for 5min at -20ºC and washed 3x with PBS+/+. Fixed samples were next incubated with PBS+/+ supplemented with 5% FBS and 0,1M glycine for 1h. After three washes with PBS+/+ supplemented with 5% FBS cells were incubated for 1h with primary antibody in PBS+/+ with 5% FBS (Table VII. B3) at 4ºC and were washed again 3x with PBS+/+, followed by a 1h incubation with secondary antibody

(Table VII. B3) at 4ºC. Finally, the coverslips were mounted, in mounting solution (fluoromount-G with 1,5µg/ml 49,6-diamidino-2-phenyl-indole, dihydrochoride (DAPI) for nuclear detection) on glass slides and visualized by Leica SPE confocal microscopy.

CFBE cells polarized at midium on snapwell filter inserts were washed three times each for 5min with PBS and fixed for 30 min using 4% (v/v) formaldehyde in PBS. After three washes with PBS the cells were permeabilized by adding 0,5% (w/v) Triton-X for 15min to apical and basolateral compartments, washed three times with PBS and blocked with 1% BSA for 20min. Subsequently, the filter with polarized cells was removed from its snapwell support using a scalpel and 1/8 membrane was incubated for 1h with primary antibodies diluted in PBS with 1% BSA at 4ºC (Table VII. B3). Cells were washed three times for 5 min in PBS with 0,05% triton–X and incubated with secondary antibodies (Table VII. B3) at 4ºC for 1h. Finally, cells were washed three times for 5 min in PBS with 0,05% triton-X and were mounted, on glass slides in flouromount/DAPI and visualized on a confocal microscope.

4. Enzyme-linked immunosorbent assay method

In all experiments the growth media recovered after 24h was frozen at -80ºC and used for determination of cytokine levels by ELISA using Multi-Analyte ELISArray^{TM} kit (SABiosciences, USA, #MEH-004A) according to the manufacturers' instructions.

5. Statistical analysis

Data are presented as a means ± standard error of the mean (SEM). Data were analyzed using student's *t*-test, as indicated in figure legends, with $p<0,05$ accepted as the level of statistical significance.

Table VII. B1 - Sequence of primers used in the real time PCR

Genes	PrimerBank ID		Sequence of primers[a] 5' → 3'	Target sites on genes	Target (bp)
β-Actin		F:	CTCTTCCAGCCTTCCTTCCT		116
		R:	AGCACTGTGTTGGCGTACAG		
Smurf1	31317290a1	F:	AGATCCGTCTGACAGTGTTATGT	41-63	92
		R:	CCCATCCACGACAATCTTTGC	132-112	
Smurf2	12232397a3	F:	GTCCAGAGACCGAATAGGCAC	411-431	102
		R:	CCAGAGGCGGTTCTCCTTTC	512-493	
Nedd4l	21361472a1	F:	GAGCCGGTCTATGGACTTTCC	19-39	8
		R:	GCGAGATCAATTCCAGAAACAAC	98-76	

[a]F, forward primer; R, reverse primer

Table VII. B2 - Primary and secondary Antibodies used in Western blotting

Primary antibodies	Supplier	Dilution	Secondary antibody	Supplier	Dilution
Mouse monoclonal IgG_1 anti-E-cadherin	BD Transduction Laboratrories 6101181	1:10000	Goat Anti-Mouse IgG (H+L) HRP conjugate	BIO-RAD 170-6516	1:3000
Mouse monoclonal IgG_1 anti-Z0-1	Invitrogen 339100	1:10000	Goat Anti-Mouse IgG (H+L) HRP conjugate	BIO-RAD 170-6516	1:3000
Mouse monoclonal IgG_2 anti- CK18	Santa cruz sc-32329	1:100	Goat Anti-Mouse IgG (H+L) HRP conjugate	BIO-RAD 170-6516	1:3000
Mouse monoclonal[2] IgG_{2a} anti-CK14	Santa cruz sc-53253	1:100	Goat Anti-Mouse IgG (H+L) HRP conjugate	BIO-RAD 170-6516	1:3000
Rabbit polyclonal IgG_1 anti-TβRII (C-16)	Santa cruz sc-220	1:100	Goat Anti- Rabbit IgG (H+L) HRP conjugate	BIO-RAD 172-1019	1:3000
Mouse polyclonal [1] Anti-CFTR 596	CFF	1:5000	Goat Anti-Mouse IgG (H+L) HRP conjugate	BIO-RAD 170-6516	1:3000
Rabbit polyclonal[2] Anti-IL-8RB	Aviva system biology ARP41956_P010	1:3000	Goat Anti- Rabbit IgG (H+L) HRP conjugate	BIO-RAD 172-1019	1:3000
Rabbit polyclonal[1] Anti-NEDD4L	Aviva system biology ARP43192_P010	1:3000	Goat Anti- Rabbit IgG (H+L) HRP conjugate	BIO-RAD 172-1019	1:3000
Rabbit polyclonal[2] Anti- SMAD2	Aviva system biology ARP32004_P010	1:3000	Goat Anti- Rabbit IgG (H+L) HRP conjugate	BIO-RAD 172-1019	1:3000
Rabbit polyclonal[2] Anti- SMAD7	Aviva system biology ARP32008_T100	1:3000	Goat Anti- Rabbit IgG (H+L) HRP conjugate	BIO-RAD 172-1019	1:3000
Rabbit polyclonal[2] Anti-PELLINO1	Aviva system biology ARP57429_P050	1:3000	Goat Anti- Rabbit IgG (H+L) HRP conjugate	BIO-RAD 172-1019	1:3000
Rabbit polyclonal[2] Anti-STAT1	Aviva system biology ARP33373_P050	1:3000	Goat Anti- Rabbit IgG (H+L) HRP conjugate	BIO-RAD 172-1019	1:3000
Mouse nonoclonal Anti-Alpha-tubulin[1]	Sigma T5168	1:10000	Goat Anti-Mouse IgG (H+L) HRP conjugate	BIO-RAD 170-6516	1:3000

[1] 7% gel running, 30µl protein loaded

[2] 12,5% gel running, 60µl protein loaded

Table VII. B3 - Primary and secondary Antibodies used in immunofluorescence

Primary antibodies	Supplier	dilution		Secondary antibody	Supplier	dilution	
		M/A	F			M/A	F
Mouse monoclonal IgG anti-E-cadherin	BD Transduction Laboratrories 6101181	1:200	1:100	Alexafluor 488 IgG anti-Mouse	Invitrogen A21202	1:1000	1:500
Mouse monoclonal IgG anti-Z0-1	Invitrogen 339100	1:200	1:100	Alexafluor 488 IgG anti-Mouse	Invitrogen A21202	1:1000	1:500
Rabbit polyclonal IgG anti-TβRII (C-16)	Santa cruz sc-220	1:50	1:50	Alexafluor 488 IgG anti-Rabbit	Invitrogen A21206	1:1000	1:500
Mouse monoclonal anti-CFTR 570	CFF	/	1:100	Alexafluor 488 IgG anti-Mouse	Invitrogen A21202	/	1:500

M/A: methanol-acetone method; F: Formaldehyde method

Solutions

Modified Lowry assay:

CTC – 0,10g $CuSO_4.5H_2O$; 0.20g $K_2C_4H_4O_6$; 10g Na_2CO_3

Solution A - 10% (w/v) SDS; 0.8M NaOH

Solution B - Folin-Ciocalteau reagent diluted 5-fold in water

Western blotting method:

Stacking Buffer – 0,5M Tris ph 6,8; 30,25g Tris in 500ml H_20 pH 6,8 with conc. HCL

Separating Buffer – 1,5M Tris pH 8,8; 90,85g Tris in 500ml H_20 pH 8,8 with conc. HCL

Running Buffer – 0,025M Tris; 0,192M glycine; 0,1% (w/v) SDS

Transfer Buffer – 20% (v/v) methanol; 3,0g/l Tris; 14,4g/l glycine

Immunocytochemistry:

PBS +/+ - 1x PBS pH 7; 0,9mM $CaCl_2$; 0,5mM $MgCl_2$

C. Results supplementary

Affymetrix ID	Gene Name	Description	RP/Rsum	FC	PFP	P Value
BM976134_at	NEDD4L	Neural precursor cell expressed; developmentally down-regulated 4-like	651.4	2.74	0.0086	0.0000
BU608977_at	TGFBR2	Transforming growth factor; beta receptor II (70/80kDa)	920.9	1.90	0.0188	0.0002
CB054710_at	SMURF2	SMAD specific E3 ubiquitin protein ligase 2	992.7	1.97	0.0227	0.0003
AA608955_at	PIAS1	Protein inhibitor of activated STAT; 1	992.8	2.09	0.0227	0.0003
AA548535_at	SMURF1	SMAD specific E3 ubiquitin protein ligase 1	1263.8	2.45	0.0409	0.0008
232528_at	UBE2E3	Ubiquitin-conjugating enzyme E2E 3 (UBC4/5 homolog; yeast)	1302.2	1.88	0.0437	0.0009

Figure VII. C1 -Gene expression in nasal epithelial cells of patients with CF (from Clarke et al, unpublished data)

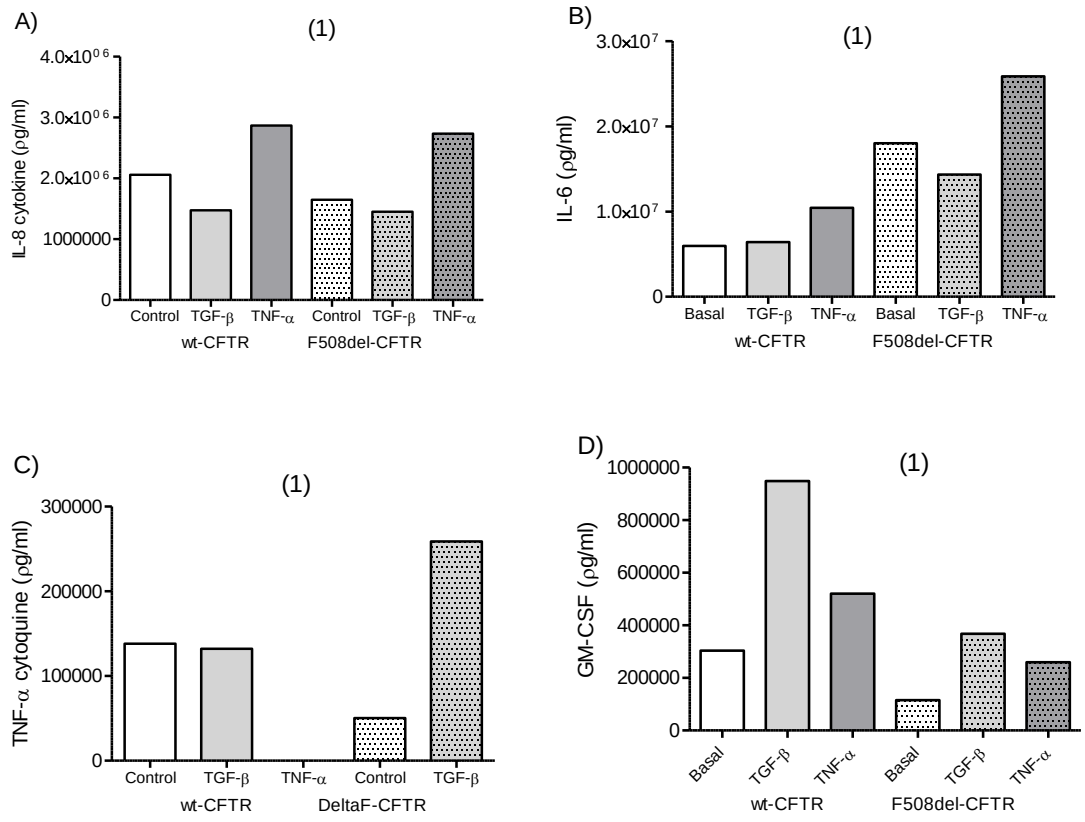


Figure VII. C2 - Cytokine concentrations in unpolarized CFBE cells. Levels of (A) IL8, (B) IL-6, (C) TNF-α and (D) GM-CSF in CFBE cells expressing wt-CFTR and F508del-CFTR treated with TGF-β (5ng/ml) or TNF-α (80ng/ml) for 24 h. Supernatant concentration are in pg/ml.