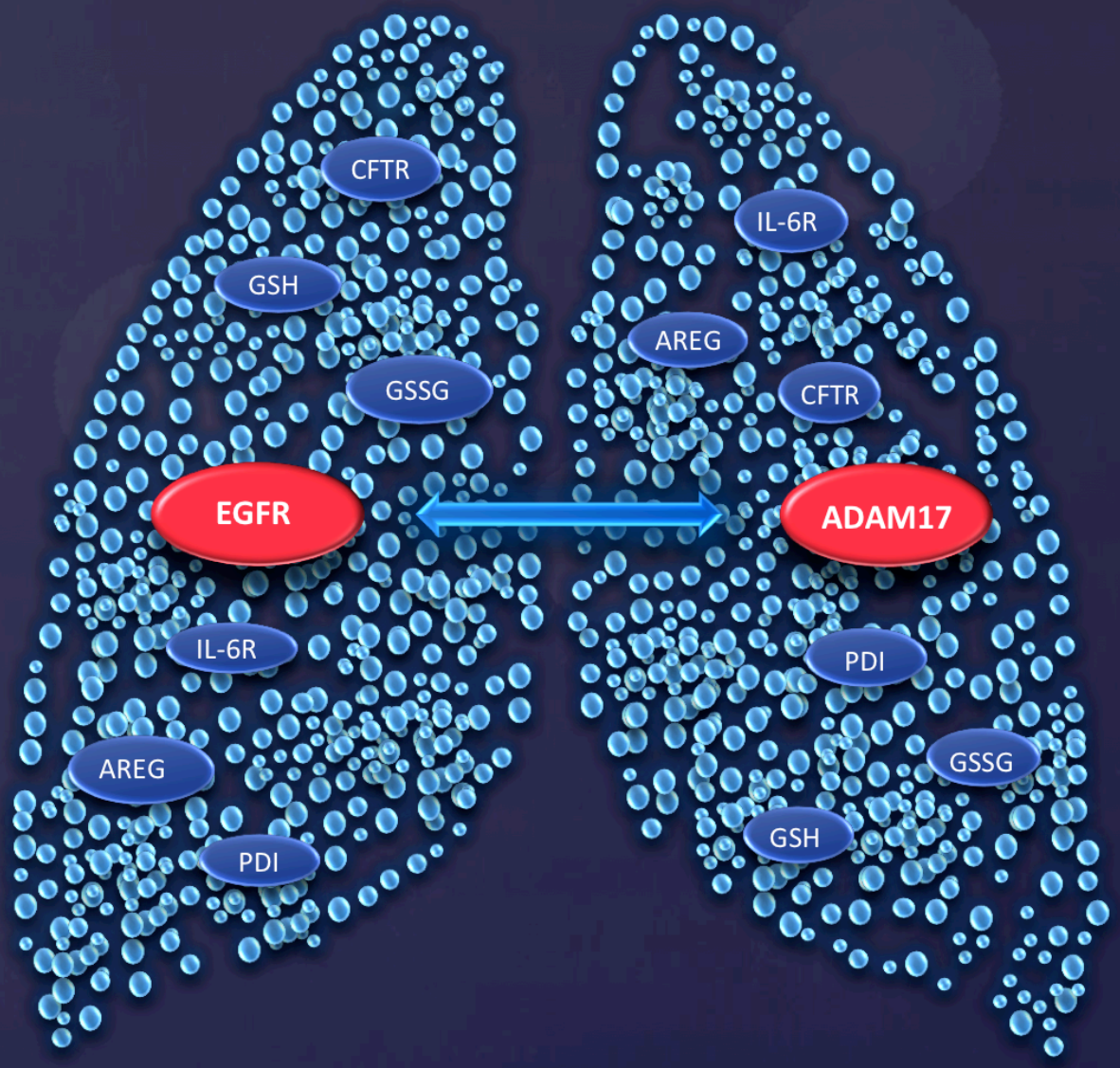
Studies on Airway Inflammation and Remodeling in Chronic Lung Disease

The EGFR-ADAM17 axis controls inflammatory responses in Cystic Fibrosis and Chronic Obstructive Pulmonary Disease



Marta Stolarczyk

Studies on Airway Inflammation and Remodeling in Chronic Lung Disease

The EGFR-ADAM17 axis controls inflammatory responses in Cystic Fibrosis and Chronic Obstructive Pulmonary Disease

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To obtain the degree of Doctor
from the Erasmus University Rotterdam
by command of the Rector Magnificus

Prof.dr.H.A.P.Pols

and in the accordance with the decision of the Doctorate Board

The public defense shall be held on
Tuesday 21 March 2017 at 13.30

By

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Born in Iława, Poland

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Co-Promotor:	**Dr. BJ Scholte**

Dla mamy Basi
i dla taty

“Once we accept our limits, we go beyond them”
Albert Einstein

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Abbreviations (in alphabetical order)

ADAM17	A disintegrin and metalloprotease 17; f.k.a TACE
ALI-CFBE41o-	CFBE41o- a CF Bronchial Epithelial Cell Line cultured at Air-Liquid Interface
ALI-HBEC	Primary Human Bronchial Epithelial Cells cultured at Air-Liquid Interface
AREG	Amphiregulin
ASL	Air Surface Liquid
ASM	Airway Smooth Muscle cells
ATP	Adenosine Triphosphate
BALF	Bronchoalveolar Lavage Fluid
cAMP	Cyclic Adenosine Monophosphate
CF	Cystic Fibrosis
CFTR	Cystic Fibrosis Transmembrane Conductance Regulator
COPD	Chronic Obstructive Pulmonary Disease
CS	Cigarette Smoke
Duox1	Dual oxidase 1, NADPH oxidase (NOX) family; transmembrane, produces extracellular peroxide (ROS)
Duox2	Dual oxidase 2, NADPH oxidase (NOX) family; transmembrane, produces extracellular peroxide (ROS)
ECM	Extracellular Matrix
EGFR	Epidermal Growth Factor Receptor
ERK1/2	Extracellular Signal-Regulated Kinase 1/2
F508del CFTR	Most prevalent pathogenic mutation in CF patients, deletion of a phenylalanine in the NBD1 domain of the CFTR gene causes abnormal folding and trafficking of CFTR protein
G551D CFTR	A missense mutation in an ATP binding pocket of CFTR, causing a channel gating defect
Gp130	Signal Transducing Glycoprotein 130, IL-6ST, CD130, cytokine receptor.
Grx-1-roGFP	Glutaredoxin-1 The reduction-oxidation sensitive Green Fluorescent Protein
Grx-1-roGFP-GPI	Glutaredoxin-1-The reduction-oxidation sensitive Green Fluorescent Protein fused to a Glycosylphosphatidylinositol anchor
GSH	Glutathione; reduced form
GSSG	Glutathione dimer; oxidized form of glutathione
IL1R	Interleukin-1 Receptor
IL-1 β	Interleukin-1 beta
IL-6	Interleukin-6
IL-6R	Interleukine-6 Receptor
IL-8	Interleukin-8
MAPK	Mitogen-activated protein kinases
MPD	Membrane proximal domain (of ADAM17)
MUC5AC	Mucin 5AC
MUC5B	Mucin 5B
NADPH	Nicotinamide Adenine Dinucleotide Phosphate (reduced)
P38 MAPK	p38 Mitogen-Activated Protein Kinases
PDI	Protein Disulfide Isomerase
ROS	Reactive Oxygen Species
S1P	Sphingosine-1-Phosphate
STAT3	Signal Transducer and Activator of Transcription 3

TACE	TNF- α -Converting Enzyme; former name of ADAM17
TGF- α	Transforming Growth Factor alpha
TIMP-3	Metalloproteinase Inhibitor 3, extracellular, regulates ADAM activity
TMI-1	4-[[4-(2-butynyloxy)phenyl]sulfonyl]-N-hydroxy-2,2-dimethyl-(3S)thiomorpholinecarboxamide; inhibitor of MMPs, ADAM10 and ADAM17
TMI-2	(2R, 3S)-2-([[4-(2-butynyloxy)phenyl]sulfonyl]amino)-N,3-dihydroxybutanamide; selective ADAM17 inhibitor
TNF- α	Tumor Necrosis Factor alpha
VX-770	Ivacaftor (trade name KALYDECO®), CFTR potentiator
VX-809	Lumacaftor, CFTR corrector

SCOPE OF THE THESIS

Cystic fibrosis (CF) and Chronic obstructive pulmonary disease (COPD) are progressive and eventually fatal lung diseases characterized by airway inflammation, bacterial infection, viscous mucus secretion and myofibroblast hyperplasia. An effective cure is not available. Though the etiology of the two diseases is obviously different, the molecular mechanisms involved are likely related, but not completely elucidated. By studying pathological processes in the CF and COPD airway model systems, we aim to establish novel therapeutic targets.

In this thesis we focus on the role of ADAM17 metalloprotease activity, which sheds a range of bioactive protein ligands, including most epithelial growth factor receptor (EGFR) ligands. We hypothesized that the ADAM17-EGFR axis is a link between CF and COPD lung pathology. When triggered by stress signals, ADAM17 releases a number of protein substrates from airway epithelial cells, including IL-6 receptor (IL-6R) and several growth factors such as amphiregulin (AREG) that activate gp130 and EGFR respectively on the epithelial cells (autocrine) and the underlying tissues (paracrine, trans-activation). Both the IL-6/IL-6R/gp130 and AREG/EGFR pathways converge in STAT3 activation, a transcription factor and genetic modulator of CF lung disease, and are involved in the lung tissue repair, inflammation and fibrosis. Thus the focal point of this thesis was to establish the role of ADAM17 and EGFR in pathology of CFTR-related lung diseases: CF and COPD.

Chapter 1 provides a general introduction to CF and COPD pathology and describes the importance of anti-inflammatory therapy in CF. In the second part, ADAM17 and EGFR are introduced and their role in lung inflammation and remodeling is underlined.

Chapter 2 shows the responses of ADAM17 and EGFR to whole cigarette smoke (CS) exposure in differentiated COPD and non-COPD primary human bronchial epithelial cells cultured at the air-liquid interface (ALI-HBEC). Further, it investigates the effect of CS exposure on localization and quantity of phospho-ADAM17/substrate interactions, and shows the involvement of the EGFR-ADAM17 axis in regulation of mRNA and protein levels of ADAM17 substrates, providing new insight into the spatiotemporal ADAM17-mediated cleavage.

Chapter 3 demonstrates that CFTR deficiency hyperactivates epithelial ADAM17 and EGFR as a cell-autonomous defect in a unique model of immortalized CFTR deficient CFBE41o- airway epithelial cells with inducible CFTR expression. Druggable targets upstream and downstream of these regulators were defined in order to understand the cascade of events leading to CFTR-mediated activation of ADAM17-EGFR axis and potentially apply them as an early intervention in CF lung therapy.

Chapter 4 provides evidence of chronic lung inflammation in a mouse model of the most common CF mutation (F508del CFTR). In unchallenged mutant mice we observe enhanced infiltration with activated granulocytes and an abnormal dendritic cell balance, consistent with basal pro-inflammatory signalling due to CFTR deficiency. Upon challenge with bacterial toxins, resolution of induced inflammation is delayed in mutant mice. This correlates with abnormal sphingosine metabolism, in particular reduced S1P in lung tissue, which is known to be associated with inflammatory processes through S1P recpetors, Oral administration of an S1P-lyase inhibitor (LX2931), corrected the S1P deficiency and reduced basal and induced inflammation in mutant mice.

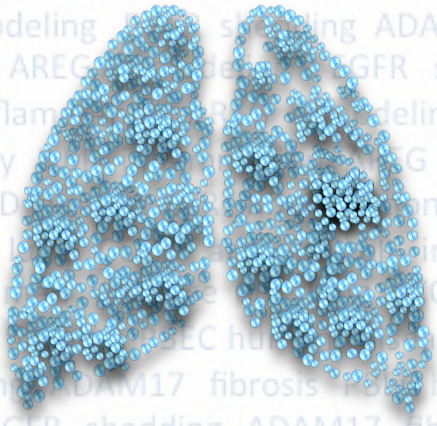
Chapter 5 Summarizes and critically discusses the studies presented in this thesis and provides possible future directions in investigation of EGFR-ADAM17 axis in the context of CF and COPD lung disease.

APPENDIX

Appendix presents further evidence that F508del CFTR mutant mice show excessive inflammation, increased IL-1β production and reduced bacterial clearance. CFTR deficient mice with the IL-1R1 knock-out (double mutant dd x IL-1R1) have attenuated inflammation, providing a rationale for targeting IL-1β/IL-1R1 pathway in CF patients. Importantly, in airway epithelial cell lines activation of IL-1R activates the ADAM17/EGFR/IL8 signaling (Kim et al, 2013), thus intervention in IL-1β/IL-R1β pathway may be also beneficial in attenuation of EGFR/ADAM17 signaling in the CF or COPD disease state, which can be further evaluated in differentiated human airway epithelial cells.

CHAPTER 1

General introduction



The EGFR-ADAM17 axis in pro-inflammatory airway responses and remodeling in CFTR related lung diseases: CF and COPD.

(to be submitted)

Stolarczyk M and Scholte BJ

INTRODUCTION

1.1 CYSTIC FIBROSIS, A CONGENITAL LUNG DISEASE WITH AN EARLY ONSET

Cystic Fibrosis (CF) is an autosomal recessive lung disease caused by more than 2000 different mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene [1] with 70-80.000 patients worldwide. The most common CF mutation is the deletion of a phenylalanine at amino acid position 508 in the NBD1 domain of CFTR protein (F508del) [2]. The CFTR gene encodes the Cystic Fibrosis Transmembrane conductance Regulator protein, a chloride channel mainly expressed in the apical membrane of secretory epithelial cells [3]. Later studies showed that CFTR is also involved in transport of other molecules, for instance bicarbonate [4] and glutathione [5] [6]. Bicarbonate is crucial to normal expression of mucins that in CF remains aggregated and poorly solubilized [7], whereas glutathione, as a natural antioxidant, reduces oxidative cellular stress [8].

CFTR has a key role in maintaining ion and water homeostasis in secretory epithelia [3], thus mutations in CFTR cause a multi-organ CF disease affecting lungs, intestine, pancreas, liver, sinuses, and reproductive organs [9]. However, under present treatment the main morbidity and mortality among CF patients is due to lung malfunction, thus most available and experimental treatments aim to prevent the progression of CF lung disease [10].

CF lung disease, an early disorder of distal airways

Advances in imaging and monitoring of the respiratory system in infant patients [11] [12] [13] have revealed that severe CF lung pathology starts early in childhood [14] [15] and progresses irreversibly over time [16] [17]. Characteristic lung functional and structural changes are observed already in pre-school patients [15] [17] [18] [19]. This early onset of lung abnormalities includes bronchiectasis, diagnosed thickening and dilation of the bronchial walls [14] [20] [21], air trapping and atelectasis (partial collapse of the lung) [14] [22]. These symptoms occur simultaneously with reduced mucociliary clearance and mucus plugging [20]. Quantitative and standardized tracking of early lung disease progression in infants with CT scans is pursued to advance the comparative analysis and provide the evaluation of the treatment [18] [23].

In a recent micro CT and histological study of end-stage CF lungs the dilatation and obstruction of distal airways and a severe reduction in the number of functional terminal bronchioles was clearly documented for the first time, confirming that obstruction and remodeling of peripheral airways is a prominent feature in CF lung disease, and therefore are prime targets of experimental therapy [24]. CF mouse models [25] (Chapter 4, Appendix) and large CF animal models, like pig [26] [27] and ferret [28] are helpful in the investigation of the mechanism involved in this early onset of CF lung disease and intervention therapy, but all have considerable limitations. In addition to state of the art clinical and biomarker studies, there is an urgent need for the development of organotypic cell culture models in which the complex molecular and cellular interactions involved in CF lung pathology can be studied (Chapter 2 and 3).

Mucus, Mucociliary transport and ciliary beat frequency

Healthy airways are protected by mucociliary transport that removes pathogens and toxic particles captured in mucus by ciliary beating. In healthy subjects this requires CFTR dependent balanced fluid and proper mucus secretion from surface cells and sub-epithelial glands. However, CF lung epithelial cells produce mucus (MUC5AC, MUC5B) with altered properties, characterized mainly by high viscosity [29] [30]. High mucus viscosity in CF is caused by reduced CFTR-dependent bicarbonate secretion, required for proper expansion of secreted mucus molecules [30] [31].

Furthermore, CF patients have intrinsically impaired ciliary beat frequency (CBF), which additionally reduces muco-ciliary transport of inhaled chemical particles and pathogens. CBF is not only dependent on CFTR-mediated bicarbonate transport, but also regulated by soluble adenyl cyclase (sAC) [32] [33].

Taken together, high mucus viscosity [29] and impaired CBF disable effective clearance of bacteria and inhaled particles [26] [34], which has been recently confirmed by less effective removal of bacteria introduced into the CF pig lung in comparison to healthy animals [35].

Air Surface Liquid

Airway epithelium is covered with a thin layer of air-surface liquid (ASL). Airway epithelial cell cultures from CF patients have reduced ASL height, presumably due to defective CFTR dependent fluid secretion [36] [37] [38]. Additionally, as a consequence of reduced bicarbonate secretion, CFTR deficiency abnormally acidifies ASL [32] [33]. Reduced pH of ASL impairs bacterial killing [39] [40], inhibits the activity of ASL antimicrobials [41], and increases ASL viscosity of newborn CF piglets, which can be improved after reducing ASL Ca^{2+} levels [26].

Taken together, the careful regulation of the balance between fluid and ion secretion and resorption at the airway surface, maintenance of the optimal liquid layer (ASL) height, pH and mucus viscosity, is the primary role of CFTR in the lungs. Impaired CFTR function leads to dehydration and acidification of ASL, which interferes with pathogen and mucus clearance [34] [36] [39] [42].

Bacterial infection and inflammation

Impaired bacterial killing and mucociliary clearance facilitate bacterial colonization in CF lungs. *Pseudomonas aeruginosa, Haemophilus influenza, Staphylococus aureus,* generally harmless in normal individuals, are pathogens frequently found in CF patient lungs [43]. Microbes that colonize airways frequently form biofilms, stimulating persistent inflammatory responses [44]. Despite activation of inflammatory responses mediated by the innate and cellular immune system, eradication of bacterial infection is impaired in CF lungs. It is plausible that persistent inflammation is caused by bacteria captured in sticky mucus, which produce a mucoid biofilm during adaptation to the CF lung environment.

Recurrent infections (mainly with *P. aeruginosa*) and concomitant chronic inflammation, recognized as main hallmarks of CF lung disease, worsen the lung pathology. In airways, bacterial infections induce a massive recruitment of neutrophils [45]. Both bacterial infections and neutrophil infiltration have been correlated with progression of lung disease [14]. This ultimately leads to irreversible airway remodeling, observed as air trapping, bronchiolar obstruction and bronchiectasis [24].

However, it still remains unknown whether elevated levels of inflammatory cytokines, growth factors and mucins always result from bacterial infection, or are an intrinsic property of CFTR deficient mucosa (Chapter 4, Appendix). Some reports argue that bacterial infections are indispensable to inflammatory responses, providing evidence that CF lungs do not have inflammation at birth in humans [46] or CF pig [35]. However, others report that CFTR malfunction leads to overexpression of pro-inflammatory molecules (such as IL-6, IL-8, IL-17, CXCL1, CXCL2) as a cell-autonomous defect occurring before *P.aeruginosa* infection [47] [48] [49] [50] [51] [52]. Thus, it is likely that mucosal inflammation is an autonomous defect of CF epithelial cells, which may occur before bacterial infection. Furthermore, it is

also unclear whether the elevated levels of inflammatory mediators in the bronchioalveolar lavage fluid (BALF) of young and adult CF patients [53] are caused by functional abnormalities in CF myeloid cells, in particular macrophages [54], dendritic cells (Appendix: CF mouse studies), and neutrophils [55] or are primarily related to abnormal cytokine signaling by CFTR deficient airway epithelial cells. This thesis aims to address the question whether inflammation is an inherited property of CF airway epithelial cells, which is covered by Chapter 2.

In summary, due to the early onset of the lung disease and its irreversible nature, it is clear that CF patients require early intervention therapy [17]. Excessive lung inflammation and tissue remodeling observed in CF may be an inherent property of CFTR deficient lung mucosa. Therefore, it is important to establish whether alleviation of inflammatory responses is beneficial in management of CF lung disease [56].

1.2 STRATEGIES OF CF THERAPY

The most prominent cause of death of CF patients is lung malfunction caused by frequent airway infections, persistent inflammatory responses, mucus plugging and airway remodeling (bronchiectasis, scarring) [24]. Indirect pharmacological management targets these different pathophysiological aspects of the disease and mainly focuses on anti-inflammatory agents [57] [58], antibiotics [59], and mucolytic agents [60] [61] [62]. Direct pharmacological management of CF disease, which is being developed at this time, intends to restore the functional expression of mutated CFTR at the plasma membrane by correcting its folding and gating defect [63] and thus reverse the abnormalities in chloride transport. Many attempts have been made to define correctors that aim to correct the folding defect and enable mutant CFTR to reach the apical membrane, and potentiators that increase the open-probability of the mutant CFTR channel at the plasma membrane, thereby increasing CFTR conductance [64] [65] [66] [67] [68]. On the basis that patients with residual CFTR activity have less severe disease [69] [70], it is claimed that for therapeutic strategies aimed at correcting mutant CFTR function or gene therapy around 10 % of normal CFTR activity is required to restore chloride transport [71].

So far, a potentiator Ivacaftor (VX-770, trade name KALYDECO®) was tested with excellent results in G551D CFTR patients [72] [73] and has been approved by the U.S Food and Drug Administration (FDA) for patients with gating mutations (G551D, G1244E, G1349D, G178R, G551S, S1251N, S1255P, S549N, or S549R), which together account for 4-5% of all CF alleles [3]. In clinical trial, the corrector VX-809 (lumacaftor) resulted in significant improvement of lung disease, albeit only in a subpopulation of homozygous F508del CFTR mutants [68]. In 2015 the FDA approved ORKAMBI® for homozygous F508del CFTR patients, the combination of CFTR corrector Lumacaftor (VX-809) and potentiator Ivacaftor (VX-770) in one pill. This therapy improves lung function in patients homozygous for the F508del mutation, although modestly and not in all patients [10] [74], so further investigations with different combinations of new correctors and potentiators are in progress to find more effective therapy [75] [76]. Long-term effects on lung function after treatment with correctors and potentiators are still not well investigated. Current developments use stem cell based intestinal organoids as a new test platform for high throughput screening and personalized evaluation of available compounds and combination therapies [77] [78] [79]. Gene therapies (viral and non-viral gene transfer, gene editing, mRNA repair) as well as strategies targeting protein repair other than potentiators and correctors (like premature termination codon (PTC) read-through drugs or proteostasis regulators) are also under investigation, but so far with limited success in clinical trials [65] [66] [67] [80] [81]. Editing the CFTR gene using the CRISPR-Cas9 system in stem cells is a potential next step to improve CF therapy

[82]. However, this approach has to overcome similar issues of efficiency, delivery and safety as conventional gene therapy.

Since the 1990s excessive airway inflammation, which positively correlates with the progression of CF lung malfunction, is recognized as the major factor in the pathogenesis of CF lung disease [83]. However, so far the effects of correctors and potentiators on infections and the release of inflammatory mediators have not been broadly investigated in clinical studies. *In vitro* studies by Pohl et al. show that Ivacaftor improves extracellular *P.aerugnosa* killing by neutrophils isolated from F508/G551D or F508del homozygous patients and corrects neutrophilic degranulation and Rab27a activation [84]. Rowe et al. showed that Ivacaftor also reduces *P. aeruginosa* isolated from CF patients after 6 months treatment. However, the free neutrophil elastase and other inflammation markers like IL-1β, IL-6 and IL-8 in sputum samples remained unchanged [73]. Therefore anti-inflammatory, anti-bacterial and other additional therapies are still important targets of investigation [85].

In summary, recent breakthroughs in the development of small-molecule compounds targeting the mutant CFTR protein have raised hope to find a cure for CF. However, the presently available CFTR correctors and potentiators are not sufficiently effective in a majority of CF patients, and the search for new compounds and additional therapies is still highly relevant. Targeting the CFTR gene by gene editing in stem cells is a next development. At this point, anti-inflammatory and anti-bacterial therapies remain important targets of investigation.

1.3 COPD, ACQUIRED CF?

Chronic obstructive pulmonary disease (COPD), the 5th ranking cause of death worldwide, is a complex inflammatory lung condition that interferes with normal breathing due to obstruction of the lung airflow, excessive sputum production and a chronic cough. Usually COPD is characterized by chronic bronchitis (inflammation of the bronchi and bronchioles) and emphysema [86]. Similar to CF, bronchiectasis and peripheral airway thickening are also observed in the lungs of COPD patient [87]. Phenotypic expression of COPD is highly variable. Some individuals develop lung disease dominated by emphysema, while others exhibit chronic bronchitis. This heterogeneous phenotype likely reflects the contribution of multiple pathogenic mechanisms. Once COPD starts to develop, it tends to worsen over time, and so far its progress cannot be controlled effectively in most patients.

The most prominent etiological factor leading to COPD is cigarette smoke, but also exposure to fumes, chemicals and dust [88]. Although COPD and CF differ in primary cause, the spectra of the pathological events overlap considerably (Figure 1). Both diseases are characterized by excessive mucus production and insufficient clearance, leading to lower airways obstruction with chronic neutrophilic infiltration. In CF and COPD airway surface liquid (ASL) dehydratation and viscous mucus secretion impair mucociliary and bacterial clearance, causing chronic inflammation and facilitating recurrent infections [89]. Furthermore, during chronic CF lung disease, *P.aeruginosa* clones adapt, resulting in a highly diverse bacterial community that is extremely difficult to eradicate therapeutically. This is also observed in severe COPD, but not in mild disease [90] [91]. Recent publications show the involvement of a wide variety of inflammatory mediators in COPD (like IL8, IL-6, CCL18), that overlap with CF related mediators [85] [92]. There are also parallels on the cellular level that include goblet cell metaplasia, hyperplasia of myoblasts, extensive extracellular matrix production [93].

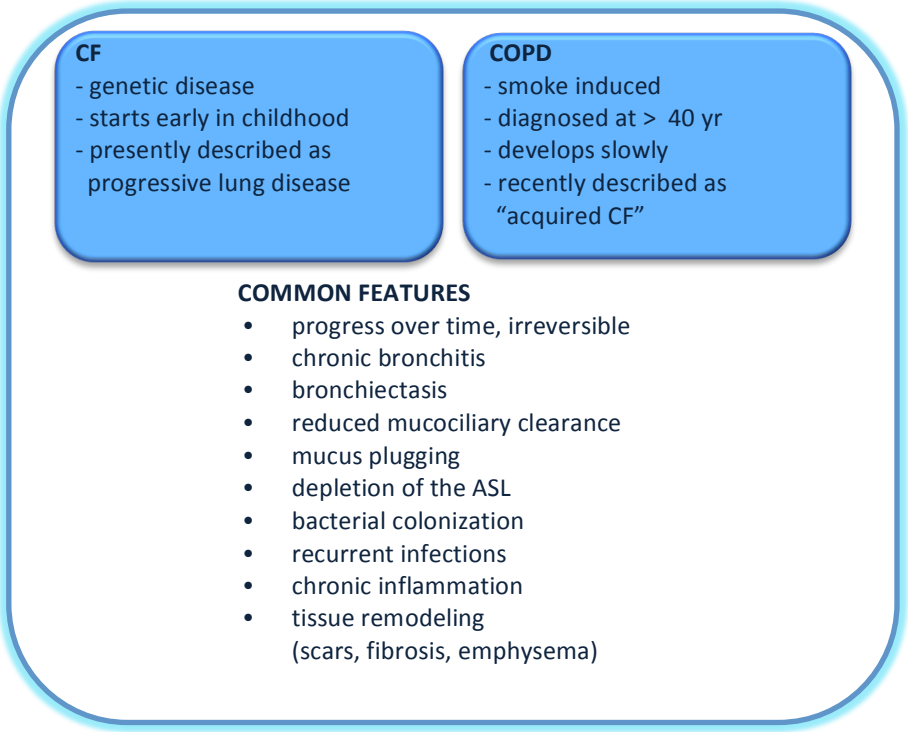


Figure 1. Common features of CF and COPD. CF and COPD despite the distinct nature of the disease development share many common clinical manifestations. Mutations in CFTR gene contribute to the CF outcome. Recently CS has been shown to affect CFTR channel activity and since is recognized as a factor that may contribute to a CF-like phenotype in COPD. Thus COPD has been recently described as acquired CF.

The broad spectrum of common features and events observed in CF and COPD encouraged researchers to seek for the common factors for both diseases. Studies showed that cigarette smoke decreases CFTR mRNA expression, and reduced CFTR protein level through accelerated degradation and altered channel activity [94] [95] [96] [97]. CFTR dysfunction and a clinical phenotype similar to mild CF developed by COPD patients has made researchers to describe COPD as an "acquired CF" [89] [93] [98] [99]. Nasal potential differences (NPD) in patients with no detectable CFTR gene mutation reflected a pattern typical of CFTR deficiency [100]. As a result of CS exposure, CFTR rapidly internalizes leading to its decreased activity, diminished CFTR-mediated anion transport, impaired mucociliary clearance and depleted ASL *in vitro* and *in vivo* [94] [95]. However, the effect presented in these papers is transient, so whether CS-induced inhibition of CFTR contributes to the chronic COPD in ex-smokers remains to be investigated.

1.4 STRATEGIES OF THERAPIES IN COPD

The CF-like symptoms of COPD, impaired CFTR function observed in COPD patients, and the direct evidence that CS exposure inhibits CFTR activity have led to a trial in which smokers were treated with a CFTR potentiator. Sloane et al. showed that Ivacaftor (VX-770) activated CFTR-dependent chloride transport in non-CF patients, increased ASL height and mucociliary transport [95]. These findings contrasted with the results of Cholon et al showing that chronic treatment with VX-770 reduced non-CF CFTR function and the amount of mature CFTR [101]. These results thus discredit the possibility to use VX-770 as an additional therapy to enhance CFTR function in COPD.

Inflammation is recognized as the major pathophysiological mechanism of COPD progression, with molecular targets overlapping those of CF (IL-6, IL8, CCL18) [85]. It is also chronic and persists even after cessation of smoking, suggesting that apart from the role of CFTR also epigenetic changes may play a role [102] [103]. Thus airway inflammation remains an important therapeutic target in COPD management. Presently, several compounds targeting inflammatory responses in COPD are under investigation [104] [105] [106] [107], though none of these have been shown to be beneficial in COPD patients as yet.

COPD is a complex multifactorial disease, with large variation in the patient population due to undefined genetic and environmental factors. Therefore, a personalised approach using multiple treatments will likely be required, but a robust trial strategy is elusive. This also applies to CF anti-inflammatory treatment, though this disease is considered monogenetic, the downstream responses to CFTR deficiency, and responses to therapeutic intervention are highly variable in the population. The perfect corrector and potentiator combination tailored to the individual patient, supported by additional anti-inflammatory medication, would likely be the best solution.

1.5 EPITHELIAL EGFR/ADAM17 AXIS, A POTENTIAL THERAPEUTIC TARGET IN CF AND COPD LUNG DISEASE

For decades the design of anti-inflammatory agents aimed mainly to decrease the neutrophil influx into the lung and concomitant inflammatory responses mediated by eosinophils, macrophages, basophils, mast cells, NK, dendritic cells, B cells and T cells [108]. Recently the importance of airway epithelial cells in inflammatory responses has been also recognised [109]. Airway epithelium serves as a first barrier and acts as defence against daily inhaled air pollutants and microbes by mucociliary clearance and secretion of a range of cytokines, cytokines receptors, growth factors, growth factor receptors and antimicrobial peptides [110] [109]. Airway epithelial cells not only release inflammatory mediators to ASL, but they also signal to the underlying tissues (myocytes or fibroblasts).

A disintegrin and metalloproteinase 17 (ADAM17) is involved in the immune defence mechanisms mediated by epithelial cells. ADAM17, also known as a Tumor Necrosis Factor-α Converting Enzyme (TACE), is an enzyme with proteolytic activity that releases extracellular domains of transmembrane proteins to produce soluble bioactive signaling proteins.

Mature ADAM17 consists of metalloprotease domain (catalytic), a disintegrin domain, membrane proximal domain (MPD) rich in cysteine residues comprising 5 disulfide bonds (this cysteine-rich segment is shorter in comparison to other ADAMs), “Conserved ADAM-seventeeN Dynamic Interaction Sequence” (CANDIS), unlike other ADAM family members it lacks EGF-like domain [111] [112] [113] [114] [115]. These extracellular domains are followed by transmembrane region and cytoplasmic tail with phosphorylation sites that potentially are involved in ADAM17 activation (Figure 2).

Ectodomain shedding mediated by the metalloprotease domain of ADAM17 provides a mechanism for both membrane protein downregulation and subsequent initiation or inhibition of autocrine/paracrine signaling. So far 76 proteins have been identified as substrates of ADAM17 [116]. They encompass membrane bound cytokines (TNF-α), cytokine receptors (IL-6R, TNF-R), growth factors (AREG), ligands of EGFR (TGF-α, AREG, EREG, HB-EGF, Epigen), adhesion proteins (L-selectin, ICAM-1) and transmembrane mucins (MUC-1). The shed soluble forms of these proteins are bioactive transducers of cell signaling via activation of cellular receptors on underlying cells (transactivation/paracrine activation), but they also are involved in activation of the shedding cells and neighbouring cells

(autocrine activation) (Figure 3).

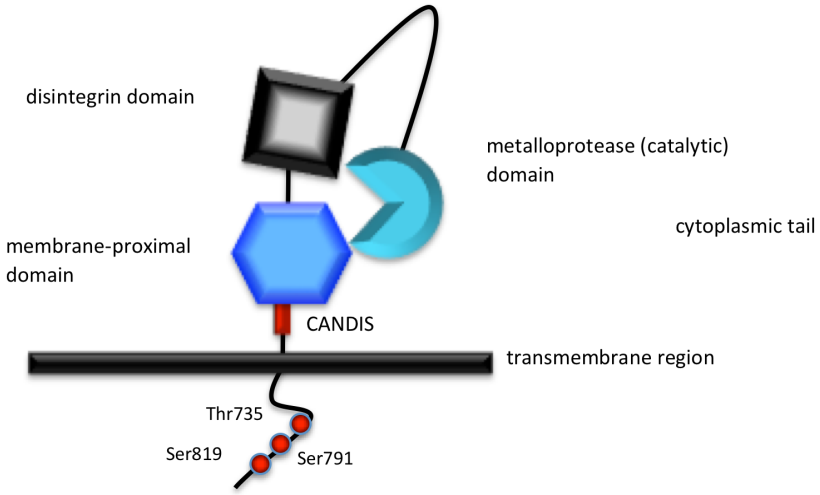


Figure 2. ADAM17 domain structure.
ADAM17 (and its close relative ADAM10) is an atypical member of the ADAM family. It has additional disulfide bonds in the metalloprotease domain, it lacks two calcium binding sites in disintegrin domain. The Membrane Proximal Domain (MPD), replacing the cysteine-rich and EGF-like domains, with a novel alpha/beta fold has a shorter cysteine-rich segment [111] [113] [114]. The MPD has cysteine residues determining the ADAM17 conformation (open/closed) and ADAM17 protease activity (active/inactive switch). The MPD is in close proximity to the active site likely due to a C-shaped conformation of the extracellular part of mature ADAM17 [111] [112]. ADAM17 lacks an EGF-like domain, so the MPD is followed by the juxtamembrane region "Conserved ADAM17 Dynamic Interaction Sequence" (CANDIS) involved in some substrate recognition (IL-6R, but not TNFα) [117], transmembrane region and a cytoplasmic tail [111] [112] [113] [114] [115] with phosphorylation sites, which are likely important for ADAM17 activity.

Because most of the epidermal growth factor receptor (EGFR) ligands are cleaved by ADAM17, this sheddase has emerged as an important transducer of the airway epithelial autocrine and paracrine signaling (Figure 3). EGFR and ADAM17 are both involved in the broad spectrum of events that is characteristic of both CF and COPD lung disease, like excessive mucus expression [118] [119] [120], cytokine secretion [121], airway epithelial cell wound healing [122] [123], abnormal airway proliferation [124], maintenance of barrier integrity and progressive lung tissue scarring [125]. They both are activated upon bacterial or viral infection and during inflammation [126] [127] [128]. While this is an effective and necessary response, it is suggested that exaggerated airway epithelial signaling in the chronic state may enhance inflammation and may lead to damage of the lung structure.

EGFR functions as a sensor of airway epithelial integrity [129]. When cells have intact tight junctions EGFR is not activated. But disruption of the epithelial cell integrity, either by mechanical injury or cytokine treatment (TNF-α/IFN-γ), leads to EGFR phosphorylation and concomitant inhibition of protein phosphatase 2A activity [130]. Cigarette smoke exposure of differentiated HBEC also leads to damage of the lung tissue observed as destruction of epithelial cell integrity, loss of E-cadherin/β-catenin complex and disappearance of cilia [42]. This coincides with phosphorylation and peri-nuclear trafficking of EGFR [42] suggesting the importance of EGFR in maintenance of epithelial cell barrier integrity. The response of ADAM17 to loss of pulmonary epithelial cell integrity has been also shown by neuregulin-1 (NRG-1) shedding and concomitant activation of human epidermal growth factor receptor-2

(HER2) [131]. This raises the question whether and how EGFR and ADAM17 co-operate in sensing responses to airway injury.

Taken together, EGFR and ADAM17, expressed by airway epithelial cells, have emerged as potential therapeutic targets in COPD and CF lung disease [125] [132]. Therefore, we aimed to understand the mechanism that provides a link between the loss of functional CFTR expression and the EGFR/ADAM17 axis. In order to do this, we studied the involvement of EGFR and ADAM17 in models of COPD and CF disease together, since it seems likely that they share molecular mechanisms and therapeutic options.

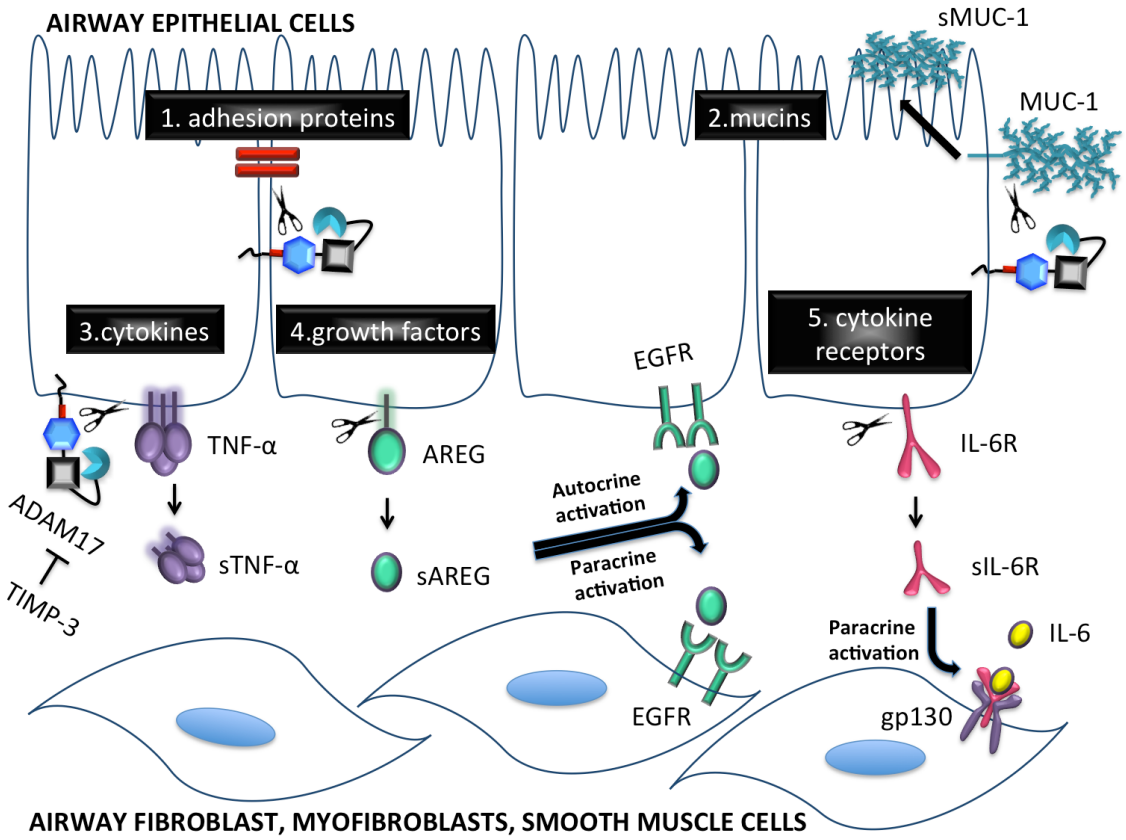


Figure 3. ADAM17 dependent paracrine and autocrine signaling.
A Disintegrin and Metalloproteinase 17 (ADAM17), also known as a Tumor Necrosis Factor-α converting enzyme (TACE), is involved in the immune defense mechanisms mediated by the epithelial cells. ADAM17 releases extracellular domains of transmembrane proteins to produce soluble bioactive signaling proteins taking part in autocrine (activation of receptors within the same epithelial cell layer) and paracrine signaling (activation of cellular receptors on underlying neighbouring cells, also termed trans-activation). Among ADAM17 substrates there are (1) adhesion proteins (L-selectin, ICAM) (2) transmembrane mucins (MUC-1), (3) membrane bound cytokines (TNF-α), (4) growth factors (AREG) and other ligands of EGFR (TGF-α, EREG, HB-EGF, Epigen), (5) cytokine receptors (IL-6R, TNF-R); in this picture the examples of these proteins are presented. Ectodomain shedding provides the mechanism for membrane protein downregulation and subsequent initiation or inhibition of autocrine/paracrine signaling. For instance, shed IL-6R from epithelial cells transactivate gp130 on the underlying myofibroblasts, whereas shed AREG from epithelial cells autocrinally activate EGFR on the neighboring epithelial cells or paracrinally on the underlying fibroblasts. ADAM17 natural inhibitor TIMP-3 inhibits ADAM17 proteolytic activity.

EGFR and ADAM17 are important in developmental processes

ADAM17 knockout mice survive between 17.5 embryonic days up to a few days after birth, underlining the need of the ectodomain shedding in development. The few survivors have defects in epithelium and lung and also in vascular system, eye, hair, heart, and skin [133]. Tissue specific deletions of ADAM17 or hypomorphic ADAM17 knock-in demonstrate an *in vivo* role of ADAM17 in controlling inflammation and regeneration [134] [135]. Similar to ADAM17-KO mice, EGFR-KO mice survive for up to 8 days after birth and suffer from impaired epithelial development in several organs, including lung, indicating the need of a functional ADAM17/EGFR axis for proper functioning and development of lung epithelium [136].

ADAM17 and EGFR crosstalk

ADAM17 works in association with a tyrosine kinase receptor EGFR by shedding most of its ligands (e.g. AREG, HB-EGF, TNF-α, EPGN (Epigen), Epiregulin) [116]. Only two EGFR ligands, EGF and betacellulin are shed by ADAM10 [137] [138], a close relative of ADAM17 [116]. Crosstalk of ADAM17 and EGFR in inflammatory signaling transduction is also defined by the establishment of a positive ADAM17/EGFR feedback loop likely involving activation of ADAM17 via the EGFR/MAPK pathway [126] [139] (Figure 4). The exact molecular mechanism of this feedback signaling has not been firmly established.

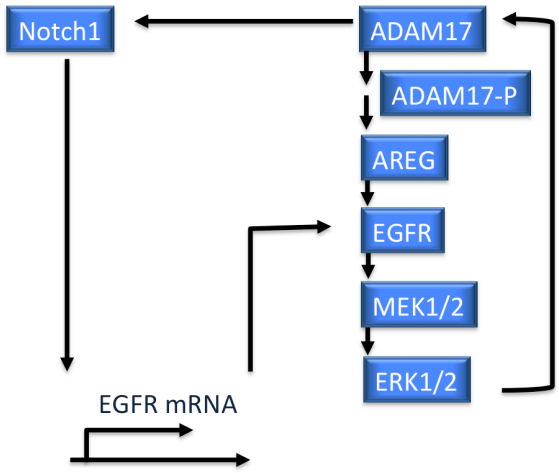


Figure 4. A positive ADAM17/EGFR feedback loop. ADAM17/EGFR positive feedback loop likely involves activation of ADAM17 via the EGFR/MAPK pathway and direct interaction of ERK1/2 with ADAM17. Additionally, by cleavage of Notch1 from a non-small lung carcinoma cell line ADAM17 regulates transcription of EGFR mRNA and increases EGFR expression on the cell surface, providing other mechanism contributing to positive feedback regulation of the ADAM17/EGFR axis.

ADAM17 regulates transcription of EGFR mRNA by cleavage of Notch1 and increases EGFR expression on the cell surface, as shown in a non-small lung carcinoma cell line [140], providing another positive feedback mechanism of the ADAM17/EGFR axis. In our studies we aimed to understand the mechanism of cigarette smoke induced activation of the EGFR/ADAM17 cascade (Chapter 2), and the role of CFTR in this process (Chapter 3).

The role of ADAM17 and EGFR in balanced regulation of lung inflammation and regeneration

ADAM17, expressed in human bronchial epithelial cells from the large and small bronchi, in lung smooth muscle cells, lung muscular vessels, alveolar macrophages, perivascular leukocytes and lung endothelial cells, is an important regulator of lung tissue homeostasis [132] [141] [142] and development [133], pro- and anti-inflammatory responses [116] and is involved in tissue regeneration [139] [143] [144].

The anti- and pro-inflammatory properties of the EGFR/ADAM17 signaling pathway are very much context dependent [116]. For instance, *in vitro* in airway epithelial cells ADAM17 together with EGFR induces mRNA expression and protein release of IL8, a neutrophil chemotactic factor that promotes inflammation [121] [145]. Whereas, by shedding TNF receptor type 2 (TNFR2) [146], which antagonises TNF-α, ADAM17 exhibits also anti-inflammatory properties [147]. Thus it is likely that the type of stimulus and substrate selection determine the nature of the EGFR and ADAM17 activity [148] [149] [150].

The involvement of EGFR/ADAM17 signaling in tissue regeneration encompasses wound healing [139], proliferation [143] [144], differentiation [151] [152] and cell migration [153]. However, when pathogenic stimuli or cell stress induces ADAM17 and EGFR activity, this contributes to cancer development [116] [154] [155] [156] and cardiac tissue remodeling after an infarct [157].

Due to the involvement of ADAM17/EGFR paracrine and autocrine signaling in several lung disorders [125] [132], modulation of ADAM17 and EGFR activation in COPD and CF is important, to keep the balance between anti-inflammatory processes and promotion of inflammation, and also between regeneration and excessive tissue remodeling. However, the mechanism of this modulation is still not fully understood.

Does CFTR deficiency affect the ADAM17/EGFR axis?

EGFR activation has been observed in NCI-H292 cells after inhibition of CFTR (inh-172) [49]. This CFTR-dependent induction of EGFR activity was likely ADAM17 dependent [49]. In CFTR deficient IB3 cells EGFR phosphorylation was markedly diminished by inhibition of ADAM17 (siRNA knockdown and broad spectrum MMPs inhibitor TAPI-1), plausibly due to reduced shedding of EGFR substrates. Interestingly, the inhibitory effect was much lower in C38 cells with corrected CFTR expression [49], suggesting that aberrant ADAM17 activity may contribute to induced activation of EGFR in CFTR deficiency. However, the relationship between ADAM17/EGFR signaling and CFTR deficiency is still poorly understood [158] and may involve a variety of actions. As a consequence of ADAM17-mediated release of EGFR ligands, the activation of downstream signaling occurs (i.e. MEK/ERK, PI3K/AKT, STAT3 or mTOR), which suggest that EGFR/ADAM17 pathway may be also abnormal in CF and COPD lung diseases [125] [132].

In our laboratory it was observed that F508del CFTR mutant mice compared to normal mice develop spontaneous and progressive chronic lung inflammation [25] (Chapter 4, Appendix). Additionally, naphthalene provoked injury induced abnormal responses in mutant mice compared to normal [159] with stronger expression of AREG, IL-6, elastin and collagen mRNA levels seven days after injury. Exaggerated responses mediated by the EGFR/ADAM17/ERK1/2/MAPK pathway have also been reported in CFTR deficient cells. Martel et al. showed that CFTR deficient cell line (CuFi-1) in response to heat-inactivated *P. aeruginosa* produce more IL-8 than non-CFTR deficient cell line (NuLi-1) and this involves

EGFR phosphorylation and ERK1/2 activation [160]. However, CuFi and NuLi are independent subclones and genetically different, and therefore not comparable models. Further studies by Kim et al. showed that inhibition of CFTR with CFTR-inh172 induces IL-8 production in ADAM17 and EGFR dependent manner and involves binding of IL-1α to IL-1R [49]. These observations led to the hypothesis that ADAM17 dependent IL-6/IL-6R/gp130 and AREG/EGFR pathways contribute to abnormal resolution of injury and inflammation in CF lung disease.

These data together suggest altered responses of the ADAM17-EGFR signaling pathway in CFTR deficiency. However, most of the studies cited here use undifferentiated, submerged immortalized cell lines [161], compare different cell lines (like CuFi and NuLi or IB3 and C38 cells) [49] [160] [162] [163] or use a CFTR inhibitor [49] with reported off-target effects [164]. Therefore, we addressed the mechanism by which CFTR deficiency activates the EGFR/ADAM17 axis in differentiated CFBE cells with inducible CFTR expression (Chapter 3), thereby overcoming both the high donor variation observed in primary airway cell culture, and the genetic and epigenetic variability of distinct immortalized cell lines.

Release of cytokines, growth factors and mucins depends on ADAM17/EGFR signaling

External stress factors like oxidative stress, viral and bacterial toxins, and CS exposure activate the EGFR/ADAM17 signaling pathway. Several studies showed that pathogens inhaled into the airways, or exposure of airway epithelial cells to other external stimuli activate toll-like receptors (TLR) [165] [166] [167] and G-coupled receptors (GPCR) [168] that cross talk with downstream EGFR/ADAM17 signaling. As a result of cigarette smoke extract exposure, secretion of downstream proinflammatory cytokines (including IL8) [121] [169] [145], growth factors (TGFα, AREG, HB-EGF) [121], mucins (MUC5AC) and phosphorylated MUC1 is induced in an ADAM17/EGFR dependent manner [119] [170] [171] [42]. Rhinovirus infection of airway epithelial cells showed that this cascade of events involves ERK1/2, MAPK and p38 MAP kinase [127] [172]. Particular matter exposure also induces IL-8 secretion in an EGFR/ERK1/2/MAPK dependent manner, but does not involve p38 MAP kinase [173]. Upon activation of the ERK or p38 MAPK pathway, ADAM17 dissociates from an endogenous extracellular tissue inhibitor of metalloproteinase-3 (TIMP-3) [174] [175] [176] [177], accumulates on the cell surface [157] [178] and induces release of TGF-α [157] [178]. Acrolein, an active component of cigarette smoke, which induces MUC5AC mRNA in an ADAM17 and EGFR dependent manner, also decreases TIMP-3 transcript levels, suggesting a role of TIMP-3 in ADAM17 activation. However in TIMP3-KO cells PMA-stimulated shedding of ADAM17 is not affected, which contradicts the model in which pre-bound TIMP3 needs to be removed for ADAM17 activation [138] and supporting that ADAM17 can be rapidly activated by conformational changes [138] [179] [180]. Thus, the role of TIMP-3 in HBEC signaling is still unclear.

Is Redox potential a link between CFTR deficiency and ADAM17/EGFR signaling?

The intra and extracellular redox potential changes in response to physiological processes and in pathophysiological conditions [181]. Reactive oxygen species (ROS), produced during cellular stress [85], is an important regulator of redox state and is also involved in activation of the EGFR/ADAM17 signaling pathway, affecting TGF-α and AREG release and mucin expression [119] [120] [165] [173]. Some studies point towards the role of NADPH oxidases (NOX), in particular dual oxidase 1 (DUOX1) [120] [165] [182] or dual oxidase 2 (DUOX2) [166], which produce ROS at an extracellular or possibly intra-vesicular domain. ATP-mediated DUOX1 activation has been shown to involve a TGF-α/ADAM17/ERK1/2/EGFR signaling pathway [182]. Other studies point towards intracellular NOX/ROS dependent SRC activity in EGFR/ADAM17 activation [139]. Recent studies indicated the involvement of

DUOX1 in allergen dependent SRC/EGFR activation in airway cells [183]. However, the mechanism by which ROS affects the EGFR/ADAM17 signaling pathway in intact airway cells is likely highly complex and still remains not well understood.

ROS not only affects signaling molecules in the EGFR/ADAM17 pathway, but it has been shown that ADAM17 [180] and EGFR [184] are redox sensitive proteins. EGFR has intracellular cysteine residues in the active site that are direct targets of ROS and determine EGFR kinase activity, likely through association of EGFR with NADPH oxidase, NOX2 [184]. ADAM17 activity, however, is regulated by thiol-disulfide isomerisation in the extracellular MPD domain mediated by protein disulfide isomerase (PDI), an oxidoreductase sensitive to redox changes [179]. PDI, by direct interaction with the membrane proximal domain (MPD) [180], changes the disulfide bridge pattern and thus the conformation of the extracellular protease domain from open, active to closed, inactive state, leading to the inhibition of ADAM17 proteolytic activity (Figure 5) [111] [112] [113] [114] [115] [117] [179] [180]. Redox dependent conformational changes likely make ADAM17 sensitive to the extracellular redox potential [180]. These findings are in line with previous observations showing that ADAM17 activity is redox sensitive [185] and blocking PDI induces ADAM17-mediated L- selectin shedding [186].

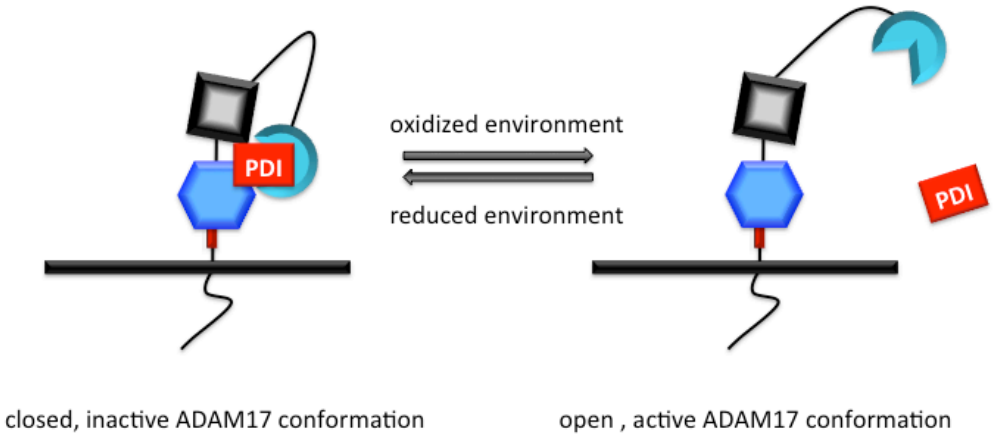


Figure 5. ADAM17 is a redox sensitive protein.
The ADAM17 membrane proximal domain (MPD), which is in close proximity to the active site, is sensitive to extracellular redox changes. This redox-sensitive ADAM17 activity is regulated by thiol-disulfide isomerisation mediated by protein disulphide isomerase (PDI), an oxidoreductase sensitive to redox changes. PDI by direct interaction with membrane proximal domain (MPD) changes the disulfide bridge pattern and thus the conformation of the extracellular protease domain from open, active to closed, inactive state, leading to the inhibition of ADAM17 proteolytic activity. Redox sensitive conformational changes likely make ADAM17 sensitive to the extracellular redox potential.

Since CFTR deficiency is thought to increase ROS levels [187], which in turn may activate ADAM17 and EGFR [139] and inactivate protein phosphatases [188], we propose that it is a link between EGFR/ADAM17 signaling and CFTR deficiency. CFTR deficiency may also affect the redox potential of ASL, due to CFTR mediated trans-epithelial glutathione transport at the apical membrane, which serves as a natural antioxidant [8]. Thus, by using the ratiometric fluorescence measurements [181], we questioned whether extracellular or intracellular redox potential is affected by CFTR and takes part in CFTR-mediated activation of EGFR/ADAM17 pathway (Chapter 2).

All in all, these studies show the involvement of different regulatory molecules leading to activation of EGFR/ADAM17 signaling, however, the exact cascade of events is still not well defined. In order to understand the regulation of EGFR/ADAM17 signaling, we aimed to define the molecules regulating ADAM17 shedding activity in CF and COPD. SRC [139], ROS [139], P38MAP [143] and ERK1/2 [189] appear to be the most important modulators of the ADAM17/EGFR pathway. Therefore, this thesis focuses on the role of these molecules in EGFR/ADAM17 signaling cascade in advanced COPD and CF cellular models (Chapter 2 and Chapter 3).

1.6 ROLE OF AREG IN EGFR/ADAM17 DEPENDENT MUCUS AND CYTOKINE SECRETION, AND REMODELING

Amphiregulin (AREG) is an ADAM17 substrate and EGFR agonist, expressed ubiquitously in the human lung [190] by human airway epithelial cells [121], smooth muscle cells [191] and fibroblasts [192]. AREG is also expressed by infiltrating and resident lung myeloid cells, including activated macrophages [193], eosinophils [194], dendritic cells [195], neutrophils [196] and mast cells [197]. Human AREG is synthetized as an N-glycosylated trans-membrane precursor (50 kDa) [198] with a basolateral sorting motif [199] and shed mainly by ADAM17 [126]. Proteolytic cleavage of the AREG extracellular domain releases several AREG soluble forms, predominantly an α-N-glycosylated 43 kDa form [198], which is one of the EGFR ligands, with lower affinity to EGFR than EGF and TGF-α .

AREG is induced in lung disease

AREG is involved in inflammation and repair responses through autocrine and paracrine activation of EGFR, and generally induced in lung disease [200]. In CF sputum samples, elevated levels of AREG have been shown in airway blood neutrophils [196]. Until now, the airway epithelial secretion of AREG has not been investigated in CF and COPD condition in comparison to controls (Chapters 2 and 3). However, in lung biopsies from asthma patients more AREG is expressed than in healthy controls [190], suggesting that this is also a case in CF and COPD lung disease. Other studies showed that in sputum of asthma patients AREG is upregulated only during an acute attack, which contrasts with the presence of EGF up to 7 days after an acute asthma attack [201], suggesting its role in a quick cellular responses to the triggers. Increase of AREG in sputum samples from children with asthma negatively correlates with lung function [202] and positively correlates with the number of eosinophils [203].

Regulation of AREG transcription and shedding

In vitro AREG mRNA expression and protein release are induced upon exposure to different stress factors like histamine [190], diesel exhaust particles [204], cigarette smoke extract exposure [121] [124] [205] and rhinoviruses [206] [127]. Human airway trypsin-like protease, a serine protease found in the sputum of patients with chronic airway diseases, induces AREG mRNA by the protease-activated receptor-2 (PAR-2)/ERK pathway in the human airway cell line (NCI-H292) [207]. Du et al showed that cigarette smoke extract triggered transcription of AREG is cAMP and EGFR dependent in the epithelial cell line MSK-Leuk1, and involves binding of the cAMP-responsive element binding protein to the AREG gene promoter in an EGFR dependent manner [208]. Also, AREG protein secretion is dependent on the EGFR/ERK1/2/MAPK pathway in an airway epithelial cell line treated with particulate matter [173].

These data together suggest the role of EGFR/ADAM17 signaling in regulation of AREG and IL-6R mRNA production and shedding in COPD human airway epithelial cells and in CFBE41o- cells with inducible CFTR expression, which is covered by Chapter 2 and 3,

respectively.

AREG affects mucus and cytokine secretion

In asthmatic patients AREG produced by mast cells enhances mucus production [209]. *In vitro* the AREG-dependent MUC5AC mRNA level induction has been shown upon exposure to particulate matter in NCI-H292 cells [210]. Importantly, prolonged exposure to AREG induces MUC5AC expression in NHBE-ALI [201] and promotes also TNF-α-induced IL-8 secretion from NCI-H292 cells [211]. Interestingly, *in vitro* eosinophil-airway epithelial cell line interaction induces AREG dependent secretion of MUC5AC, TGF-β1, and IL-8 in culture supernatants [203]. All these findings show an important role of AREG in mucus secretion and cytokine release in airway epithelial cells.

AREG in paracrine signaling and tissue remodeling

The role of airway epithelial AREG in activation of underlying tissues has also been studied. Conditioned media from AREG-stimulated airway epithelial cells induced expression of IL8, VEGF, COX-2 and AREG in human airway smooth muscle cells (HASMC), providing proof for crosstalk between epithelial cells and HASMC in an AREG-dependent manner [191], and underlined the important role of AREG in paracrine signaling. Inhibition of AREG or EGFR in TGF-β1-stimulated lung fibroblasts diminished AREG-dependent fibroblast proliferation, expression of α-smooth muscle actin and collagen [212], strongly suggesting a role of AREG/ADAM17/EGFR signaling in pulmonary fibrosis *in vivo*. AREG stimulation also induces airway smooth muscle cell (ASM) proliferation, which leads to airway remodeling *in vivo* [213]. The AREG/EGFR/ERK1/2/MAPK signaling pathway is also involved in cell migration through extracellular matrix (ECM), due to plasmin-dependent degradation of matrix proteins in MSK-Leuk1 cells treated with a saline extract of cigarette smoke (CSE) [214]. CSE and exogenous AREG treatment also lead to induction of DNA synthesis in an EGFR dependent manner, providing further proof of AREG and EGFR involvement in cell proliferation [208].

These data together suggest that exaggerated and chronic AREG release may contribute to mucus plugging and excessive inflammation and tissue remodeling in CF and COPD. Therefore, the AREG/EGFR/ADAM17 signaling pathway is a potential therapeutic target to regulate both inflammation and lung cell proliferation and migration, which may prevent lung tissue inflammation, remodeling and fibrosis in COPD and CF. However, AREG is only one of the many players involved in lung tissue autocrine and paracrine signaling leading to lung pathology. There is no available literature presenting intervention in AREG function in clinical trials.

The role of AREG and EGFR trafficking in cellular signaling

The majority of EGFR signaling is believed to occur at the plasma membrane. However, many studies show that upon ligand-dependent activation, EGFR is rapidly internalized into endosomes, where hypothetically it may continue to signal [215] [216] [217] [218] [219]. Up till now, the endocytic transport of EGFR has been interpreted as a signal attenuation, due to lysosomal degradation [220]. However, recent reviews elaborate on the role of EGFR endocytic trafficking and recycling in active signal transduction [220] [221] [222] [223] [224].

The full-length forms of AREG and other EGFR substrates have also been observed in intracellular vesicular compartments, i.e. lysosomes [225] and endosomes [199]. Mono-ubiquitination of pro-AREG at Lys240 (pro-AREGmUb) leads to quick endocytosis of AREG from the cell surface and its localization into early endosomes [226]. AREG has been also observed in exosomes negative for the ER marker calreticulin [224]. Higginbotham et al.

showed the importance of paracrine exosomal AREG-mediated signaling in breast cancer cells [224]. Recipient LM2-4175 cells rapidly take up AREG containing exosomes in an EGFR-dependent manner. In comparison to other EGFR substrates, namely TGF-α and HB-EGF, exosomal AREG uptake enhanced invasion of LM2-4175 cells through the Matrigel to the recipient cells, which were placed under the Transwell filter [224]. These exosomes also improve wound healing.

Taken together, presented data indicate the existence of a non-canonical trafficking pathway of EGFR-mediated signaling after EGFR endocytosis. So far, these studies largely focus on deregulated EGFR trafficking in the context of carcinogenesis. However, the EGFR trafficking in CFTR deficiency and its effect on the signaling cascade has not been investigated yet. As a first step to study the role of ADAM17 substrate and receptor trafficking, we investigated the intracellular localization of EGFR, ADAM17 and its substrates in CS treated ALI-HBEC and in CFBE41o- cells with induced CFTR activity (Chapter 2 and 3).

1.7 ROLE OF IL-6-R IN HUMAN LUNG INFLAMMATION AND FIBROSIS

Pro and anti-inflammatory responses of IL-6R

The IL-6 co-receptor (IL-6R) is a transmembrane receptor type I that in classic signaling binds to IL-6, which evokes an association with the homodimer of signal transducing glycoprotein, gp130. This trimer dimerises to form a hexameric complex composed of IL-6, IL-6R and gp130 [227] (Figure 6). IL-6R can also evoke a trans-mediated signaling cascade, when it is shed close to the transmembrane region by ADAM17 [228] or ADAM10 [229] [228] [230]. Subsequently, γ-secretase cleaves the IL-6R C-terminal fragment, which is rapidly degraded, so intracellular part does not take part in further signaling [231]. sIL-6R can be also generated by alternative splicing [232] [233] [234], however it is thought that shedding rather than the alternative splicing takes part in regulated generation of the sIL6-R [146]. Both the alternatively spliced form and the shed form of sIL-6R can create functional complexes with IL-6 (IL-6/sIL-6R) to dimerize and trans-activate gp130 finally creating a hexameric complex on the underlying myofibroblasts [227] [232] [235] [236] (Figure 6). The soluble form of gp130 (sgp130) selectively blocks trans-signaling and is described as a natural inhibitor of IL-6R trans-signaling *in vivo* [237] [238] [239].

It is thought that IL-6 activated membrane bound IL-6R has anti-inflammatory, anti-apoptotic and regenerative properties [146] [235] [240] [241]. In contrast to classic IL-6R signaling in which IL-6 binds to membrane bound IL-6R associating with gp130, trans-signaling responses mediated by IL-6/sIL-6R complexes binding to gp130 are thought to maintain inflammation and promote inflammation-associated cancer [146] [235] [242] [243] (Figure 6). IL-6R-mediated signaling leads to activation of STAT3 [239] [244], ERK [245] and PI3K [246], linking this signaling pathway with the EGFR/ADAM17 axis, which also activates STAT3 through a different pathway.

IL-6R expression is restricted to specific cell types

IL-6R expression is restricted to several cell types, such as lymphoid cells (megacaryocytes, neutrophils, monocytes/macrophages, leukocytes, certain lymphocytes), hepatocytes and airway epithelial cells [146] [246] [247] [248]. Cells that do not express IL-6R, but do express gp130 can still transduce IL-6 signals via binding of IL-6/sIL-6R complexes. Many cells including smooth muscle cells [249] and endothelial cells [250] are only responsive to IL-6 through IL-6/sIL-6R trans-signaling [249] [250] (Figure 6).

The role of IL-6R in inflammation and remodeling

Bacterial toxins, bacterial metalloproteinases and apoptosis are known to stimulate release of sIL-6R. It has been shown that in polarized airway epithelial cell lines bacterial treatment

induces shedding of sIL-6R [248]. Expression of IL-6 and sIL-6R is increased significantly in serum and lung biopsies from adenocarcinoma patients [243]. Further, in an adenocarcinoma mouse model blocking of the sIL-6R mediated trans-signaling with an anti-IL-6R antibody or the inhibitor sgp130Fc alleviates lung cancer pathogenesis showing the importance of IL-6R signaling in tumour signaling [243], proliferation and migration [251].

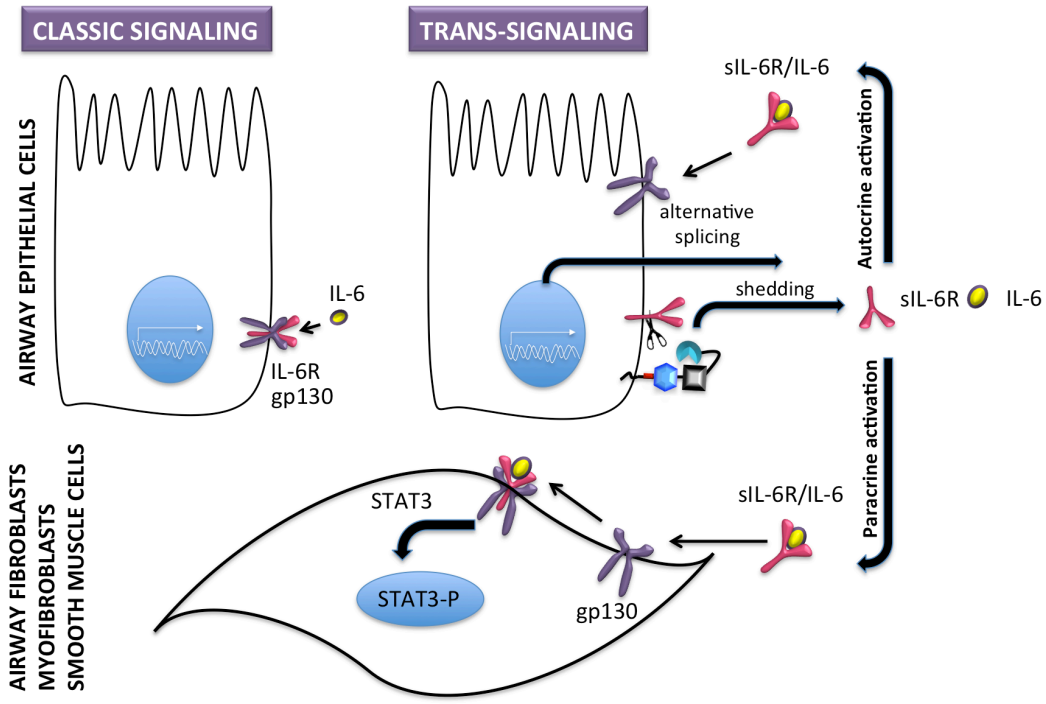


Figure 6 Classic and trans-signal activation of IL-6R signaling. In classic signaling membrane bound IL-6R stimulated with autocrinally or paracrinally secreted IL-6 associates with the homodimer of signal transducing glycoprotein, gp130 to activate downstream molecules, like STAT-3. In trans-signaling soluble form of IL-6R (sIL-6R) generated either by ADAM17 or by alternative splicing binds to IL-6 and this IL-6/IL-6R complex activates gp130 on the airway epithelial cells (autocrine IL-6R trans-signaling) or underlying fibroblasts/myoblasts/smooth muscle cells that do not express IL-6R (paracrine IL-6R trans-signaling), but express gp130 to evoke activation of downstream molecules, for instance STAT-3 signaling.

However, the role of IL-6R in CF and COPD has not been studied in detail. In COPD patients, elevated levels of IL-6R have been observed in peripheral blood leukocytes [252] and sputum samples [189]. Recently, genetic variants of IL-6R have been linked with lung function [253] and COPD severity [254]. Elevated levels of IL-6R were also detected in protein lysates from lungs of the patients with idiopathic pulmonary fibrosis (IPF), which is a lethal lung disease with progressive fibrosis [255]. In contrast to elevated IL-6R levels in sputum of COPD patients [189] and in bronchoalveolar lavage fluids (BALF) from patients with allergic asthma [256], the levels of sIL-6R in BALF form CF patients were not different in comparison to control [257]. This was explained by enhanced degradation of sIL-6R by serine proteases. Furthermore, it is not evident what the impact of luminal IL6-R on the activity of the sub-epithelial tissue is, which is separated from the lumen by tight junctions.

Shedding of IL6-R from epithelial cells occurs mainly towards the basal side (Chapter 2) [248]. Importantly, in bleomycin challenged murine lungs inhibition of sIL-6R by intraperitoneal injection of a recombinant decoy receptor (gp130Fc) attenuates pulmonary fibrosis observed as reduction in myofibroblasts, fibronectin, and collagen, whereas activation of IL-6 trans-signaling in cell lines enhances fibroblast proliferation and extracellular matrix protein production [255], which are the hallmarks of the pulmonary fibrosis progression.

Together these data show the importance of IL-6R trans-signaling in inflammation and lung remodeling [24]. We focused our studies on the mechanism regulating the IL-6R mRNA and protein shedding from COPD airway epithelial cells (Chapter 2).

Species specificity of IL-6R signaling

IL-6R is a substrate for both ADAM17 and its close relative ADAM10 [229]. IL-6R needs to bind IL-6 to evoke downstream signaling, neither IL-6R alone nor IL-6 has affinity for gp130 [258]. Species differences in IL-6R mediated signaling have been observed. Human IL-6 stimulates human and murine cells, whereas murine IL-6 only stimulates murine IL-6R signaling [259]. Because of this species specificity transgenic mice expressing human sIL-6R from a liver promotor did not bind the endogenous murine IL-6, and as a consequence the transgenic animals do not have a transgene specific phenotype [259] [260]. Garberts et al for the first time questioned the species specificity of IL-6R shedding by showing that human IL-6R is a substrate of human ADAM17, but murine IL-6R is a substrate of murine ADAM10 [261]. Subsequently, Schumacher et al. revealed that trigger-induced shedding of both human and mouse IL-6R is mediated by murine ADAM17, but constitutive release of IL-6R is largely mediated by ADAM10 in mice [230]. Additionally, in humans, but not in mice, the sIL-6R can be generated by translation from an alternatively spliced mRNA [262] [230]. Of note, the soluble gp130 form, which circulates in human plasma, blocks IL-6R trans signaling responses and does not show species specificity, meaning that it interacts with human and mouse sIL-6R complexes [263]. All of this has implications in the extrapolation of IL-6R data obtained in mouse models to human. Thus, to provide clinically relevant data, we focused our studies on primary and immortalized human cells from COPD and CF patients.

1.8 IS ADAM17 PHOSPHORYLATION IMPORTANT FOR CS-INDUCED AREG AND IL-6R SHEDDING?

The serine- and threonine-rich ADAM17 cytoplasmic tail [130] has three phosphorylation sites that have been proposed to activate ADAM17: Thr735, Ser791 and Ser819 (Figure 2). Phosphorylated ADAM17 is detected under basal conditions [264]. Stimulation of ADAM17 further increases its phosphorylation, without changing the total protein level [130] [264], suggesting that phosphorylation plays a role in the regulation of the proteolytic activity of ADAM17. However, mutation of the phosphorylation sites individually or in combination and even removal of the whole cytoplasmic tail has no significant effect on stimulated shedding suggesting that phosphorylation may be an additional but not a crucial regulatory mechanism of ADAM17 proteolytic activity, which does not exclude that it plays a role in transport, processing, and maturation [130] [138] [185] [265] [266].

Different stimuli affect distinct phosphorylation sites on the ADAM17 molecule. Cigarette smoke extract increases phosphorylation of Ser and Thr, but not Tyr residues on ADAM17 molecule in a PKC-ε dependent manner. ADAM17 ***Ser819*** residue, but not on Thr or Tyr, is phosphorylated when ERK/MAP kinase signaling pathway is induced by growth factor (GF) stimulation [130]. However, an ADAM17-Ser819 mutant does not lose its proteolytic activity when simulated with fibroblast growth factor (FGF) [130], suggesting that this phosphorylation is not required for ADAM17 proteolytic activity. To date, the kinase

responsible for phosphorylation of this site has not been identified. Another phosphorylation residue on ADAM17 molecule, a ***Ser791*** is phosphorylated in resting cells, whereas in response to GF stimulation ***Ser791*** undergoes dephosphorylation, which is independent of the ERK/MAPK pathway [130]. Pro-ADAM17 and mature ADAM17 are also phosphorylated at ***Thr735*** under resting condition. Phorbol esther (PMA) treatment further increases phosphorylation at this site [264]. ERK 1/2 co-immunoprecipitates with ADAM17 and directly phosphorylates it at Thr735 [143] [264] [267], providing evidence that it regulates ADAM17 maturation, trafficking to the cell surface and activation. Also p38 has been shown to phosphorylate Thr735 on ADAM17 [264] [267]. Stimulation of ERK and PKC with PMA also activates ADAM17, leading to shedding of IL-6R [143]. P38α MAP kinase interacts with the cytoplasmic domain of ADAM17 and phosphorylates it on Thr735 resulting in release of TGF-α and enhanced cell proliferation [143], which is likely dependent on the association of ERK and ADAM17 [264].

Taken together, some studies show that GF stimulation induces ADAM17 phosphorylation at Thr735 [264], while others find contradicting data [130], which may be cell or context dependent. In Chapter 2 we addressed the association of Thr735 phosphorylated ADAM17 with its substrates (IL-6R and AREG) in CS treated airway epithelial cells, and correlated this with ADAM17 shedding activity.

1.9 Murine models to study activity of ADAM17 in airway epithelia

Inactivation of ADAM17 by deletion of the zinc binding domain through homologous recombination in mice led to severely hypoplastic lungs at birth, reduced branching morphogenesis and alveolar development, impaired epithelial cell proliferation and differentiation and delay in vasculogenesis [268]. Due to the early mortality of ADAM-KO mice, several alternative ADAM17 mouse models have been developed, with reduced activity (hypomorphic) or conditional mutants [132]. Conditional knockout ADAM17 mice can serve as a model to investigate the role of ADAM17 in organogenesis, in specific cell types, and in inflammatory responses and tissue remodeling.

Induced dysfunction of ADAM17 (ADAM17flox/flox SPC-rtTA TetO-Cre) in developing lung epithelial cells reduced saccular formation, decreased cell proliferation and reduced lung epithelial cell differentiation, but the mice were born without severe respiratory distress [152]. Phenotypic differences were reduced after alveolarisation of the immature lung (P14). Knocking out the gene in mesenchymal cells using a *Dermo1-Cre* transgene did not produce a detectable phenotype [152]. This suggests that epithelial, but not mesenchymal ADAM17 plays a prominent, but not decisive role in lung development.

Conditional knockout mice in the hematopoietic system (Adam17flox/ΔZn driven by Vav-Cre) treated with LPS have significantly reduced LPS-induced alveolar TNF-α and L-selectin levels, but IL-6R levels are reduced by only 25% [269], consistent with reports that in mice IL-6R is a preferred substrate of ADAM10, but not ADAM17 as in human [261] [230]. In the mutant mice alveolar neutrophil counts are reduced after LPS challenge, but initial infiltration of neutrophils occurs quicker, in comparison to control animals [269]. Other studies show that conditional knockout mice (ADAM17flox/flox with R26Cre ER) treated with human neutrophil elastase have attenuated goblet cell metaplasia in comparison to the wild-type mice [270], suggesting that ADAM17 is involved in the airway metaplasia.

In our laboratory we took an effort to generate CF ADAM17 conditional knockout mice in lung epithelial cells (SPC-rtta/Tet-O-Cre/Adam17flox/flox), which can serve as a model of inflammatory and repair responses in normal and CF airway epithelial cells, by crossing *Cftr*^{tm1EUR} F508del CFTR mutant mice with ADAM17flox/flox [271] and SPC-rtTA/tetO-cre [272].

HYPOTHESIS:

ADAM17 is hyperactive in CF and COPD, promoting inflammation and tissue remodeling by shedding of EGFR binding growth factor AREG and the cytokine receptor IL-6R from airway epithelial cells

We hypothesized that both CF and COPD lung disease are associated with hyperactivity of the EGFR/ADAM17 signaling cascade in airway epithelial cells, causing enhanced or sustained release of growth factors, pro-inflammatory cytokines and cytokine receptors. This may contribute to epithelial metaplasia, fibroblast and smooth muscle cell activation, and net deposition of extracellular matrix. Interventions in this pathway may therefore reduce CF and COPD lung pathology.

We focused our studies on two ADAM17 substrates: growth factor AREG and pro-inflammatory IL-6 receptor (IL-6R). AREG, a type I trans-membrane protein [273], proteolytically cleaved by ADAM17 from airway epithelial cells, activates EGFR not only on the airway epithelial cells, but also on the underlying fibroblasts. IL-6R trans-signaling requires ADAM17-mediated shedding of the extracellular domain [228] [230] or alternative mRNA splicing [232] [233] [234]. The shed form and alternatively spliced form bind to IL-6 creating IL-6/IL-6R complexes that dimerize and trans-activate gp130 on the underlying myofibroblasts [232] [235] [236]. gp130 is ubiquitously expressed, meaning that cells that do not express IL-6R, but do express gp130 can still transduce IL-6R signal by binding IL-6/IL-6R complexes. Many cells including smooth muscle cells [249] and endothelial cells [250] are only responsive to IL-6 through IL-6/IL-6R trans-signaling.

Taken together, we hypothesised that pathology driven trans-signaling at least in part depends on airway epithelial AREG and IL-6R shedding. Importantly, signals transduced by shed AREG and IL-6R from airway epithelial cells may converge in activation of the transcription factor STAT3 in lung fibroblasts, myofibroblasts and smooth muscle cells (Figure 7), which is involved in fibrotic responses and inflammatory lung disease [274]. This aspect has not been investigated during the course of this thesis; however, it is the subject of ongoing investigations in our institute.

Considering the similarity of the pathology of COPD and CF, we investigated the influence of COPD disease on the basal and CS-induced EGFR/ADAM17 signaling in differentiated primary bronchial epithelial cells. Furthermore, by using CFBE41o- cells with inducible expression of CFTR, we aimed to establish whether CFTR deficiency hyper-activates EGFR/ADAM17 signaling as a cell-autonomous defect. Finally, we aimed to define the druggable targets in the EGFR/ADAM17 signaling pathway, which could be applied in management of COPD and as an early intervention CF lung therapy.

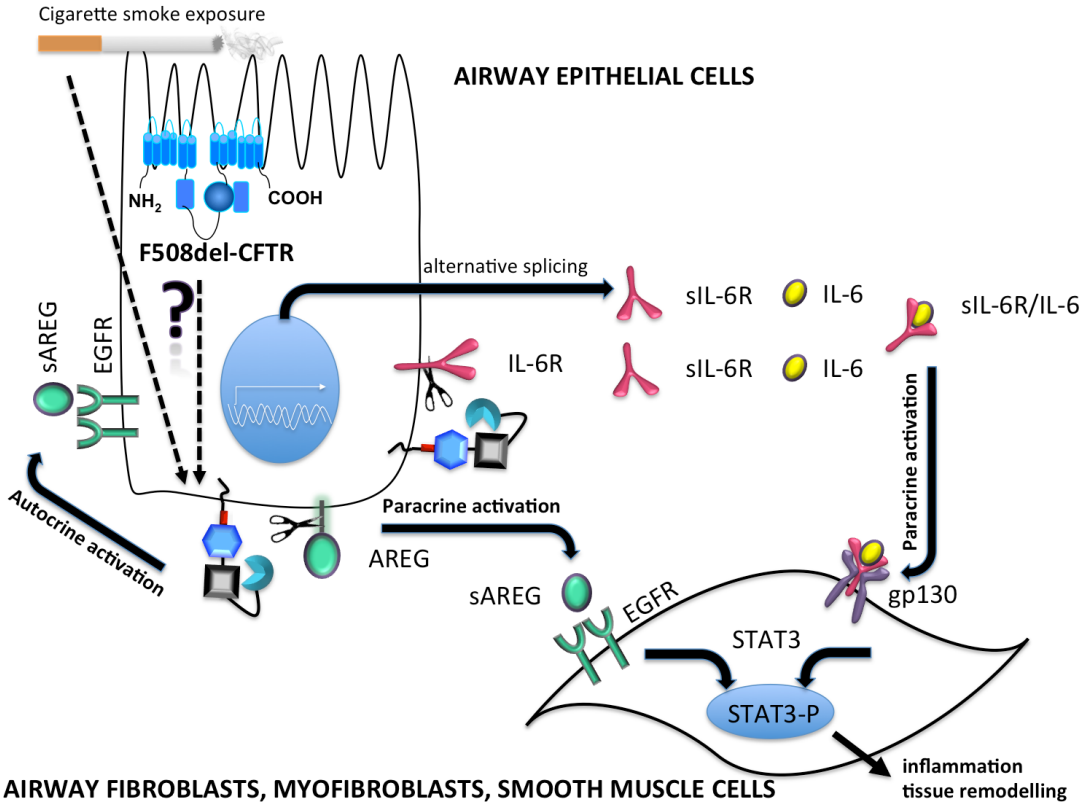


Figure 7 Is ADAM17/EGFR signaling an important player in CF and COPD lung disease?
We hypothesized that both CFTR deficiency and cigarette smoke exposure are associated with hyperactivity of ADAM17/EGFR signaling cascade in airway epithelial cells, causing enhanced release of pro-inflammatory cytokines and growth factors. We focused our studies on two ADAM17 substrates: pro-inflammatory IL-6 receptor (IL-6R) and growth factor AREG. AREG proteolytically cleaved by ADAM17 from airway epithelial cells activates EGFR autocrinally in the airway epithelial cells and paracrinally on the underlying fibroblasts. IL-6R trans-signaling requires ADAM17-mediated shedding of the extracellular domain or alternative mRNA splicing. The alternatively spliced and shed form binds to IL-6 creating IL-6/IL-6R complexes to dimerize and trans-activate gp130 on the underlying myofibroblasts. Signals transduced by shed AREG and IL-6R from airway epithelial cells may converge in activation of a transcription factor STAT3 in lung fibroblasts, myofibroblasts and smooth muscle cells, which is involved in fibrotic responses and inflammatory lung disease. Enhanced ADAM17/EGFR signaling may contribute to hyper-inflammation and epithelial metaplasia, fibroblast and smooth muscle cell activation, and net deposition of extracellular matrix (tissue remodeling) leading to lung pathology observed in COPD and CF.

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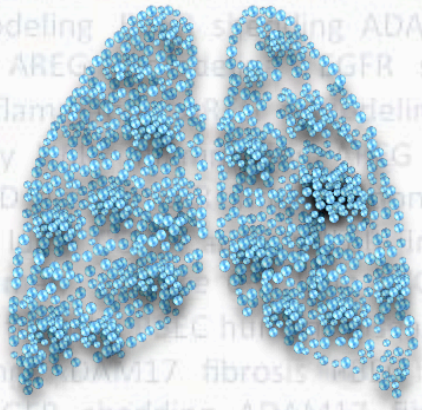
CHAPTER 2



ADAM17 and EGFR regulate IL-6 receptor and amphiregulin mRNA expression and release in cigarette smoke-exposed primary bronchial epithelial cells from patients with chronic obstructive pulmonary disease (COPD).

(published - Physiol Rep)

Stolarczyk M, Amatngalim GD, Yu X, Veltman M, Hiemstra PS, Scholte BJ

Physiological Reports

Open Access

Physiological Reports ISSN 2051-817X

ORIGINAL RESEARCH

ADAM17 and EGFR regulate IL-6 receptor and amphiregulin mRNA expression and release in cigarette smoke-exposed primary bronchial epithelial cells from patients with chronic obstructive pulmonary disease (COPD)

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Keywords
A disintegrin and metalloprotease 17 (ADAM17), amphiregulin (AREG), Chronic Obstructive Pulmonary Disease (COPD), epidermal growth factor receptor (EGFR), IL6 receptor (IL6R), TACE.

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Funding Information
This study (MS) was supported by grants from the Lung Foundation Netherlands (#3.3.10.027). the Dutch Cystic fibrosis Foundation NCFS (MV HIT-CF7) and Galapagos NV, The Netherlands.

Received: 13 June 2016; Revised: 8 July 2016; Accepted: 9 July 2016

doi: 10.14814/phy2.12878

Physiol Rep, 4 (16), 2016, e12878, doi: 10.14814/phy2.12878

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Abstract

Aberrant activity of a disintegrin and metalloprotease 17 (ADAM17), also known as TACE, and epidermal growth factor receptor (EGFR) has been suggested to contribute to chronic obstructive pulmonary disease (COPD) development and progression. The aim of this study was to investigate the role of these proteins in activation of primary bronchial epithelial cells differentiated at the air–liquid interface (ALI-PBEC) by whole cigarette smoke (CS), comparing cells from COPD patients with non-COPD. CS exposure of ALI-PBEC enhanced ADAM17-mediated shedding of the IL-6 receptor (IL6R) and the EGFR agonist amphiregulin (AREG) toward the basolateral compartment, which was more pronounced in cells from COPD patients than in non-COPD controls. CS transiently increased IL6R and AREG mRNA in ALI-PBEC to a similar extent in cultures from both groups, suggesting that posttranslational events determine differential shedding between COPD and non-COPD cultures. We show for the first time by in situ proximity ligation (PLA) that CS strongly enhances interactions of phosphorylated ADAM17 with AREG and IL-6R in an intracellular compartment, suggesting that CS-induced intracellular trafficking events precede shedding to the extracellular compartment. Both EGFR and ADAM17 activity contribute to CS-induced IL-6R and AREG protein shedding and to mRNA expression, as demonstrated using selective inhibitors (AG1478 and TMI-2). Our data are consistent with an autocrine-positive feedback mechanism in which CS triggers shedding of EGFR agonists evoking EGFR activation, in ADAM17-dependent manner, and subsequently transduce paracrine signaling toward myeloid cells and connective tissue. Reducing ADAM17 and EGFR activity could therefore be a therapeutic approach for the tissue remodeling and inflammation observed in COPD.

Introduction

Chronic obstructive pulmonary disease (COPD) is a progressive lung disorder characterized by irreversible airflow obstruction due to airway inflammation, infection, and tissue remodeling (Vestbo et al. 2013). Airway epithelial cells play a central role in the pathogenesis of COPD through a variety of mechanisms, including production of inflammatory mediators, antimicrobial peptides, and growth factors (Hiemstra et al. 2015). Exposure of the lung tissue to triggers like cytotoxic particles and gasses, including cigarette smoke, microbes, and innate immune

mediators (Koff et al. 2008; Kim et al. 2011) (Lemjabbar-Alaoui et al. 2011) (Shao 2004) (Zhou et al. 2012) activate various matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinases (ADAMs) expressed by airway epithelial cells (Dijkstra et al. 2009; Paulissen et al. 2009). The activity of MMPs and ADAMs contributes not only to proteolytic degradation of lung tissue, but also to regulation and processing of numerous receptor activating proteins (Richter et al. 2002; Gomez et al. 2005; Bell et al. 2007; Baumgart et al. 2010). Through these various activities, MMPs and ADAMs are implicated in a broad spectrum of processes ranging from inflammatory responses to airway epithelial repair. It has been proposed that their aberrant activity might lead to chronic inflammation and abnormal tissue remodeling in the lungs of COPD patients (Paulissen et al. 2009).

One of the ADAMs, a ubiquitously expressed Zn^{2+}-dependent disintegrin and metalloprotease 17 (ADAM17), formerly known as TNFα converting enzyme (TACE), is recognized as an important regulator of pulmonary inflammation, cell proliferation, and epithelial barrier function (Gooz 2010; Lemjabbar-Alaoui et al. 2011). In bronchial epithelial cells, ADAM17 modulates these processes by cleaving membrane-bound cytokines (TNFα), several EGF receptor (EGFR) agonists (TGF-α, amphiregulin, epiregulin, HB-EGF), cytokine receptors (IL6R, TNF-R), growth factor receptors (NOTCH receptors), and adhesion proteins (L-selectin, ICAM-1, E-cadherin) (Gomez et al. 2005; Bell et al. 2007; Baumgart et al. 2010; Gooz et al. 2012). Moreover, ADAM17 phosphorylation and activity is enhanced in airway epithelial cell lines and in undifferentiated primary cells upon exposure to cigarette smoke extract (Lemjabbar et al. 2003; Shao 2004; Lemjabbar-Alaoui et al. 2011).

Our studies focus on two ADAM17 substrates implicated in COPD pathogenesis: the IL-6 cytokine receptor (IL6R) and the growth factor amphiregulin (AREG), one of the EGFR agonists produced by bronchial epithelial cells (Richter et al. 2002). Elevated levels of IL6R have been observed in peripheral blood leukocytes of COPD patients (Edmiston et al. 2010), and recently genetic variants of IL6R have been linked with COPD severity (Pérez-Rubio et al. 2016). However, the regulation of shedding of IL6R and AREG from COPD airway epithelium has not been studied. Upon shedding from epithelial cells, IL6R and AREG activate the shared interleukin receptor gp130 and EGFR, respectively, on epithelial cells (autocrine), as well as on underlying myofibroblasts and myeloid cells (paracrine) (Burgel and Nadel 2008; Nechemia-Arbely et al. 2008; Kasina et al. 2009; Rose-John 2012). Both IL6/IL6R/gp130 and AREG/EGFR/ERK pathways are involved in the resolution of lung inflammation and repair of injury, but also in progression of subepithelial fibrosis and collagen deposition (Zhou et al. 2012). These signaling pathways involve the JAK kinase and/or MAP kinase pathway, which are druggable targets in COPD pathology (Barnes 2013). Excessive ligand-mediated EGFR activation results in epithelial hyperproliferation and increased production of the inducible mucin MUC5AC, processes observed in smokers with or without COPD (Lemjabbar et al. 2003; Shao 2004; Deshmukh et al. 2005; Kasina et al. 2009; Lemjabbar-Alaoui et al. 2011; Li et al. 2011; Zhang et al. 2014). Moreover, EGFR activation results in subsequent transcriptional regulation of inflammatory mediators such as IL-8 (Richter et al. 2002), a chemokine that has been implicated in COPD development.

So far, studies on the effect of cigarette smoke on epithelial ADAMs activity has largely relied on the use of airway epithelial cell lines or undifferentiated primary cell cultures, stimulated with an aqueous extract of cigarette smoke. However, whole cigarette smoke exposure and primary differentiated airway cell cultures represent more relevant physiological conditions. Firstly, fresh whole cigarette smoke (CS) contains unstable active components and particulate matter that are largely absent from extracts. Furthermore, immortalized epithelial cells are poor models of bronchial epithelium in situ, since they are frequently karyotypically unstable and heterogeneous, do not show characteristic features of differentiation and inherently carry multiple mutations in pathways essential for growth, differentiation, cell-cell interaction, and polarization. Furthermore, submerged cultures of primary airway epithelial cells fail to differentiate. Finally, using cell lines does not allow a comparison of patient populations. Therefore, we examined the effect of whole CS exposure on shedding of the soluble interleukin-6 receptor (sIL6R) and the EGFR-ligand amphiregulin (AREG) by well-differentiated, air–liquid interface cultured human primary bronchial epithelial cells (ALI-PBEC).

This allowed us to compare CS-induced ADAM17-mediated protein shedding and mRNA expression of sIL6R and AREG in well-differentiated ALI-PBEC from COPD and non-COPD (ex)smokers. Moreover, we established in this model the involvement of both EGFR and ADAM17 not only in shedding of ADAM17 substrates, but also in the regulation of mRNA levels of ADAM17 substrates and IL-8. Finally, for the first time, we observed intracellular CS-induced phosphorylated ADAM17-substrate interaction via an in situ proximity ligation assay. Overall, our results provide novel insights into the activation of airway epithelial cells by cigarette smoke in COPD, and highlight a possible role of ADAMs and EGFR in COPD pathology.

Materials and Methods

Air–liquid interface cell culture of human primary bronchial epithelial cells

Human airway epithelial cells were obtained from macroscopically normal, anonymous bronchial tissue obtained from lung cancer patients undergoing resection surgery for lung cancer at LUMC. This material was be used for research according to the "Code of Conduct for Responsible Use" (FEDERA code) based on the condition that the patient has no objection against such use. Primary bronchial epithelial cells (PBEC) were isolated from tumor-free lung resection material (Wetering et al. 2000), and passage 2 expanded cells were cultured at the air–liquid interface (ALI) to achieve mucociliary differentiation as previously described (Amatngalim et al. 2015). Briefly, 40,000 cells were seeded on 0.65 cm Transwell inserts (Corning Costar, Cambridge, MA) with a 0.4 μm pore size, which were coated with 30 μg/mL PureCol (Advanced BioMatrix, San Diego, CA), 10 μg/mL Bovine serum albumin (Sigma-Aldrich, St. Louis, MO), and 10 μg/mL Fibronectin (isolated from plasma). Cells were cultured in Bronchial epithelial growth medium (BEGM) (Lonza, Verviers, Belgium) and Dulbecco's modified Eagle's medium (DMEM) (Gibco, Bleiswijk, The Netherlands) (1:1 mixture) containing 1 mmol/L Hepes (Lonza) and supplemented with SingleQuot supplements and growth factors according to the manufacturer's instructions (bovine pituitary extract, hydrocortisone, human epidermal growth factor, epinephrine, transferrin, insulin, T3 and retinoic acid; all from Lonza), additional 15 ng/mL retinoic acid (Sigma–Aldrich), 1 mg/mL BSA (Sigma–Aldrich), 100 U/mL penicillin, and 100 μg/mL streptomycin (Lonza). PBEC were initially cultured on inserts in submerged conditions until cell layers were confluent. Next, apical medium was removed and cells were cultured at air-exposed conditions for at least 2 weeks to allow mucociliary differentiation. Clinical history and lung function data were obtained from anonymized patients (Table 1), and COPD disease status was based on lung function data according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) classification (Vestbo et al. 2013). Donor cells were randomly allocated to experimental groups.

Table 1. COPD and non-COPD patient characteristics.

	COPD	non-COPD	*P*-value
Number of donors	15	11	
Gender (females/males)	4/11	2/9	
Age, years	70 ± 8	66 ± 6	0.1667
FEV_1, % predicted	65 ± 16	81 ± 16	<0.01
FEV_1/FVC %	55 ± 9	79 ± 9	<0.0001

Characteristics of PBEC donors. Age in years, and lung function as FEV_1 (% predicted) and FEV_1/FVC are shown as means ± SD. The mean differences were compared using the nonparametric Mann–Whitney test. COPD, chronic obstructive pulmonary disease, FEV1, Forced expiratory volume in one-second, FVC, forced vital capacity.

Cigarette smoke exposure

Air–liquid interface cultured human primary bronchial epithelial cells were exposed to whole cigarette smoke (CS) in an exposure model, adapted from (Beisswenger et al. 2004) and previously described in more detail (Amatngalim et al. 2015). In this model, ALI-PBEC cultures were placed in either a CS- or air (negative control) exposure chamber located in a tissue incubator at 37°C and 5% CO_2. Smoke derived from one cigarette (3R4F reference cigarettes [University of Kentucky, Lexington, KY]) was infused into the exposure chamber by a mechanical pump with a constant flow of 1 L/min, and equally distributed by a ventilator inside the chamber. After infusion (approximately 4–5 min), residual smoke was removed by infusion of air from the tissue incubator for 10 min. Directly after CS exposure, the basal medium of the cell cultures was refreshed and cells were incubated for the indicated periods of time. Untreated cells used as controls were subjected to the same procedure omitting the smoke (AIR).

Inhibitors

TMI-2 (1 μmol/L; PF-5480090), a highly selective inhibitor of ADAM17 activity (Zhang et al. 2004), was obtained from Wyeth inc. (Philadelphia, Pennsylvania) and the selective EGFR inhibitor AG1478 (1 μmol/L) was from Sigma Aldrich. Cells were preincubated for 1 h with inhibitors before CS exposure, and directly after CS exposure media were replaced and inhibitors were freshly added.

ELISA

ALI-PBEC conditioned culture media were collected from the basolateral side of the inserts (1 mL), and apical washes were obtained by washing the apical surface with 100 μL PBS at different time points, dependent on the experiment. Collected samples were diluted 1:1 with BEGM media and analyzed for IL6R and AREG by human IL6R or AREG ELISA kit, R&D. Further steps were performed according to the manufacturer's protocol. Data were corrected for the dilution factor and insert size, and the amount of the shed IL6R and AREG was expressed as pg/mL per cm^2.

RNA isolation and quantitative real-time PCR

RNA was isolated using the miRNeasy Mini Kit (Qiagen) according to the manufacturer's instructions, and cDNA was synthesized by reverse-transcription PCR using oligo (dT) primers (Qiagen) and Moloney murine leukemia virus (M-MLV) polymerase (Promega, Leiden, The Netherlands). mRNA expression was determined by quantitative real-time PCR as described previously (Amatngalim et al. 2015) with primer pairs presented in Table 2. mRNA expression was quantified using the standard curve method (Larionov et al. 2005), in which arbitrary expression levels were normalized to the housekeeping genes RPL13A and ATP5B. The housekeeping genes were selected based on stable expression using the "Genorm method" (Vandesompele et al. 2002).

Proximity ligation assay

Chronic obstructive pulmonary disease ALI-PBEC were-fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton-X100 in PBS twice for 15 min, blocked with 1% BSA and 0.15% glycine. Next, they were incubated overnight in 4°C with two different first antibodies simultaneously: against ADAM17 (rabbit polyclonal, C-terminal ADAM17, 25 μg/mL, ab78162, Abcam) or ADAM17P^{T735} (rabbit polyclonal, phospho-ADAM17 in position T735, 25 μg/mL, ab60996, Abcam) and IL6R (goat anti-human IL6R recognizing extracellular domain, 20 μg/mL, AF-227-NA, R&D) or AREG (polyclonal goat anti-human Areg, 25 μg/mL, AF-262, R&D). All washing steps were repeated three times with 0.5% Triton-X100 in PBS (Sigma-Aldrich). Further steps were performed according to the DuoLink manufacturer's protocol. Briefly, Proximity ligation assay (PLA) probes for anti-goat PLUS and anti-rabbit MINUS were incubated at 37°C for 1 h. Ligation and amplification steps were performed with Detection Red Reagent. Finally, inserts with ALI-PBEC were cut out and mounted on the slides with DAPI (Vectashield mounting medium for fluorescence with DAPI, H-1200). *z*-stacks were acquired using confocal microscopy (Leica604).

PLA image analysis

The number of dots was counted in the whole *z*-stack with Image J software. The threshold values were adjusted with the *Intermodes* algorithm (the filter size set between 10 and 437 microns to exclude the small and large dots, which were in the range of 10% of the total dot count). The objects on the edges of the culture inserts were excluded from the analysis. The number of nuclei was counted in each *z*-stack by hand to express the number of dots per nucleus.

Statistical analysis

Data were analyzed with GraphPad Prism Software, using the appropriate statistical test, as indicated underneath each figure. Cells from various donors (number indicated by n in the legends of figures) were used for each experiment, samples were collected from two or three wells from a single donor, averaged and represented as a single dot in the figure. Statistical analysis was performed on the averaged data. Values are presented as mean with SEM values. Differences at *P*-values <0.05 were considered to be statistically significant. ns > 0.05, $*P < 0.05$, $**P < 0.01$, $**P < 0.001$, $****P < 0.0001$.

Results

CS induces shedding of IL6R and AREG by ALI-PBEC into basolateral medium, but not apical

We first examined the effect of cigarette smoke (CS) exposure on the release of sIL6R and AREG by ALI-PBEC

Table 2. Quantitative-PCR primers.

Primer name		[ref]
IL8	FW 5′-CAGCCTTCCTGATTTCTG-3′ RV 5′-CACTTCTCCACAACCCTCTGC-3′	Amatngalim et al. (2015)
AREG	FW 5′GTGGTGCTGTCGCTCTTGATA 3′ REV 5′ACTCACAGGGGAAATCTCACT3′	Clarke et al. (2013)
IL6R full-length form (full-IL6R)	FW 5′GCTGTGCTCTTGGTGAGGAAGTTT3′ REV 5′CTGAGCTCAAACCGTAGTCTGTAGAAA3′	Rath et al. (2010)
IL6R alternatively spliced variant (spliced IL6R)	FW 5′GCGACAAGCCTCCCAGGTT3′ REV 5′CCGCAGCTTCCACGTCTTCTT3′	Rath et al. (2010)

FW, Forward primer; REV, Reversed primer.

at the apical surface and in the basal medium, which contains a maintenance level of EGF, associated with a basal level of EGFR activity. This was done, using a previously described whole CS exposure model (Amatngalim et al. 2015), in which CS caused a transient disruption in the airway epithelial barrier integrity, accompanied by minor cytotoxic effects measured at the apical surface. Both sIL6R and AREG were barely detectible in the apical washes collected from ALI-PBEC of 17 COPD donors at different stages of disease, following exposure to either CS or air (Fig. 1A and B). In contrast, sIL6R and AREG were markedly released into the basal medium in both conditions. CS significantly increased release of sIL6R into the basal medium at 12 h postexposure, while AREG levels were increased at 12 and 24 h after CS exposure. These results demonstrate that shedding of sIL6R and AREG by ALI-PBEC occurs mainly to the basolateral compartment, and is enhanced by CS exposure.

CS significantly induces shedding of IL6R and AREG in COPD ALI-PBEC but not in non-COPD ALI-PBEC

Next, we explored whether shedding of sIL6R and AREG differs between ALI-PBEC isolated from COPD patients and non-COPD (ex)-smokers upon CS and air exposure. Based on the previous result, the release was only determined in the basal medium 24 h after exposure. Shedding of sIL6R and AREG did not differ between COPD and non-COPD ALI-PBEC exposed to air (Fig. 2A and B), indicating no differences at baseline conditions. In contrast, shedding of sIL6R (Fig. 2A) and AREG (Fig. 2B) was significantly higher after CS exposure only in COPD ALI-PBEC, and not in non-COPD ALI-PBEC. These data show that CS-induced release of sIL6R and AREG was more pronounced in airway epithelial cells from COPD in comparison to non-COPD donors.

CS-induced IL6R and AREG mRNA expression is lower in COPD ALI-PBEC compared to non-COPD cultures

We further determined mRNA expression of IL6R and AREG in CS and air exposed ALI-PBEC cultures from COPD and non-COPD patients. The soluble form of IL6R can be generated either by shedding of the membrane anchored form or by de novo synthesis of the alternatively spliced isoform that differs at the C-terminus (Rose-John 2012). Therefore, we determined mRNA expression levels of both IL6R variants: the membrane-anchored (full-IL6R mRNA) and the alternatively spliced (spliced-IL6R mRNA) variant.

Time-course analysis revealed that CS increased full-IL6R mRNA 3 h after exposure, but not at later time points (Fig. 3A). In contrast, baseline expression of spliced-IL6R mRNA did not differ from the expression after CS treatment (Fig. 3B), suggesting that the increase in sIL6R protein levels in culture supernatants did not result from alternative splicing. Similar to full-IL6R mRNA, CS significantly induced AREG mRNA expression 3 h after exposure, but not at later time points (Fig. 3C). These findings suggest that the CS-induced increase in

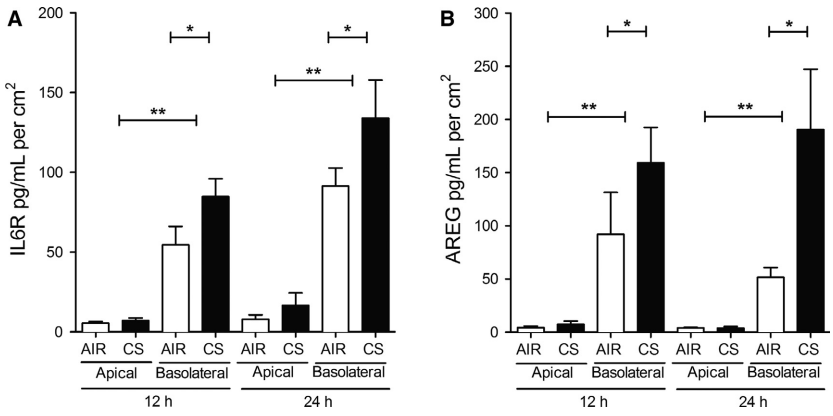

Figure 1. Cigarette smoke induces shedding of IL6R and AREG by ALI-PBEC into basolateral medium. IL6R (A) and AREG (B) were mainly shed to the basolateral compartment in ALI-PBEC (COPD donors at different stages, n = 12 for IL6R and n = 5 for AREG). Basolateral media (basolateral) were collected and apical PBS washes (apical) were performed 12 h and 24 h after CS or air exposure. Both IL6R and AREG were readily detectable in the basolateral compartment, and barely present in the apical washes. The response of cells from each donor was analyzed within one experiment using duplicate or triplicate inserts. Statistical analysis was performed by two-way ANOVA (Bonferroni) on the averaged data from each donor, comparing apical versus basolateral shedding at air and CS exposure, and basolateral shedding at air versus CS exposure.

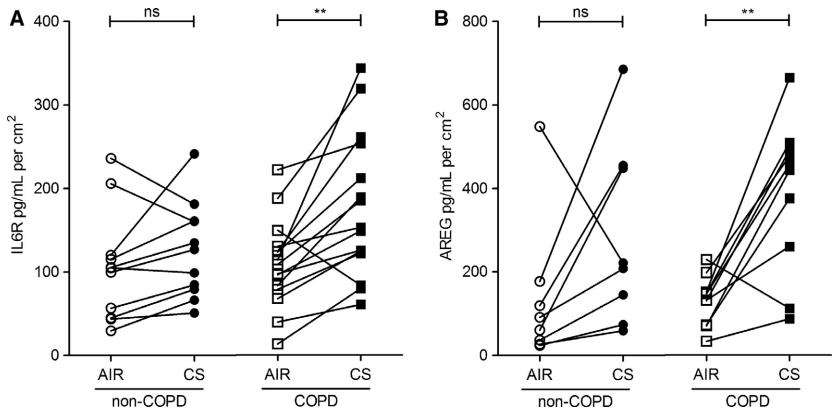

Figure 2. Cigarette smoke significantly induces shedding of IL6R and AREG into basolateral medium by COPD ALI-PBEC. Soluble forms of IL6R (A) and AREG (B) shed into the basolateral compartment were detected 24 h after CS or air exposure in ALI-PBEC derived from non-COPD and COPD donors (Table 1). (A) IL6R levels were significantly increased 24 h after CS treatment in COPD-ALI-PBEC (n = 15), but this increase was not significant in the non-COPD group (n = 11 donors). (B) Similarly, CS exposure significantly increased AREG levels in ALI-PBEC cells derived from COPD donors (n = 10), but not in non-COPD ALI-PBEC (n = 8 donors). Statistical analysis: paired t-test. n refers to the number of donors, duplicate or triplicate data were averaged per donor. Statistical analysis was performed on the averaged data from each donor.

IL6R and AREG shedding is mediated at least in part *via* regulation of their mRNA expression levels.

Baseline expression of full-IL6R and AREG mRNA did not differ between COPD and non-COPD ALI-PBEC (Fig. 3D and F). After CS exposure, full-IL6R and AREG mRNA were expressed at higher levels in both non-COPD and COPD ALI-PBEC. Interestingly, after CS induction, COPD cells expressed full-ILR and AREG at lower levels on average but this did not reach statistical significance (Fig. 3D and F). Spliced-IL6R mRNA expression did not differ between investigated groups either after CS or air exposure (Fig. 3E). These findings suggest that COPD patients may have impaired transcriptional or posttranscriptional responses to inflammatory and tissue regenerative triggers. The apparent contrast with the more pronounced shedding from COPD cells after CS challenge (Fig. 2) suggests that posttranslational mechanisms determine shedding rate, rather than substrate mRNA levels.

ADAM17 is required for CS-induced release of IL6R and AREG in ALI-PBEC

To confirm the previously established involvement of ADAM17 in the shedding process of IL6R and AREG in our model, we used the selective ADAM17 inhibitor TMI-2 (Wyeth) (Zhang et al. 2004). TMI-2 only partially decreased baseline IL6R release at all investigated time points (Fig. 4A), plausibly because release of the product of spliced-IL6R mRNA, which cannot be distinguished from shed IL-6R with the available antibodies, is not sensitive to inhibitors of ADAMs (Vermes et al. 2012). In contrast, TMI-2 significantly decreased baseline AREG shedding at all time points (Fig. 4B). Importantly, CS-induced shedding of IL6R and AREG was significantly inhibited by TMI-2 at all time points after CS exposure, indicating that ADAM17 activity is involved in CS-induced ADAM17 substrate release (Fig. 4).

ADAM17- and ADAM17P-substrate interactions are increased after CS exposure in an intracellular compartment of ALI-PBEC

Next, we explored the interactions of IL6R or AREG with ADAM17 3 h after CS treatment in ALI-PBEC with an in situ proximity ligation assay (PLA) (Fredriksson et al. 2002), using antibodies against ADAM17 phosphorylated at Thr735 (ADAM17-P^{T735}) or total ADAM17. Protein IL6R/AREG-ADAM17 and IL6R/AREG-ADAM17-P^{T735} interactions were visualized as fluorescent red dots in x-y confocal sections (representative confocal pictures shown in Fig. 5A and B). In air-exposed cells, PLA signals were largely confined to the basal region, as in the control incubations, and not significantly higher than background (data not shown), as indicated by red lines in Figure 5C–F (relevant control data are shown in Figure S1). Interestingly, CS exposure significantly increased the total number of PLA signals for interactions of IL6R or AREG with ADAM17 (Fig. 5C and E). We observed that CS strongly enhanced interactions of IL6R or AREG with ADAM17-P^{T735} (Fig. 5D and F), which further extends previous findings showing that ADAM17 is phosphorylated after smoke extract (CSE) exposure in submerged immortalized NCI-H292 cells (Lemjabbar-Alaoui et al. 2011). CS-induced PLA signals of substrate-ADAM17 and substrate-ADAM17-P^{T735} were primarily detected in the apical

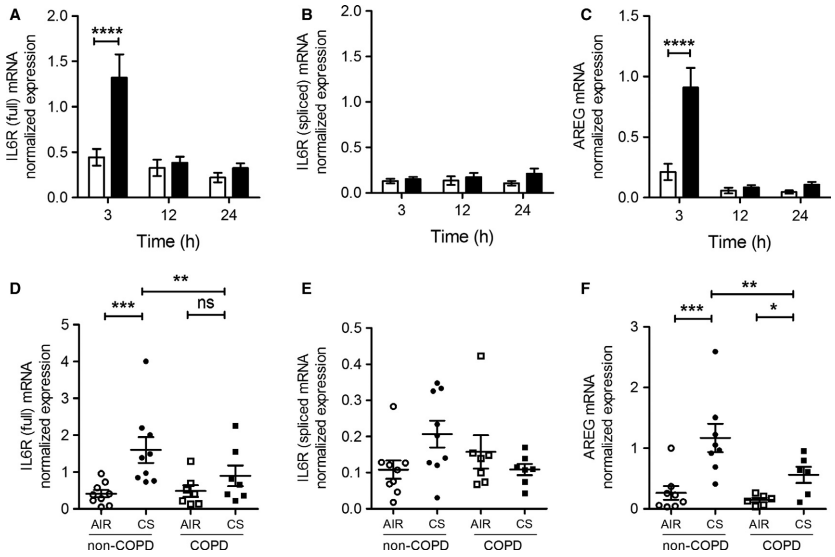

Figure 3. CS exposure transiently enhances IL6R and AREG mRNA expression in COPD and non-COPD ALI-PBEC. mRNA levels of the IL6R full-length variant (full-IL6R) (A), the IL6R splice variant (spliced-IL6R) (B) and AREG (C) were determined by qPCR 3, 12, and 24 h after CS (black bars) or air exposure (open bars) (n = 14 unspecified donors). A transient induction of full-IL6R (A) and AREG (C), but not spliced-IL6R (B) was observed at 3 h after CS exposure. In COPD (n = 7) and non-COPD (n = 8) ALI-PBEC, mRNA of full-IL6R (D), spliced-IL6R (E), and AREG (F) were determined 3 h after CS exposure. mRNA expression of full-IL6R and AREG, was lower on average but not statistically significant in COPD compared to non-COPD donors. Data were normalized for expression against two reference genes (ATP5B and RPL13A). n refers to the number of donors. The response of cells from each donor was analyzed within one experiment using duplicate inserts and data were averaged per donor. Statistical analysis was performed on the averaged data from each donor. Statistical analysis: Two-way ANOVA With Tukey's multiple comparison test.

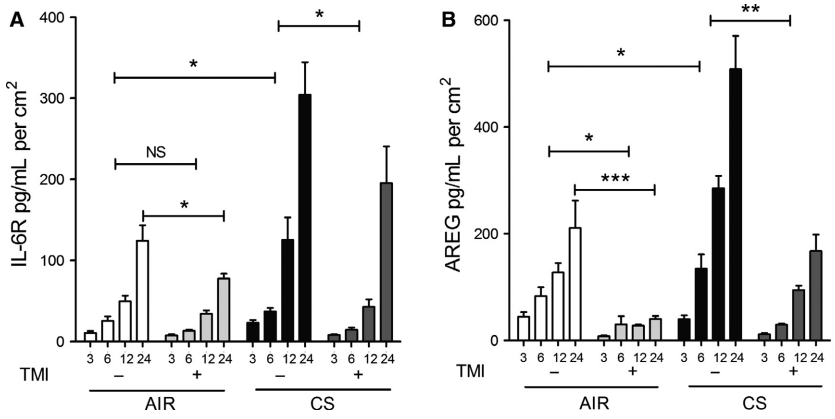

Figure 4. ADAM17 is involved in the release of soluble IL6R and AREG in ALI-PBEC. The selective ADAM17 inhibitor, TMI-2 (Zhang et al. 2004) decreases basal and CS-induced IL6R (A,B) and AREG (C,D) shedding in ALI-PBEC cells (n = 3 COPD donors) at 3, 6 (A,C), 12, and 24 h time points (B,D). n refers to the number of donors. The response of cells from each donor was analyzed within one experiment using duplicate or triplicate inserts and data were averaged per donor. Statistical analysis was performed on the averaged data from each donor, by two-way ANOVA (Bonferroni), confirming first the effect of CS on IL6R and AREG shedding at different time points, and second the effect of TMI-2 on shedding during air and CS exposure.

region of the cells and were not confined to a lateral membrane pattern suggesting an intracellular vesicular localization of protein complexes in ALI-PBEC. These data for the first time demonstrate that CS exposure strongly increases the interaction of ADAM17 and ADAM17-P^{T735} with IL6R or AREG in an intracellular vesicular compartment of ALI-PBEC, suggesting a CS induced effect on protein trafficking.

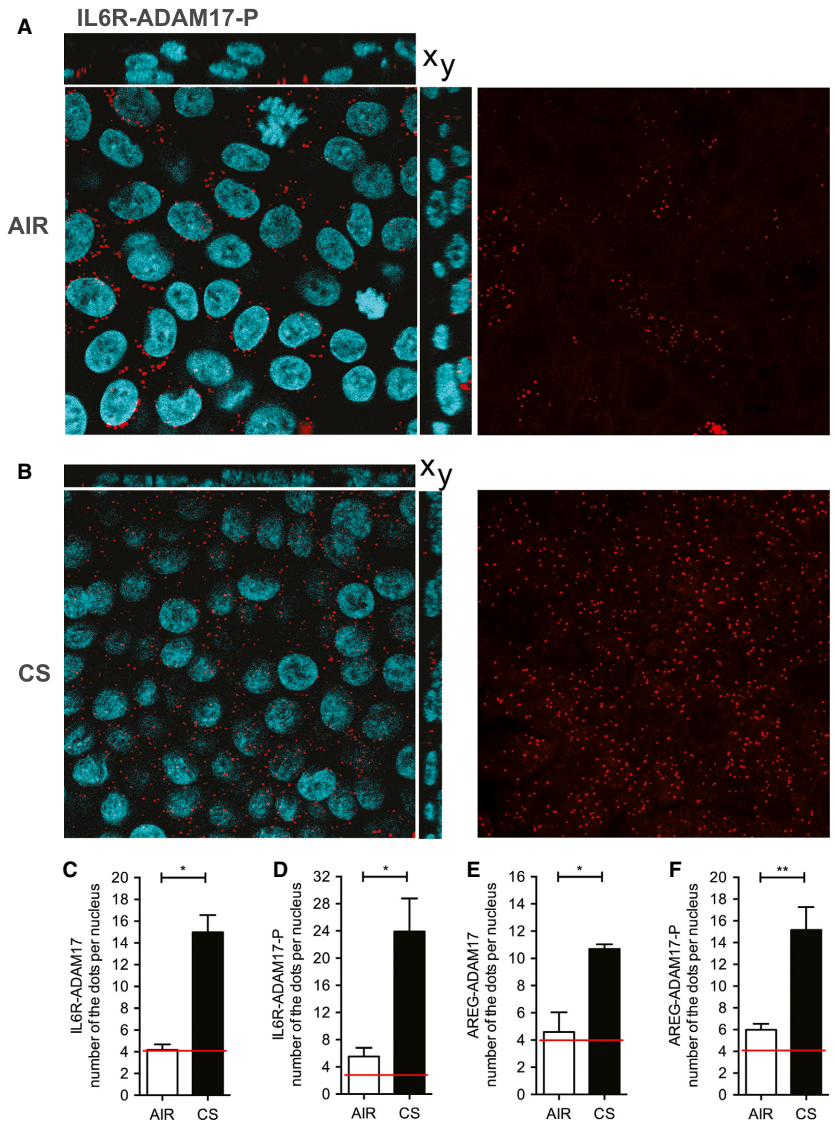

Figure 5. CS increases substrate-ADAM17 and substrate-ADAM17-P^{T735} interactions in pseudostratified COPD ALI-PBEC cells. The proximity ligation assay (PLA) signal in COPD- ALI-PBEC exposed to CS or air was performed for the following interactions: IL6R-ADAM17, IL6R-ADAM17P^{T735}, AREG-ADAM17, and AREG-ADAM17-PT^{735}. Relevant control data are shown in Figure S1. Here, we show a representative figure of IL6R-ADAM17P^{T735} 3 h after air (A) and CS exposure (B). Left panels show merged signals of nuclei (blue) and PLA (red) in the *x*–*y* sections of the confocal *z*-stack and right panel presents PLA signal in the apical region (red dots). The number of PLA interactions was counted for all interactions as described in the methods section and expressed per nucleus 3 h after CS or air exposure in the whole *z*-stack of the ALI-PBEC (C–F). The red lines on the graphs indicate the maximal dot count in the PLA assay controls, in which one of the antibodies for the interaction was omitted (background staining not shown). For each interaction, cells from one donor were analyzed. Different filters (n = 4) were used to show distinct interactions. Statistical analysis: unpaired *t*-test.

EGFR is required for basal and induced AREG shedding in ALI-PBEC

ADAM17-dependent shedding of EGFR ligands such as AREG results in activation of EGFR through an autocrine feedback loop, which modulates basal EGFR activity (DeWitt et al. 2001). This mechanism was shown to be activated by CS extract in submerged cultured PBEC and in cell lines (Lemjabbar et al. 2003). In our experimental set-up, we have previously shown that CS enhances basal EGFR activity by increasing its phosphorylation (Amatngalim et al. 2015). To illustrate the involvement of EGFR in CS-induced ADAM17-related shedding in ALI-PBEC, we assessed sIL6R and AREG shedding after starvation for

growth factors, using medium devoid of EGF and bovine pituitary extract (BPE). Removing these factors from the medium substantially reduced baseline shedding of IL6R and AREG (Fig. 6), when compared to standard culture conditions including EGF and BPE (Fig. 4A and C). Both sIL6R and AREG release were significantly increased at 3 h after CS exposure. The selective ADAM17 inhibitor TMI-2 and the EGFR tyrosine kinase inhibitor (AG1478) added prior to CS exposure, partially inhibited sIL6R shedding, consistent with a substantial contribution of the ADAM-insensitive splice variant sIL-6R levels in the basal medium (Fig. 6A). AG1478 strongly impaired AREG shedding, to a similar extent as TMI-2 (Fig. 6B). These findings together demonstrate a critical role of EGFR activation in ADAM17-mediated basal and CS-induced shedding activity.

EGFR and ADAM17 are required for CS-induced IL6R and AREG mRNA expression

We previously observed that EGFR activation is involved in CS-induced expression of several genes in ALI-PBEC (Amatngalim et al. 2015). The molecular mechanism by which CS activates EGFR are not known. Here, we explored the effect of ADAM17 and EGFR inhibition on CS-induced IL6R and AREG mRNA levels in ALI-PBEC. At 3 hours after CS exposure in the absence of EGF in the medium, both TMI-2 and AG1478 significantly impaired CS-induced expression of full-L6R mRNA (Fig. 7A), but not the splice variant (Fig. 7B). Both inhibitors strongly diminished CS-induced AREG mRNA levels (Fig. 7C) as well as IL-8

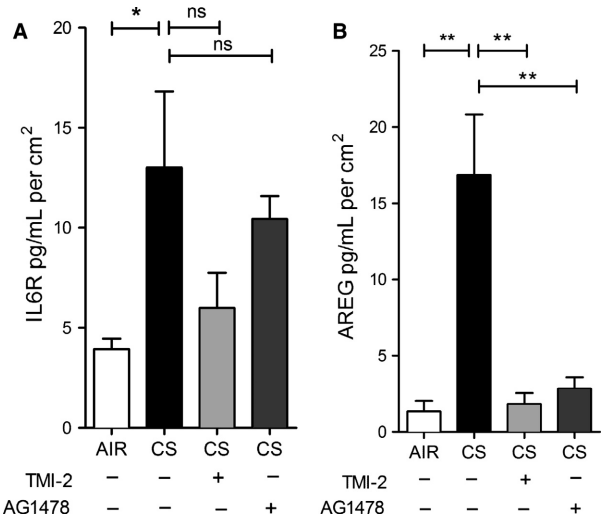

Figure 6. IL6R and AREG shedding depends on ADAM17 and EGFR activity in COPD-ALI-PBEC. COPD ALI-PBEC (n = 3 donors) were starved for growth factors for 48 h prior to CS or air exposure. Three hours after CS exposure, IL6R (A) and AREG (B) shedding were significantly increased compared to air. The ADAM17 inhibitor (1 μmol/L TMI-2) and the EGFR inhibitor (1 μmol/L AG1478) significantly reduced AREG, but IL6R shedding to a lesser extent. The response of cells from each donor was analyzed within one experiment using triplicate inserts and data were averaged per donor. Statistical analysis was performed on the averaged data from each donor by one way ANOVA (Tukey multiple comparison test), only relevant comparisons are shown, air versus CS-treated cells and the effect of inhibitors in CS-treated cells.

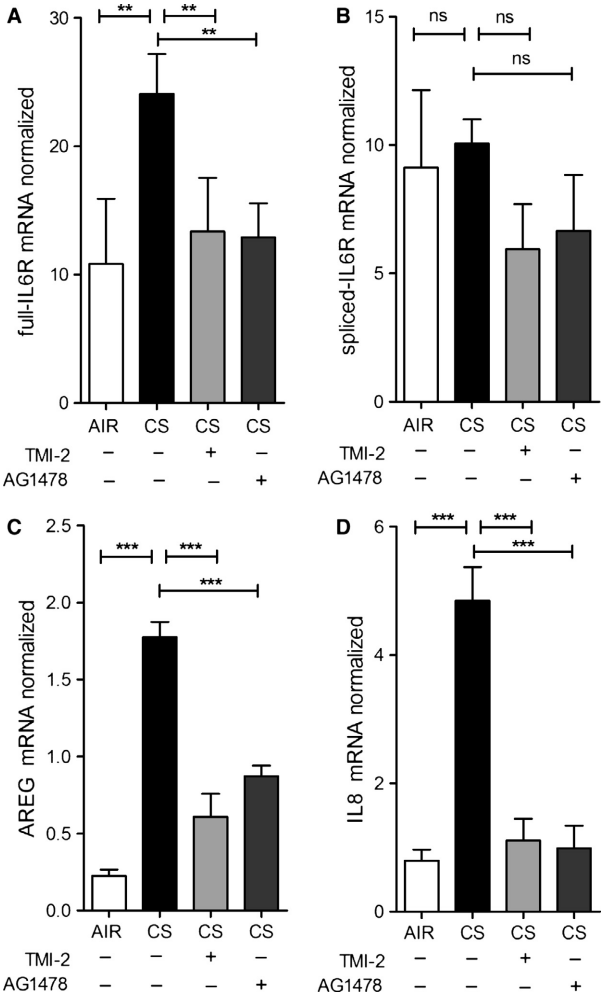

Figure 7. IL6R and AREG mRNA expression are regulated by ADAM17 and EGFR activity in ALI-PBEC. ALI-PBEC (n = 3 donors) were starved for growth factors for 48 h prior to CS exposure. At 3 h, CS-induced mRNA levels of full-length IL6R (A), AREG (C), and IL8 (D) were diminished upon ADAM17 (1 μmol/L TMI-2) and EGFR (1 μmol/L AG1478) inhibition, whereas that of the alternatively spliced form of IL6R was not affected (B). n refers to the number of donors. The response of cells from each donor was analyzed within one experiment using triplicate inserts and data were averaged per donor. Statistical analysis was performed on the averaged data from each donor, by one-way ANOVA (Tukey multiple comparison test), only relevant comparisons are shown, air versus CS-treated cells and the effect of inhibitors in CS-treated cells.

mRNA expression (Fig. 7D). Overall, these findings for the first time demonstrate that ADAM17, next to EGFR, is essential in the CS-induced mechanism regulating not only the mRNA of ADAM17 substrates (IL6R and AREG), but also IL-8 in ALI-PBEC.

Discussion

Many studies have demonstrated that airway epithelial cells are activated by exposure to environmental triggers like cigarette smoke, which contributes to COPD pathology (Rusznak et al. 2000; Schulz et al. 2004; Heijink et al. 2012; Amatngalim et al. 2015). In contrast to most studies, we used fresh whole cigarette smoke instead of (aged) cigarette smoke extract, and ALI-differentiated PBEC from COPD and non-COPD donors instead of submerged cultures of nondifferentiated primary cells or cell lines.

While the obvious advantage of this approach is that we can study well-differentiated primary cells from different patient populations, a limitation is that confirmation of data obtained with experimental pharmaceutics by, for example, gene editing or RNAi technology is not feasible in this context. Aside from efficiency issues and off-target effects in primary cells, knocking down EGFR or ADAM17 likely affects the growth and differentiation of primary bronchial epithelial cells, which essentially defeats our purpose. However, the two inhibitors that we apply here to inhibit EGFR (AG1478) and ADAM17 (TMI-2), respectively are widely used and are known to be highly selective.

Importantly, our data demonstrate for the first time that CS triggered increase of basal shedding of IL6R and AREG into the basal medium, in the presence of EGF in the growth medium providing basal EGFR activity, was more pronounced in ALI-PBEC derived from COPD patients compared to non-COPD controls. We further report the ability of CS to increase mRNA expression of these genes in an EGFR- and ADAM17-dependent way in ALI-PBEC cells, under these conditions, with a lower tendency to induction in the COPD group. These results extend previous studies showing dysregulated responses of COPD airway epithelial cells to cellular stress, and provide novel evidence for the mechanism of CS-induced and COPD-related proinflammatory and profibrotic responses (Fig. 8).

The differential effect of CS on sIL6R and AREG release between COPD and non-COPD ALI-PBEC might be related to differences in epithelial barrier function as previously described (Heijink et al. 2014). Using the current CS exposure system, we have previously shown that CS causes a transient decrease in epithelial barrier function (Amatngalim et al. 2015). However, in contrast to Heijink et al., we did not observe differences between COPD and non-COPD cultures at baseline conditions and upon CS exposure (G.D. Amatngalim et al., unpubl. data), which may be explained by the fact that Heijink et al. focused on severe (GOLD stage IV) COPD. Another explanation might be differences in epithelial cell differentiation, as it has been shown that COPD epithelial cells display a more mesenchymal phenotype due to enhanced autocrine expression of TGF-β1 (Gohy et al. 2015).

As previously shown, the EGFR-ADAM17 pathway is essential for IL-8 release from a bronchial epithelial cell line exposed to particulate air pollution (Ovrevik et al. 2011) and implicated in CS extract-induced expression of the mucin MUC5AC (Shao 2004). Further, autocrine production of EGFR ligands is involved in CS-induced IL-8 release from airway epithelial cells (Richter et al. 2002). Our studies extend these observations by showing the involvement of the ADAM17-EGFR pathway in the release of IL6R and AREG upon CS exposure of differentiated PBEC, both in the presence (Fig. 4), and absence (Fig. 6) of EGFR ligand (EGF) in the basal medium respectively. Notably, the basal shedding rates are considerably lower in cells preincubated in medium lacking EGF, resulting in a much larger ADAM17- and EGFR-dependent stimulation effect of CS (compare Figs 4 and 6). Which of these extreme conditions of basal EGFR activation apply in normal and COPD lungs in situ, and to what extent autocrine feedback signaling through ADAM-dependent EGFR ligand shedding determines EGFR activity (Fig. 8) remains to be established.

Additionally, EGFR and ADAM17 were both essential for CS-induced IL6R and AREG mRNA expression (Fig. 7). These results provide novel insights into the mechanisms of airway epithelial cell activation by cigarette smoke in COPD, and highlight a role of ADAMs and EGFR in this process (Fig. 8).

We further found that CS increases shedding of IL6R and AREG to the basal medium, but not to the apical side (Fig. 1). This is in line with report in polarized Madin-Darby canine kidney cells (MDCK cells) showing that newly synthetized AREG is directly delivered to the basolateral surface with >95% efficiency (Brown et al. 2001). However, this is in contrast to the secretion of the innate immune mediators IL-8 and ribonuclease 7, which were also detected at the apical surface (Amatngalim et al. 2015). A polarized ADAM17-mediated secretion toward underlying tissue may be relevant for lung tissue remodeling through autocrine, paracrine, extracrine (exosomal targeted receptor activation) pathways in COPD (Booth et al. 2007; Zhou et al. 2012). Further examination of this phenomenon in epithelial–mesenchymal co-culture systems is in progress.

Amphiregulin release and phosphorylation of ADAM17 after CS extract treatment in ALI-PBEC has been previously detected by ELISA or western Blotting (Lemjabbar-Alaoui et al. 2011). Our proximity ligation assay (PLA)

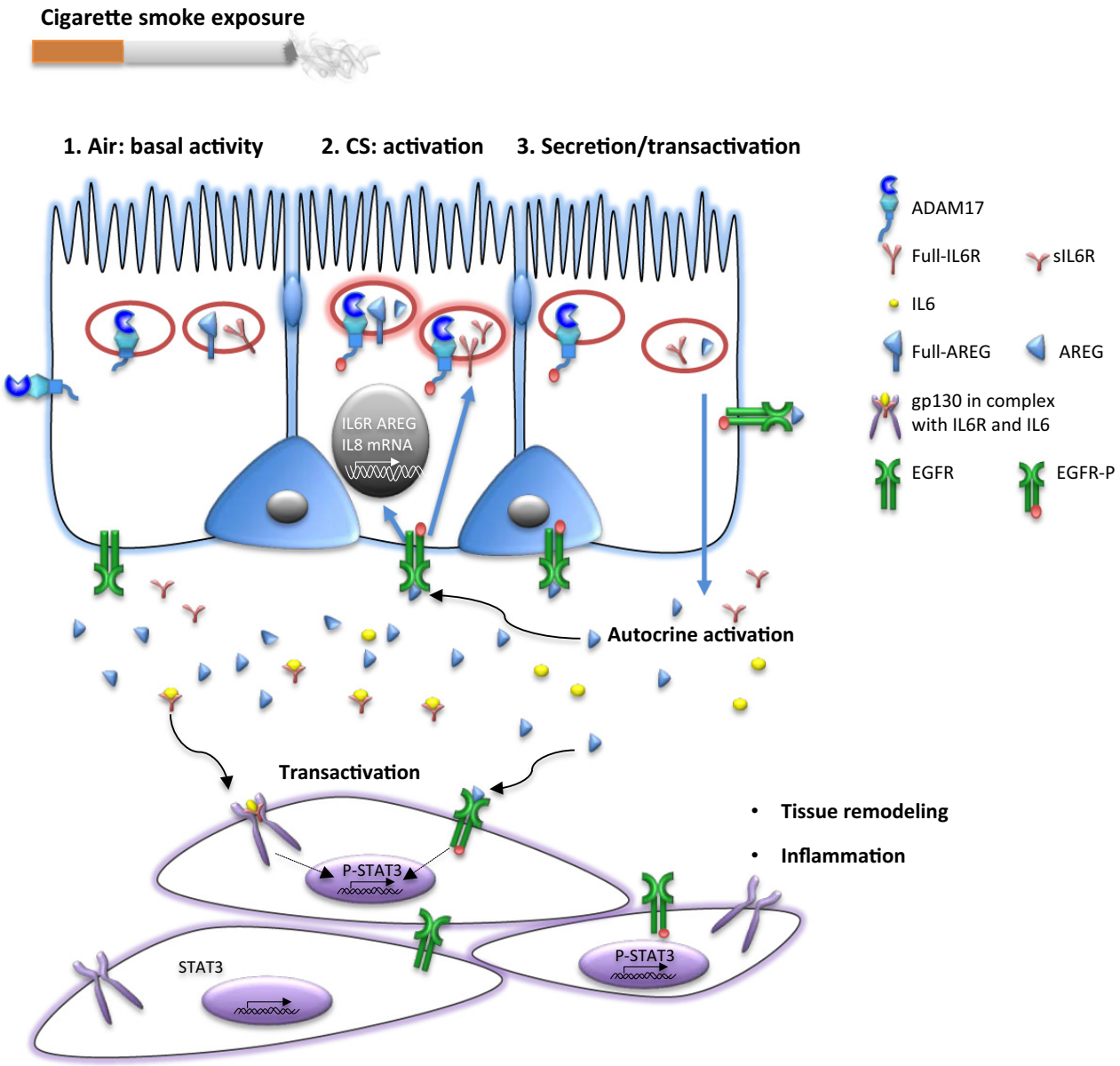

Figure 8. Cigarette smoke exposure activates EGFR-ADAM17 axis in airway epithelial cells. Under basal conditions, there is ADAM17 related AREG and IL6R shedding activity, depending on the level of EGFR activity (compare Figs 4 and 6). Cigarette smoke exposure (CS) initiates an interaction of the phosphorylated form of ADAM17 (ADAM17-P) with the full-length transmembrane forms of IL6R and AREG in an intracellular compartment of the airway epithelial cell (Fig. 5), resulting in proteolysis and subsequent secretion (shedding) of the soluble active domains of IL6R and AREG toward the basolateral compartment. This involves both ADAM17 and EGFR activity (Figs 4 and 6). CS exposure also affects IL6R and AREG gene expression or mRNA stabilization in airway epithelial cells through ADAM17 and EGFR activation. sAREG and sIL6R secreted towards the basolateral compartment may change the level of activity of EGFR and the interleukin receptor IL6st/gp130 on the airway epithelial cells (autocrine). This may contribute to the activity of the EGFR/ADAM17 axis (positive feedback), which is likely kept in check by inactivation of internalized EGFR. Paracrine activity of sAREG and sIL6R may further transactivate EGFR and the interleukin receptor IL6st/gp130 on the underlying myofibroblasts, and myeloid cells, activating downstream pathways, including STAT3, involved in inflammation, collagen deposition, and myofibroblast proliferation.

data show for the first time that CS-induced shedding involves an intracellular interaction between phosphorylated ADAM17 and its substrates (Figs 5 and 8), whereas the majority of the literature suggests that shedding occurs mainly at the plasma membrane surface. This interaction likely takes place in intracellular

membranes that sequester active phosphorylated ADAM17 and its transmembrane substrates upon activation. This process may relate to the transient change in barrier function upon CS treatment in our system and the subsequent activation of EGFR (Amatngalim et al. 2015). Our observation is supported by other reports showing the presence ADAM17 or its substrates in a vesicular compartment in lysosomes (Ebsen et al. 2015), endosomes (Gephart et al. 2011; Dombernowsky et al. 2015), and exosomes negative for the ER marker calreticulin (Higginbotham et al. 2011). Moreover, Gutwein et al. demonstrated that ADAM10-mediated L1 migration factor cleavage occurs in Golgi-derived vesicles in tumor cells (Gutwein et al. 2002). This was further supported by a recent paper suggesting that also ADAM10/ADAM17-mediated release of soluble FasL occurs from an intracellular vesicular pool of secretory lysosomes in stimulated T lymphocytes (Ebsen et al. 2015).

Moreover, after ligand binding, EGFR traffics in endosomes from the plasma membrane to an intracellular compartment to continue its signaling (Vieira et al. 1996; Teis et al. 2006). EGF-dependent MAPK signaling occurs from late endosomes and lysosomes (de Araujo et al. 2013). Interestingly, the MAPK/ERK pathway regulates trafficking of ADAM17 phosphorylated at Thr735 from the endoplasmic reticulum toward the plasma membrane (Soond 2005; Hilliard et al. 2011), which can be also activated through ligand binding to EGFR. Higginbotham et al. showed that AREG containing exosomes are rapidly internalized by recipient cells in an EGFR-dependent manner (Higginbotham et al. 2011), enhancing invasion of LM2-4175 cells through Matrigel and wound healing. In our ALI-PBEC system, we observed a predominantly lateral localization of EGFR under basal culture conditions. After exposure to CS, we observed a more cytoplasmic localization, consistent with EGFR activation (Figure S2). Therefore, in line with these and published observations, our findings suggest that in HBEC-ALI, CS triggers EGFR-mediated trafficking of ADAM17 and its substrates to a common subcellular compartment to allow proteolysis and subsequent secretion of soluble products (Fig. 8). At this time, we cannot establish to what extent autocrine signaling through shed ADAM substrates determine this response, or whether alternative mechanisms such as transactivation by intracellular kinases or oxidation or the extracellular receptor domain plays a role. Additional studies of triggered trafficking of EGFR, ADAM17-P, and its substrates in polarized airway cells are required to further establish this mechanism.

In addition to CS-enhanced release through ADAM17 enzymatic activity, we observed transiently enhanced mRNA expression of both AREG and full-IL6R in ALI-PBEC upon CS exposure (Figure 3). CS did not affect the level of the alternatively spliced form of IL6R, so we conclude that alternative splicing unlikely contributes to CS-enhanced release of IL6R. Previously, we observed upregulated IL-8 mRNA expression in ALI-PBEC exposed to CS as a result of enhanced EGFR phosphorylation and activation of the downstream MAPK/ERK1/2 signaling pathway (Amatngalim et al. 2015). Here, we report that CS-induced IL6R and AREG mRNA expression was also reduced upon EGFR inhibition. In addition, we show here for the first time that inhibition of ADAM17 has the same effect on these mRNA levels (Fig. 7). Therefore, our data suggest that CS enhances factors common for activation of IL6R, AREG, and IL-8 mRNA expression likely via an autocrine ADAM17-EGFR axis (Fig. 8).

The transcriptional and posttranscriptional regulation of these genes upon inhaled toxic substances has not been fully elucidated. Induced EGFR signaling is able to activate transcription of target genes. In addition, it has been shown that CS extract enhances HuR-mediated IL-8 mRNA stability in airway epithelial cells (Hudy and Proud 2013). Moreover, UV-exposure of keratinocytes enhances mRNA HuR-mediated stability of AREG, in an EGFR-dependent manner (Nakayama et al. 2013). These observations suggest that CS-induced activation of EGFR enhances sIL6R, AREG, and IL8 mRNA stability in ALI-PBEC. Furthermore, mRNA regulation may be altered in cultured airway epithelial cells from COPD patients (Steiling et al. 2013). CS-treated COPD ALI-PBEC expressed lower AREG and IL6R mRNA levels on average compared to non-COPD controls, but this difference was not statistically different (Fig. 3). Further studies on mRNA stability in this system are required to establish this. Nevertheless, this observation contrasts with the shedding data (Fig. 2) and suggests that ADAM17-dependent AREG and sIL6R output is not primarily regulated on the mRNA level, but involves posttranslational regulation.

Our data support the relevance of the ADAM17/EGFR pathway in COPD development and progression. Selective inhibitors of ADAM17, EGFR, and other components of this signaling pathway such as JAK and MAPK potentially expand therapeutic possibilities. The development of ADAM inhibitors for clinical use has been studied intensively (Moss et al. 2008; Duffy et al. 2011; Dreymueller et al. 2015). In cellular and animal tumor models, positive results were recorded (Witters et al. 2008). An ADAM17 inhibitor, TAPI-0, reduced bleomycin-induced lung inflammation (Lee et al. 2012). The selective inhibitor TMI-2 used in this study, reduced LPS-induced inflammation in vivo (Zhang et al. 2004). However, due to a lack of target specificity of available compounds, and side effects associated with the various other biological functions of

ADAMs, chronic and systemic application of these compounds in humans is so far prohibited (Arribas and Esselens 2009). Clearly, more advanced intervention tools are required. Our data offer new insights in the regulation of mRNA expression, secretion, and release of ADAM17 substrates in airway epithelial cells upon triggering, which in combination with state of the art molecular design and advanced organotypic cellular modeling of airways could allow development of more selective inhibitors, targeted to specific cells and subcellular domains.

In summary, this study provides evidence that ADAM17-mediated release and shedding of IL-6R and AREG is highly enhanced in airway epithelial cells in response to CS-induced injury. Next to ADAM17, we highlight the importance of EGFR in the regulation of IL6R and AREG release and mRNA expression. Moreover, CS-induced ADAM17-mediated shedding of IL6R and AREG is especially high in COPD ALI-PBEC, suggesting that reducing ADAM17 activity in COPD might be a potential therapeutic approach.

Acknowledgements

The ADAM inhibitors TMI-1 and TMI-2 were kindly provided by Wyeth Research, Cambridge, MA 02140, USA. The authors thank the Department of Thoracic Surgery at LUMC for collection of lung tissue, and Winifred Broekman and Jorn Nützinger (LUMC) for their help in collecting patient data.

Conflict of Interest

Dr. Hiemstra reports receiving research grants from Galapagos NV for the submitted work, and research grants from Boehringer Ingelheim and Grifols outside the submitted work. Dr Scholte reports a research grant from Lexicon inc not related to the submitted work.

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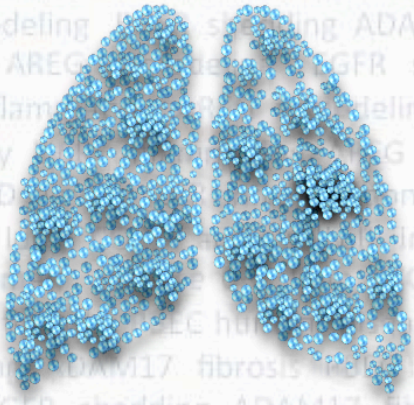
CHAPTER 3



Lack of CFTR function in polarized bronchial epithelial cells results in increased extracellular redox potential leading to augmented ADAM17 and EGFR activity and enhanced AREG release

(submitted)

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Abstract

A disintegrin and metalloprotease 17 (ADAM17) activity is involved in chronic inflammation and tissue remodeling. Since these are hallmarks of CF lung disease we aimed to establish whether CFTR deficiency affects ADAM17 in polarized CFBE41o- cells lacking functional CFTR (iCFTR-) compared to cells with induced CFTR expression (iCFTR+). We found enhanced ADAM17-mediated shedding of an EGFR ligand, amphiregulin (AREG) in iCFTR- compared to iCFTR+ cells. This difference was observed at air-liquid interface, but not in submerged culture. Expression of the G551D mutant form of CFTR does not have this effect. As previously reported, ADAM17-mediated AREG shedding is tightly regulated by tyrosine kinases and phosphatases, particularly by EGFR/MAPK and SRC. iCFTR- cells displayed enhanced apical presentation of the basolateral marker EGFR compared to iCFTR+, which suggests its abnormal trafficking in CFTR deficient cells. Furthermore, AREG shedding is controlled by extracellular redox potentials, since the non-permeant natural antioxidant glutathione (GSH), but not its permeant precursor GSH ester, significantly inhibits AREG release. The apical extracellular redox state measured with a fluorescent ROS probe (Grx1-roGFP-GPI) indicated enhanced oxidation of the apical surface fluid (ASL) in iCFTR- compared to iCFTR+ cells. In contrast, an intracellular fluorescent ROS probe (Grx1-roGFP) did not reveal a difference between iCFTR- and iCFTR+ cells. In summary our data suggest that in iCFTR- cells a hyper-oxidized state of the extracellular membrane, likely caused by defective GSH secretion, results in enhanced activity of the EGFR/ADAM17 signaling axis. This may contribute to the development of CF lung pathology, which should be investigated in future studies.

INTRODUCTION

More than 2000 different mutations in the cystic fibrosis transmembrane conductance regulator gene (*CFTR)* have been identified. Most of these mutations impair the function of the CFTR protein (68) (76), an anion channel involved in fluid homeostasis in multiple secretory epithelia (19) (46), leading to a recessive inherited disease Cystic Fibrosis (CF). The morbidity and mortality of CF patients is mainly due to chronic lung disease characterized by mucus plugging, chronic inflammation, recurrent bacterial infections, and tissue remodeling (11).

So far, the results of gene therapy and small molecule therapies aimed at correcting the activity of mutant CFTR are promising, but not sufficiently effective to correct CFTR function to the WT level in CF patients (16). Therefore, additional therapeutic strategies targeting downstream effects of CFTR deficiency are required to prevent or reduce loss of lung function, preferably as early intervention (44). This requires in depth knowledge of the relationship between the primary functional CFTR defect and disease pathology. In the present study we focused on the effect of CFTR dysfunction on airway epithelial pro-inflammatory and pro-fibrotic signaling through the EGFR/ADAM17 axis.

The transmembrane protease A Disintegrin And Metalloprotease 17 (ADAM17; formerly known as TACE) is likely involved in CF lung disease (17). ADAM17 function is closely linked to Epithelial Growth Factor Receptor (EGFR) activity. An activated ADAM17 releases a soluble EGFR binding domain of transmembrane growth factors and cytokines into the extracellular space in a process called shedding. This allows both autocrine signaling towards the epithelial cells and paracrine epithelial-mesenchymal trans-signaling (6) (8) (61). Inhibition of either ADAM17 or EGFR affect ADAM17-mediated release of EGFR ligands, suggesting that the EGFR/ADAM17 axis is established by a positive feedback loop, likely involving the MAPK pathway (5) (35) (61) (66) (64) (79).

In the lungs, the ADAM17/EGFR pathway is activated during inflammation and bacterial infections (9). EGFR and ADAM17 activities are involved in cytokine (e.g. IL-8) and growth factor (e.g. TGFα) release and in regulation of their mRNA expression levels (2) (51) (69). Among other substrates, ADAM17 sheds most of the epidermal growth factor receptor (EGFR) ligands (64), including amphiregulin (AREG). AREG autocrine signaling affects mucus expression (75) (21) and cytokine secretion (12), whereas its paracrine signaling has been linked to airway smooth muscle cell proliferation (15) and TGFβ induced fibrosis characterized by fibroblast proliferation, enhanced α-smooth muscle actin and collagen expression (88).

ADAM17/EGFR signaling is also involved in regulation of mucus production. Its hyperactivity leads to mucus plugging of distal airways, as frequently observed in CFTR deficient subjects (71) (85) (87). Importantly, the hyperactivity may also contribute not only to excessive inflammation and mucus secretion, but due to paracrine signaling also to lung tissue remodeling in CF lung disease. EGFR/ADAM17 activity regulates tissue repair by increasing epithelial proliferation and differentiation (8) (10) (40) (53). Conversely, inhibition of either AREG or EGFR diminishes proliferation of TGF-β-stimulated lung fibroblasts, expression of α-

smooth muscle actin and collagen (88) suggesting that hyperactivation of ADAM17/AREG/EGFR signaling may contribute to pulmonary fibrosis.

Altered epithelial EGFR expression and signaling have been observed in CF lung disease (55) (34), but its cross-talk with ADAM17 has not been studied in detail. We recently found that cigarette smoke (CS) leads to a more pronounced increase in EGFR dependent ADAM17 activity in differentiated primary epithelial cells from COPD patients in comparison to non-COPD controls, suggesting that chronic inflammation changes the EGFR/ADAM signaling axis (69). An inhibitory effect of CS on CFTR activity has recently been reported (13) (67), which raises the possibility that lack of functional CFTR is a contributing factor to the increased EGFR/ADAM17 signaling.

The EGFR/ADAM17 signaling pathway is sensitive to oxidative stress (ROS), which activates the PKC and MAPK pathways (52). ROS is also a direct regulator of both ADAM17 and EGFR activity by oxidizing or rearranging their cysteine residues (18) (81) (31). In CF bronchi proper oxidant/antioxidant homeostasis (25) is disrupted and increased concentration of reactive oxygen species (ROS), lower levels of glutathione (GSH) and reduced nitric oxide (NO) have been reported (25) (89). This abnormal redox signaling is possibly linked to impaired proteostasis and autophagy in CF epithelial cells (45) (74), and may enhance EGFR/ADAM17 responses in CFTR deficient airway epithelial cells.
To test this hypothesis we have investigated the ADAM17 activity in a CF cell model of airway epithelium that allows inducible expression of CFTR (77). We observed that CFTR deficiency in airway epithelial cells enhances ADAM17 dependent growth factor shedding. This activity is regulated by EGFR, MAPK and SRC. Ratiometric measurements of redox potentials show that the enhanced ADAM17/EGFR responses in CFTR deficient cells are not related to a substantial change in intracellular redox potential, but can instead be attributed to an increased oxidation state of the extracellular fluid. Oxidation of the apical lining fluid in CFTR deficient condition may be caused by defective glutathione transport (25) (38) (89). Overall, our study provides novel insights in the involvement of the EGFR/ADAM17 axis in CF pathology and suggests that intervention in this pathway may be beneficial for CF patients.

RESULTS

Absence of CFTR expression enhances AREG release in human CF bronchial epithelial cells.

We used the immortalized CF bronchial epithelial cell line CFBE41o- (CFBE) to study the effect of functional CFTR expression on the release of amphiregulin (AREG), an autocrine and paracrine factor for EGFR activation that is involved in lung morphogenesis, tissue repair following infection or injury, and the resolution of inflammation (83). CFBE cells have a CFTR F508del/F508del genotype with no detectable endogenous F508del CFTR protein expression (20). These cells were engineered to express WT-CFTR under control of a doxycycline responsive transactivator to a level that is comparable with the endogenous expression in Calu-3 (77). Apical expression and function of CFTR was confirmed by immunostaining and short-circuit current (I_{sc}) measurement, respectively (77). CFBE cells were seeded onto permeable filter supports, allowed to differentiate for 4 days post-confluence under liquid-liquid culture (LLC) conditions in presence (iCFTR+) or absence

(iCFTR-) of induced CFTR expression, followed by a shift to air-liquid interface (ALI) to mimic the native environment of respiratory epithelia. iCFTR+ and iCFTR- ALI filters do not differ with respect to protein content or total metabolic activity (Scholte, Veit unpublished data). To examine the release of AREG at the apical and basolateral plasma membrane, polarized CFBE cells were cultured at ALI for 24 h followed by medium change and re-establishment of LLC conditions for 3 h, medium collection and ELISA. The AREG release at the basolateral membrane was four times higher in comparison to the apical release, which, considering three times larger area of the basolateral in comparison to the apical membrane, suggests little polarity of AREG secretion in iCFTR cells (Fig. 1A).

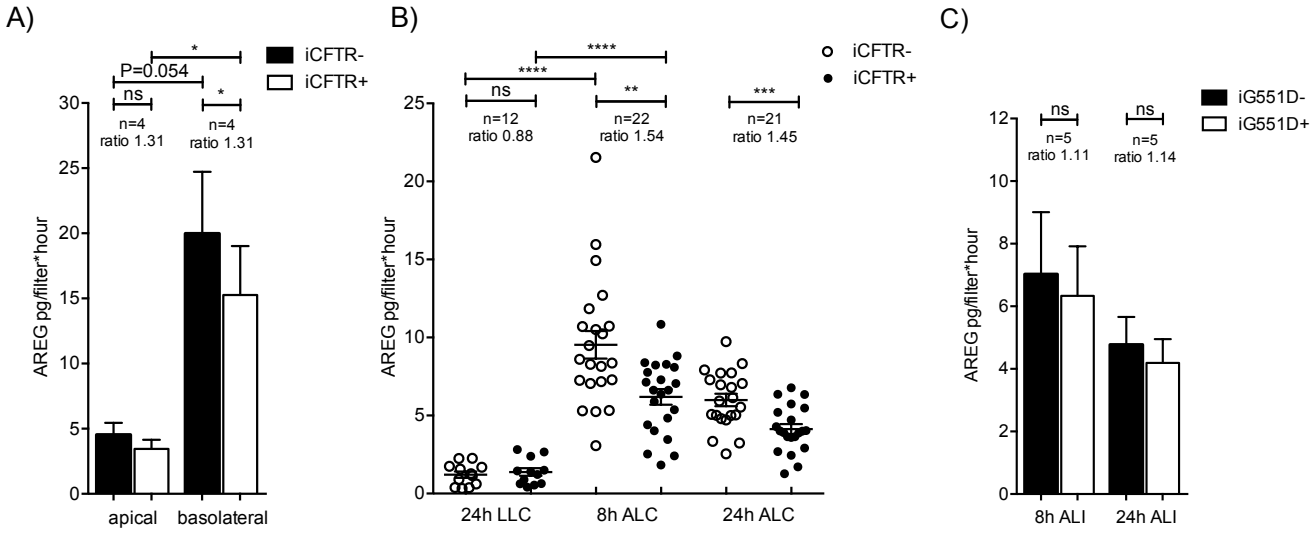

Figure 1 AREG shedding is enhanced in CFTR deficient CFBE. (A) AREG release is mainly towards the basolateral compartment. The AREG release to basolateral and apical compartment was examined (ELISA) in 24h ALI polarized iCFBE+ and iCFBE- cells followed by medium change and re-establishment of LLC conditions for 3 h. The AREG release at the basolateral plasma membrane (PM) was 3-4 times higher in comparison to the apical release. However, considering the larger area of the basolateral cellular membrane in comparison to the apical (approximately 3 times), it suggests modest basolateral polarity of AREG secretion in iCFTR cells. Statistical analysis: paired t-test. Values represent means of n independent experiments performed in triplicate (three filters in parallel within one experiment). (B) Functional CFTR expression decreases AREG release in CFBE cells. Constitutive basolateral AREG release from under LLC conditions and from 4-8h and 8-24h ALI differentiated CFBE41o- cells with (iCFTR+) and without (iCFTR-) induced CFTR expression. The low basolateral AREG release under LCC was increased upon transfer to ALI conditions. After ALI exposure, during the time span 4-8h induction of CFTR expression partially attenuated AREG release, which was maintained in subsequent 8-24h medium collections. (C) Expression of the mutant G551D-CFTR in CFBE41o- cells (iG551D+) did not significantly reduce AREG release in comparison to CFBE41o- cells not expressing G551D-CFTR (iG551D-).

AREG levels were determined by ELISA and expressed as pg AREG per filter produced per hour. Data were collected in parallel for iCFTR- and CFTR+ in triplicate (three different filters per experiment), averaged and presented as a single value in a figure. Horizontal bars indicate averages of averaged triplicates of n separate experiments with SEM values. Statistical analysis: unpaired t-test.

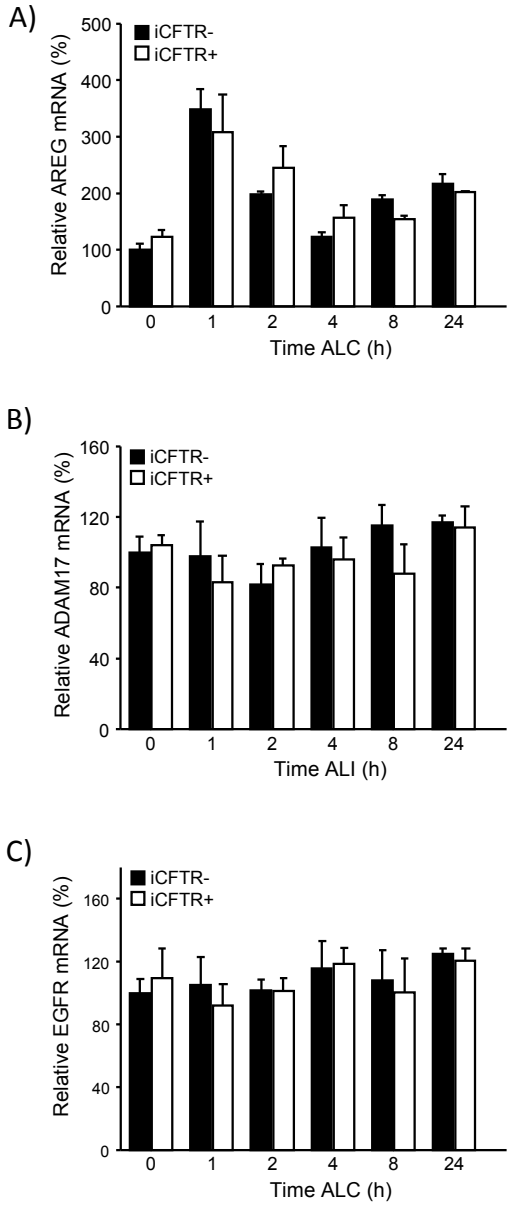

Figure 2 CFTR expression does not alter the mRNA levels of AREG, ADAM17 or EGFR. The mRNA levels of AREG (A), ADAM17 (B) and EGFR (C) were determined by qPCR in CFBE differentiated for 5 days on filter supports under LLC and shifted to ALI for the indicated times. Data are means ± SEM of three independent experiments.

The low constitutive AREG release under LLC conditions (~2 pg/filter*hour) was increased by four to five fold upon transfer to ALI conditions (Fig. 1B). This was associated with a transient increase in AREG mRNA expression (Fig. 2A). The increase in AREG release was attenuated by induction of CFTR expression, which reduced the AREG secretion rate by ~35% from ~9 to 6 pg/filter*hour in the basolateral medium (Fig. 1B). The CFTR suppressive effect was maintained in subsequent medium collections (8-24h) under ALI, however it was absent in epithelia under LLC (Fig. 1B). Expression of the mutant G551D-CFTR, which has a comparable biosynthetic processing and stability to the WT CFTR, but functionally is severely impaired (77) (3), did not significantly reduce AREG release (Fig. 1C). Furthermore, this result excludes a direct influence of doxycycline on AREG shedding activity in this system. Similarly, removing doxycycline from the medium for 24h before the experiment did not affect shedding activity (n=1 in triplicate, data not shown). Also, doxycycline did not affect the relative expression levels of AREG in these cells (Fig. 2A). These data indicate that constitutive WT CFTR activity is necessary and sufficient to limit the excess secretion of AREG in CF epithelia (Fig. 1B).

The increased AREG release of CFBE epithelia is mediated by ADAM17

The increased release of AREG in iCFTR- compared to iCFTR+ CFBE cells (Fig. 1B) was not reflected by a difference in AREG mRNA levels (Fig. 2A), suggesting that the CFTR may influence the shedding, rather than the expression of AREG. AREG is expressed as a type-I transmembrane protein (pro-AREG) that is predominantly directed to the basolateral membrane by a cytosolic sorting motif, where it undergoes constitutive internalization and recycling (28). The ectodomain shedding of pro-AREG is mediated by the metalloproteinase

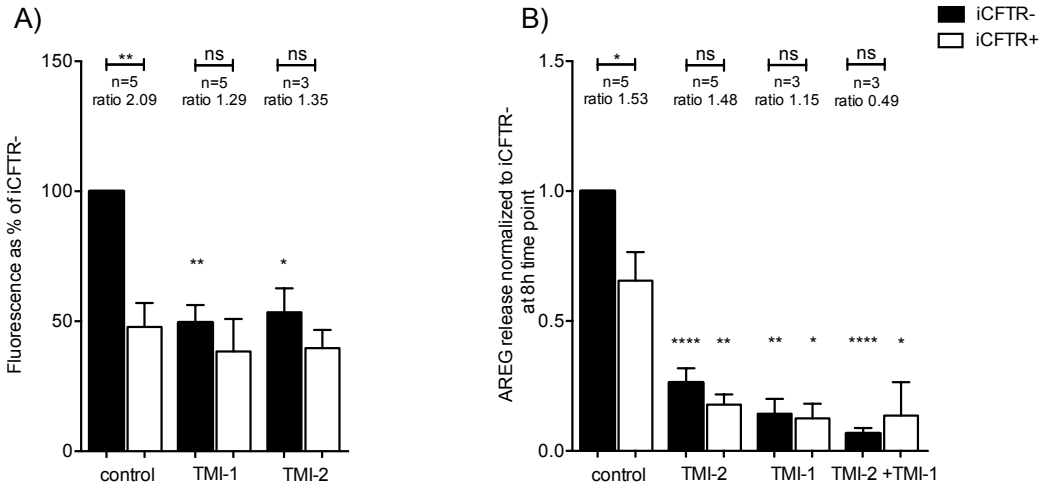

Figure 3 Increased CFTR-dependent basolateral AREG release is mediated by ADAM17
(A) Differentiated CFBE were shifted to ALI for 24 h followed by addition of the fluorogenic ADAM17 substrate (10 µM, 1h) to the basolateral medium in presence or absence of TMI1 (2 µM, n = 3), TMI-2(1 µM, n = 5) or an equivalent amount of carrier (DMSO, n =5). Data are means ± SEM of averaged independent experiments normalized to iCFTR- (100%), statistical analysis was performed by one way ANOVA. (B) 8h treatment with the selective ADAM17 inhibitor, TMI-2 (1µM, Wyeth) decreased AREG release to the same extend as treatment with an inhibitor targetting a broad spectrum of metalloproteases, TMI-1 (2µM, Wyeth), providing the involvement of ADAM17 in AREG shedding in 24h ALI differentiated iCFTR+ and iCFTR- CFBE41o- cells.

ELISA data were normalized to iCFTR- condition. Values represent means of n independent experiments in triplicates (three different filters per experiment). Statistical analysis: unpaired t-test. Statistic of differences between iCFTR+ and iCFTR- indicated at the top of the graph, differences between control condition and treatment are indicated just above the bars.

ADAM17 (60) and occurs mainly intracellularly in differentiated airway epithelial cells, likely in endosomal compartments (69).

To measure the ADAM17 activity in polarized CFBE monolayers under ALI conditions, a fluorogenic ADAM17 substrate was added to the basolateral medium for 2 h. In the absence of CFTR expression significantly higher amounts of substrate were cleaved (Fig. 3A). The highly specific ADAM17 inhibitor TMI-2 (1µM) (86) reduced production of the fluorescent product to a similar extent as a broad specificity ADAM/MMP inhibitor (TMI-1) (2µM) confirming the involvement of ADAM17 in this activity (Fig 3A). Since CFTR expression or culture conditions did not alter the ADAM17 mRNA levels (Fig. 2B), these results suggest that under ALI conditions active CFTR decreases the ADAM17 enzyme activity. Consistent with this observation and similar to the fluorogenic ADAM17 substrate release, inhibition of the ADAM17 activity with the highly specific inhibitor TMI-2 (1µM) (86) also reduced AREG release to a similar extent as a broad specificity ADAM/MMP inhibitor (TMI-1) (2µM), confirming the involvement of ADAM17 in this activity (Fig 3B) (85). These data suggest that, as in other epithelial cell lines (9) and primary human bronchial epithelia (69), ADAM17 is the main shedding protease involved in AREG release in CFBE cells. Moreover, inhibition of ADAM17 reduced the CFTR-dependent differences in proteolysis of a fluorescent substrate (Fig 3A) and in AREG release (Fig 3B), suggesting that CFTR deficiency increases ADAM17 dependent shedding activity.

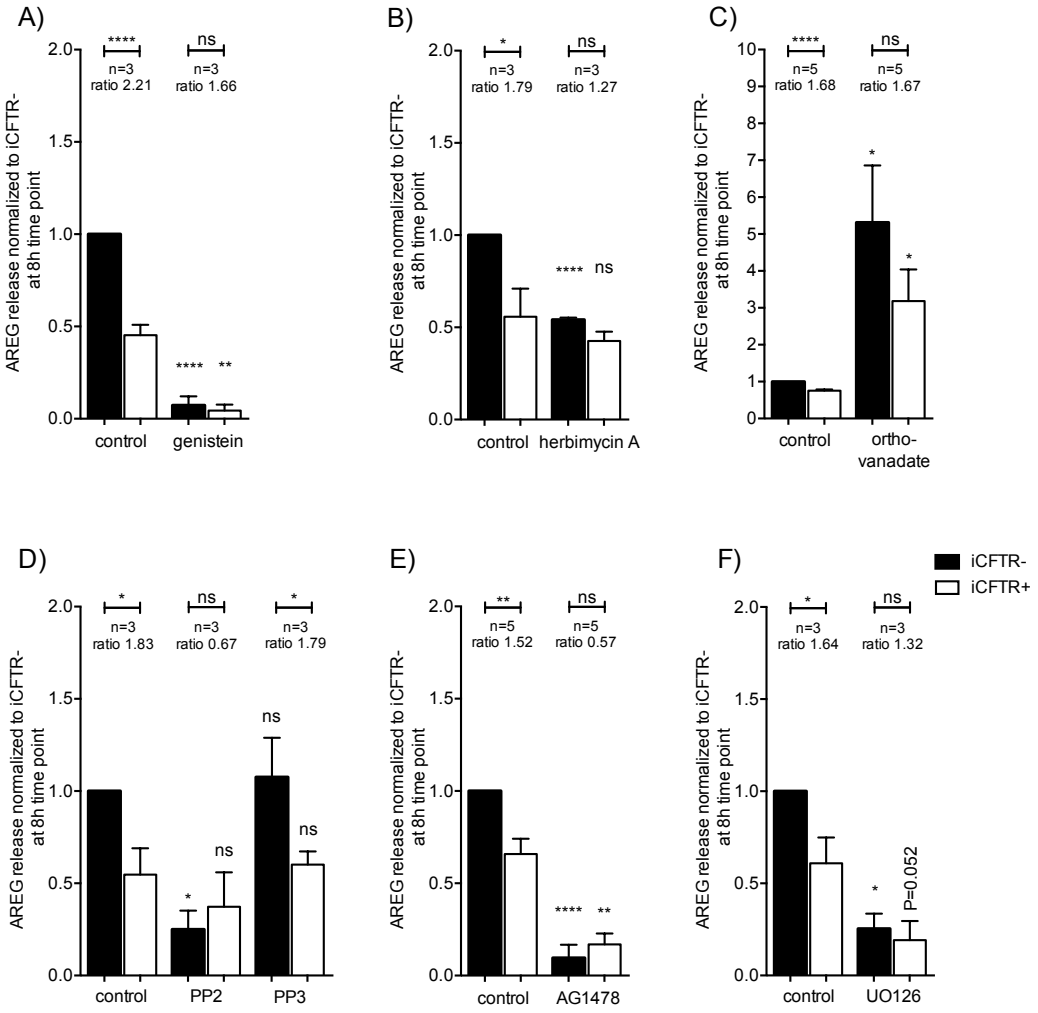

Figure 4 Basolateral AREG shedding is tightly regulated by protein phosphatases and kinases, in particular EGFR/MAPK

(A) The broad range tyrosine kinase inhibitor genistein (100 µM) strongly reduces the AREG shedding in iCFBE41o- cells. (B) Herbimycin A (1 µM), as a consequence of Hsp90 inhibition destabilizes multiple kinases and results in decreased AREG release, particularly in iCFTR- CFBE41o- cells. (C) Conversely, the tyrosine phosphatase inhibitor sodium orthovanadate (25 µM) induces AREG shedding, also in particular in iCFTR- CFBE41o- cells. (D) SRC kinase family inhibitor, PP2 (10 µM) decreased AREG shedding, while the inactive analog PP3 had no inhibitory effect (10 µM) (E) The canonical inhibitor of EGFR AG1478 (1µM) strongly inhibits AREG release, to the similar extent as selective ADAM17 inhibitor TMI-2 presented in Figure 2 (F) Inhibition of downstream MAP kinase MEK1/2 with U0126 inhibitor (10 µM) also reduces AREG shedding. Reduction of AREG shedding upon inhibition of EGFR/MAPK pathway proofs the existence of positive feedback loop in EGFR/ADAM17 axis.

ELISA data were normalized to iCFTR- condition. Values represent means of n independent experiments in triplicates (three different filters per experiment). Statistical analysis: unpaired t-test. Statistic of differences between iCFTR+ and iCFTR- indicated at the top of the graph, differences between control condition and treatment are indicated just above the bars.

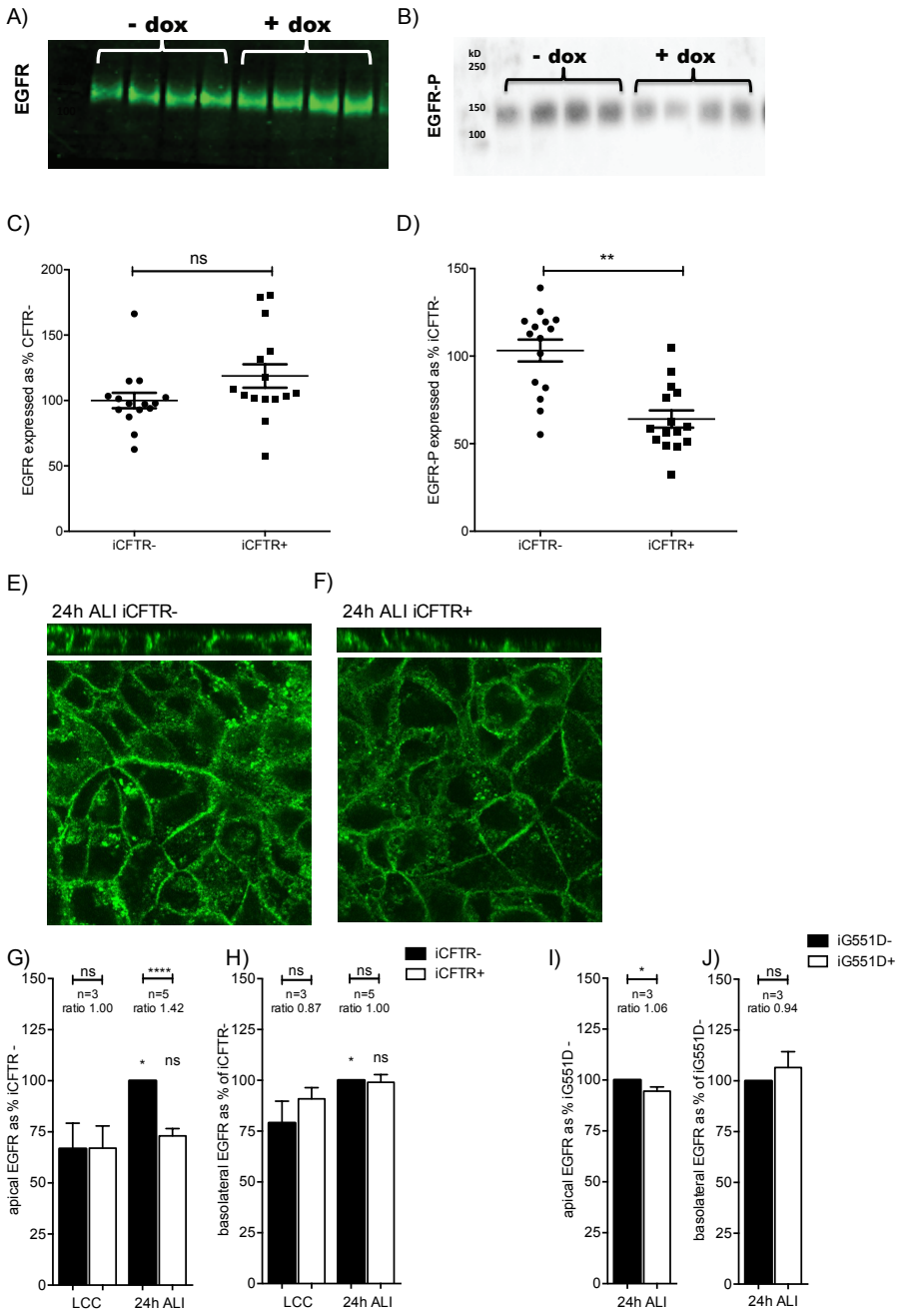

Figure 5

CFTR deficiency increases apical localization and phosphorylation of EGFR in CFBE41o- cells cultured at ALI

(A-D) Western blot analysis. In four separate independent experiments iCFTR- and CFTR+ cells were grown in quadruplicate for 8h at ALI and analyzed (see Material and Method section for details) (A) EGFR (green fluorescence) and (B) EGFR-P (luminescence) show representative results of blots from a single experiment. Quantitative analysis of EGFR (C) and EGFR-P (D) of all data, normalized to the average percentage for iCFTR- cells per experiment show no significant effect of CFTR expression on total EGFR expression, but a significant reduction of EGFR phosphorylation. (E,F) Laser confocal fluorescence microscopy pictures of EGFR immunostaining in 24h ALI exposed CFTR- and CFTR+ iCFBE41o- cells showed that EGFR is predominantly, but not exclusively localized to lateral membrane. Particularly in iCFTR- CFBE41o- cells apical EGFR staining is localized to the apical membrane. (G) EGFR apical plasma membrane density (PMD, cell surface labeling ELISA, see Material and Methods) is higher in iCFTR- 24h ALI CFBE41o- cells than in iCFTR+, and this difference is not present in LCC cells (H) EGFR basolateral PMD in CFBE41o- cells does not show differences in EGFR expression between iCFTR- and iCFTR+ CFBE41o- cells neither under ALI nor LCC condition. (I) EGFR apical and (J) basolateral PMD did not show the differences in apical and basolateral EGFR expression in iG551D- and iG551D+ CFBE41o- cells (n=number of independent experiments, in triplicate each). Statistical analysis: unpaired t-test.

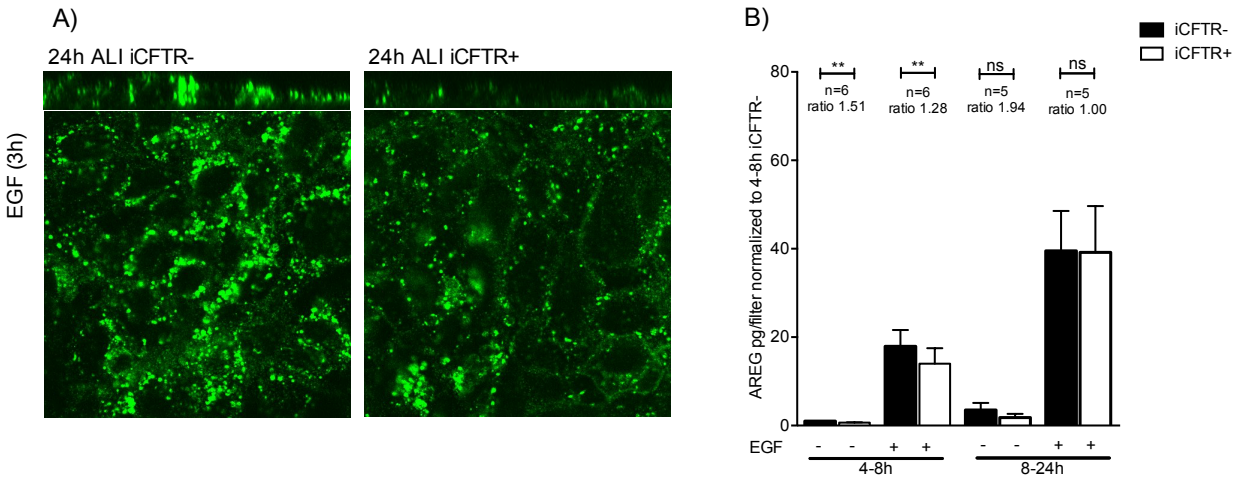

Figure 6
EGF stimulation causes EGFR internalization and AREG shedding
(A) EGFR aggregates 3h after EGF (10ng/ml) stimulation in 24h ALI exposed CFBE41o- cells (untreated control: Fig. 5A), (B) This is accompanied by strong induced AREG shedding at the 8h and 24h time points (nearly 20 and 40-fold respectively).

ELISA data were normalized to the iCFTR- condition. Values represent means of n independent experiments in triplicates (three different filters per experiment). Statistical analysis: unpaired t-test. Statistic of differences between iCFTR+ and iCFTR- indicated at the top of the graph, differences between control condition and treatment are indicated just above the bars.

Inhibition of EGFR signaling abrogates the CFTR-dependent differences in AREG release

Since it is not obvious how the loss of an apical anion transporter could directly lead to an increase in ADAM17 activity, which is active in intracellular compartments (69) and at the plasma membrane (8) (63), we measured the involvement of signaling pathways upstream of ADAM17. Among other factors, ADAM17 activity is regulated by receptor tyrosine kinases (RTKs) (23). The broad-range tyrosine kinase inhibitor genistein (100 µM) strongly reduced the AREG shedding and diminished the CFTR-dependent differences in cells under ALI conditions (Fig. 4A). Similarly, treatment with herbimycin A (1 µM), which inhibits Hsp90 (80) leading to the destabilization of multiple kinases (70), resulted in decreased AREG release in non-CFTR expressing cells (Fig. 4B). Consistent with the notion that tyrosine kinase signaling is involved in the augmented AREG shedding in the absence of CFTR, the tyrosine phosphatase inhibitor sodium ortho-vanadate (25 µM) increased AREG release (Fig. 4C).

Next, we inhibited specific candidate tyrosine kinases that are involved in ADAM17 activation. The SRC-kinase in part regulates ADAM17 activity (63). The SRC family inhibitor PP2 decreased AREG shedding (10 µM, Fig. 4D; lower concentrations 1 µM and 3 µM; were equally effective, data not shown), while the inactive analog PP3 (10 µM) had no effect. The SRC-kinase may also directly phosphorylate and activate the EGFR, bypassing EGFR ligand binding, which results in downstream MAP kinase activation and in turn ADAM17 activation (27) (43). The canonical inhibitor of EGFR signaling AG1478 (1µM) strongly inhibited AREG

release (Fig. 4E). Similarly, inhibition of the downstream MAP kinase MEK1/2 with the inhibitor U0126 (10µM) reduced AREG release (Fig. 4F). These data are consistent with the existence of a positive feedback loop in the EGFR/ADAM17 axis that involves the MAPK pathway (9) (63) (69). Most importantly, inhibition of SRC, EGFR or MEK1/2 abrogated the CFTR-dependent differences in AREG shedding, indicating that non-functional CFTR activates the EGFR-ADAM17 axis.

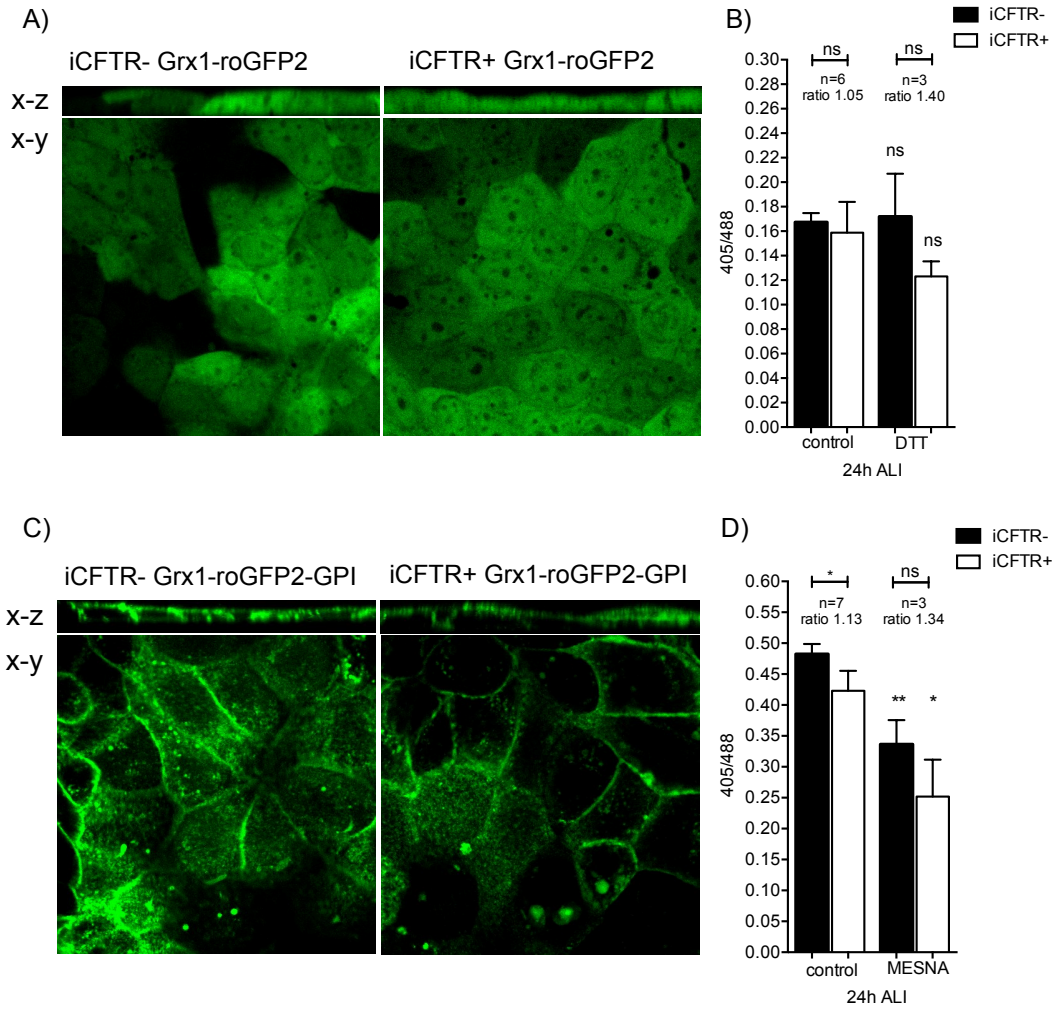

Figure 7 CFTR mediates oxidation of the air surface liquid (ASL), but not cytosol in CFBE41o- cells
(A) Laser confocal fluorescence microscopy pictures of iCFBE41o- cells lentivirally transduced with glutaredoxin-1-redox sensitive GFP (Grx-1-GFP) (B) The ratiometric fluorescence measurements (the ratio of 405 nm emission and 488 nm excitation) of Grx-1-GFP probe expressed in iCFBE41o- cells revealed no difference in cytosolic glutathione redox potential between iCFTR+ and iCFTR- cells. Cell permeable reducing agent DTT (0,5 mM) was used as a control for ratiometric fluorescence measurements. (C) Laser confocal fluorescence microscopy pictures of iCFBE41o- cells lentivirally transduced with glutaredoxin-1-redox sensitive GFP fused to C-terminal GPI anchor and N-terminal signal sequence (Grx-1-GFP-GPI) (D) The ratiometric fluorescence measurements of apically expressed Grx-1-GFP-GPI probe in iCFBE41o- cells measured as the ratio of 405 nm emission and 488 nm excitation of the probe revealed oxidation of ASL in CFTR deficient cells. The cell impermeable reducing agent MESNA (50 µM) was used as a control.
Statistical analysis: unpaired t-test; n indicates the number of independent experiments in duplicate or triplicate, meaning that two or three different filters were used per experiment.

CFTR deficiency increases apical localization and phosphorylation of EGFR in CFBE cells cultured at ALI

To test whether the EGFR inhibitor-sensitive and CFTR modulated AREG release is the result of increased EGFR expression, the mRNA and protein levels of the receptor were determined by qPCR (Fig. 2C) and immunoblot (Fig. 5A-D), respectively. The levels of EGFR mRNA at different times before and after shift to ALI conditions were not significantly different between CFTR and non-CFTR expressing cells (Fig. 2C). Consistent with this, the total EGFR antigen, as determined by western blotting, did not differ significantly between iCFTR- and iCFTR+ (Fig. 5A, C).

Indirect immunostaining and laser confocal fluorescence microscopy showed EGFR was predominantly confined to the basolateral membrane in both iCFTR+ and iCFTR- expressing cells (33), but a small proportion of the EGFR staining was localized to the apical membrane (Fig. 5E,F). The specificity of the EGFR immunofluorescence signal was confirmed by the intracellular localization of EGFR antigen after 3 h stimulation with EGF (Fig. 6A), a known trigger for internalization and lysosomal degradation of the receptor (57). To establish an effect of CFTR deficiency on EGFR localization, the plasma membrane density of EGFR was determined at the apical and basolateral cell surface (Fig. 5G,H). Here an anti-EGFR antibody recognizing the extracellular domain of EGFR, followed by an HRP-coupled secondary antibody was used to detect the cell surface localized receptor by fluorescence spectroscopy with the cell impermeable Amplex Red as HRP substrate (see Methods). The apical EGFR PM density was increased by ~30% in non-CFTR expressing cells cultured for 24h under ALI conditions compared to CFTR-expressing cells (Fig. 5G). In contrast, at the basolateral membrane in ALI cultured cells, and in cells grown under LLC conditions no CFTR-dependent differences in the EGFR PM expression were observed (Fig. 5H). Furthermore, expression of G551D-CFTR in cells cultured under ALI did not suppress apical (Fig. 5I) or basolateral EGFR PM density (Fig. 5J).

Taken together, these results indicate that functional CFTR deficiency alters the net polarity of EGFR under ALI conditions. However, the majority of EGFR is still localized at or near the basolateral membrane in iCFTR- cells (Fig 5A).

The constitutive phosphorylation of EGFR at Y1173, a measure of EGFR activity, was decreased in CFTR expressing cells compared to CFTR deficient cells cultured for 8 h under ALI as determined by phospho-immunoblotting (Fig. 5 B,D,H), whereas the total amount of EGFR was not significantly affected by CFTR induction (Figure 5C), consistent with the EGFR mRNA results (Figure 2A). Activation of EGFR by exogenous stimulation with 10 ng/ml EGF at both the apical and basolateral PM resulted in a massive CFTR-independent increase in AREG release, and gradually abolished the CFTR-dependent differences (Fig. 6B). Taken together, these data indicate that CFTR expression modulates EGFR trafficking and the level of EGFR constitutive activity, but not the expression or maximal signaling capacity of the EGFR pathway.

CFTR mediated shift of the extracellular redox potential attenuates AREG release.

Since the expression of functional CFTR is sufficient to limit the augmented AREG release in CF cells, it is plausible to assume that CFTR-mediated ion transport at the apical membrane attenuates the constitutive signaling that leads to AREG shedding. CFTR transports chloride, bicarbonate and reduced glutathione (GSH) that regulate the height, pH and redox potential

of the airway surface liquid (ASL), respectively (62). It was previously established that oxidation activates ADAM17 directly, by changing its conformation (18) (81) and indirectly, by initiating upstream signaling that increases ADAM17 activity through activating SRC (63) or EGFR (54). To determine if CFTR expression leads to changes in the cytosolic and ASL redox state, which may lead to induced ADAM17 activity, we used the glutaredoxin1–redox-sensitive GFP (Grx1-roGFP) fusion protein that allows for measurement of the glutathione redox potential by ratiometric fluorescence measurement (31). The unmodified (Grx1-roGFP) or the N-terminal signal sequence and C-terminal GPI-anchor signal fused (SS-Grx1-roGFP-GPI) redox probes were delivered by lentiviral transduction into the CFBE-iCFTR cells for expression in the cytosol or tethered to the outer leaflet of the plasma membrane, respectively (Fig 7A).

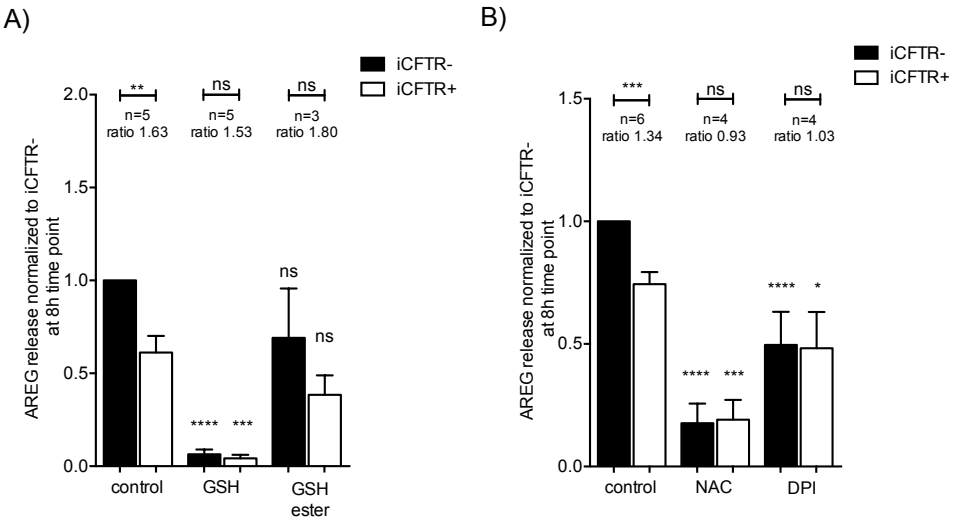

Figure 8 ROS scavengers decrease AREG release from CFBE41o- cell
(A) Glutathione (10 mM GSH), but not the cell permeable GSH ester (1 mM), strongly inhibits AREG release in iCFTR CFBE41o- cells abrogating differences between CFTR- and CFTR+ condition (B) A ROS scavenger, N-acetyl cysteine (5 mM NAC) and NADPH oxidase inhibitor diphenylene-iodonium (5 µM DPI) significantly decreases AREG release in CFTR+ and CFTR- iCFTR CFBE cells, showing the role of ROS in regulation of ADAM17 activity.
ELISA data were normalized to iCFTR- condition. Values represent means of n independent experiments in triplicates (three different filters per experiment). Statistical analysis: unpaired t-test. Statistic of differences between iCFTR+ and iCFTR- indicated at the top of the graph, differences between control condition and treatment are indicated just above the bars.

In submerged non-polarized CFBE the cytosolically confined Grx1-roGFP redox probe was in a relatively reduced state, as measured with a fluorescence plate reader (data not shown). The redox probe was slightly further reduced by the addition of the cell-permeant reducing agent dithiothreitol (DTT), but not the cell impermeable 2-mercaptoethanesulfonic acid sodium salt (MESNA). Grx1-roGFP was strongly oxidized by addition of hydrogen peroxide (H_2O_2) (data not shown). In contrast, the GPI-anchored Grx1-roGFP was in oxidized state under similar conditions and was only slightly oxidized further by H_2O_2, but strongly reduced by both the cell-permeant DTT and the non-permeant MESNA

addition, confirming the extracellular accessibility and redox sensitivity of the probe (data not shown).

On polarized filter grown CFBE at ALI, the apical Grx1-roGFP fluorescence was measured by ratiometric laser confocal fluorescence microscopy using an upright confocal microscope after shifting the cells for 24 h to ALI (Fig 7A). Under these conditions, CFTR expression had no significant effect on the redox state of the cytosolic probe in iCFBE cells (Fig. 7B), which already was almost at the maximally reduced state, as revealed by DTT addition (Fig. 7B). In contrast, induction of CFTR expression resulted in a more reduced state of the ASL probe Grx1-roGFP-GPI (Fig. 7C), approaching the minimal fluorescence ratio detected in the presence of MESNA (Fig. 7D).

Supplementation of the basolateral and apical medium with GSH shortly before establishing ALI strongly inhibited the AREG release in non-CFTR and CFTR expressing cells, and abrogated the CFTR-dependent differences in AREG shedding (Fig 8A). Addition of extracellular GSH (3 mM as in Fig 8) inhibited AREG shedding to the same extent, but did not significantly reduce EGFR Y1173 phosphorylation, suggesting that the effect was not due to a change in the activation of EGFR (n=3 independent experiments in fourfold, data not shown). In contrast to GSH, the cell permeable GSH ester, which is cleaved by phospho-diesterases and causes an increase in the intracellular GSH concentration did not diminish AREG shedding significantly (Fig. 8A). N-acetyl cysteine, a cell permeant precursor of GSH synthesis that by itself has an antioxidant effect (59), reduced AREG release (Fig. 8B). Complementary to these results, reducing the cellular reactive oxygen species (ROS) production using diphenyleneiodonium (DPI), an inhibitor of intracellular NADPH oxidases (NOX) and dual oxidases (DUOX), also reduced AREG release, although to a lesser extend (Fig. 8B).

Taken together, our data suggest that high extracellular GSH does not exert its inhibitory effect on AREG shedding through inactivation of EGFR, but rather switches ADAM7 to its closed, inactive conformation (18). Reduced extracellular GSH in CFTR deficient epithelia would have the opposite effect.

DISCUSSION

ADAM17 shedding of AREG is enhanced by CFTR-deficiency.

Here we report that dysfunctional CFTR in differentiated CFBE airway epithelial cells cultured at air-liquid interface induces activity of ADAM17 (Fig 1B). Importantly, this effect was not observed in a normally expressed but inactive form of CFTR (G551D) (Fig 1C), which shows that CFTR activity is required for ADAM17 regulation. The increased ADAM17-mediated AREG shedding caused by CFTR deficiency observed in our model likely contributes to mucus hypersecretion, inflammation and tissue remodeling in CF lung disease. Further studies should test whether ADAM17 activation is also observed in the CF airways *in situ*, and whether attenuation of ADAM activity reduces CF pathology.

Polarity of shedding.

In differentiated ALI-CFBE41o- cells, we found that total AREG shedding towards the basal compartment is fourfold higher than towards the apical side (Fig 1A). This is consistent with the relative membrane surface area of the two compartments, suggesting that there is no

strong preference for shedding polarity. Importantly, induction of CFTR does not have a significant effect on the polarity of secretion (Fig 1A). In ALI differentiated primary bronchial epithelial cells the basolateral to apical ratio is substantially higher (69), which may be explained by the fact that these cells have a higher columnar morphology compared to the low cubical CFBE cells. Since the volume of apical lining fluid is small, the actual apical concentration of ligand, which determines the rate of receptor binding, is potentially high. Therefore, we have to consider that also ADAM ligands shed apically from epithelial cells contribute to luminal signaling in the lung, where myeloid cells likely play a major role in determining the downstream effects of AREG signals, in particular activated macrophages during acute injury response (82). Furthermore, enhanced expression of AREG mRNA was reported in alveolar neutrophils from CF patients, consistent with a heightened state of inflammation (1).

Regulation of ADAM17 mediated shedding activity by EGFR.

To establish the mechanism by which CFTR deficiency changes the level of ADAM17 dependent agonist shedding in iCFTR CFBE cells, we analyzed the involvement of several known effectors. First we showed that in this model ADAM17-mediated shedding is tightly regulated by protein phosphatases and tyrosine kinases, particularly by SRC, EGFR and MAPK (Fig 4). Under basal conditions EGFR emerged as a potent ADAM17 regulator. When inhibited, it reduced ADAM17 mediated shedding to the same extent as the selective ADAM17 inhibitor TMI-2 (Fig 3B), whereas addition of the EGFR agonist EGF to the medium strongly enhances AREG release (Fig 6B). This is consistent with results with other epithelial models (9) (56) and our data in primary ALI-HBEC (69). In the latter, inhibition of EGFR reduced ADAM17 dependent AREG and IL-6R shedding, as well as AREG, IL6R and IL8 mRNA levels to the same extent as an ADAM17 selective inhibitor (69). Therefore, there is a close link between EGFR and ADAM17 activity in airway cells. It is likely that the EGFR/ADAM17 axis is established by a feedback loop in which EGFR agonists such as AREG are shed by ADAM17, activating EGFR and downstream MAPK pathway, which in turn activates ADAM17 (9), or ADAM17 regulates EGFR expression by activation of Notch1 (5). Hyperactivity of this EGFR/ADAM17 axis in CFTR deficient epithelia may affect CF lung pathology (24).

EGFR phosphorylation is enhanced by CFTR-deficiency.

Exaggerated epithelial EGFR expression and abnormal signaling are observed in chronic lung disease such as COPD and asthma (2) (34) (55), which emphasizes its potential relevance in CF. Here we show that induction of functional CFTR expression in CFTR deficient airway cells does not affect total EGFR protein (Fig. 5) or mRNA expression (Fig 2), however it significantly reduces the level of EGFR Y1173 phosphorylation, a measure of EGFR activation (Fig. 5D). Our data are consistent with increased phosphorylated EGFR (EGFR-P) levels reported in airway epithelium and inflammatory cells from CF patients in comparison to non-CF controls (47). Furthermore, our data show increased EGFR-dependent AREG shedding in iCFTR- cells (Fig 4E and Fig. 6B) suggesting that CFTR deficiency enhances cellular signaling through the EGFR/ADAM17 axis. In accordance with our results, several reports suggest that active CFTR suppresses other EGFR related inflammatory responses, like IL8 secretion and mucin production (7) (56) (37) (48).

CFTR-deficiency affects EGFR trafficking.

As summarized in the introduction, one of the factors regulating EGFR activity is its localization relative to its target molecules. Upon ligand binding EGFR dimerizes and is internalized to intracellular compartments where it is either inactivated or continues signaling (36) (73) (90) (72). We showed that in CFBE-ALI EGFR is primarily, but not exclusively localized at the basolateral membrane under basal ALI conditions (Fig 5 E,F). Surface ELISA showed an increase of EGFR trafficking to the apical membrane in CFTR deficient ALI-CFBE cells (Fig 5G). Addition of an inhibitory antibody against EGFR (cetixumab) to polarized iCFTR did not reduce AREG shedding, whereas basal addition was effective (Scholte et al, unpublished data). This does not support a major role of apical EGFR in this process, however, abnormal trafficking of EGFR would likely be an important factor in the regulation of the EGFR/ADAM17 axis.

EGF binding leads to EGFR internalization and lysosomal degradation, whereas AREG stimulation causes EGFR internalization and recycling to the membrane (24) (57). Thus, CFTR dependent enhanced shedding of AREG (Fig. 1A) may lead to activation of EGFR (Fig. 5B,D) and more frequent recycling to the membrane (Fig. 5 G), sustaining enhanced EGFR dependent ADAM17 activity through the feedback loop.

As expected, EGF induced EGFR internalization towards a perinuclear compartment in iCFTR (Fig. 6A,B) and caused a massive increase in ADAM17 shedding activity (Fig 6B), gradually reducing the difference between iCFTR+ and iCFTR-. However, further investigations are needed to establish whether abnormal polarisation and trafficking of EGFR plays a decisive role in CFTR dependent EGFR/ADAM17 activity (39). Furthermore, we need to establish whether this is specific for EGFR or reflects a general imbalance of proteostasis and deficient autophagy in reported in CF epithelial cells (22) (45). Our data support the notion that CFTR deficiency changes the kinetics of EGFR/ADAM17/AREG trafficking and activity (24). Further studies in primary bronchial epithelial cells with well-defined tight junctions and preferably in CF lung biopsies need to be performed to confirm our observations.

Regulation of shedding activity by SRC.

The ubiquitous tyrosine kinase c-SRC reportedly causes ROS dependent activation of EGFR and ADAM17 in immortalized epithelial cells (41),(63) suggesting it has a regulatory role in the EGFR/ADAM17 signaling cascade. Indeed, in CFTR-deficient CFBE cells inhibition of SRC (PP2) reduced AREG release to the level observed in cells with induced CFTR activity (Fig. 4D).

A role of other SRC family members in the enhanced AREG shedding in CFTR deficient CFBE cells cannot be ruled out, considering the limited specificity of PP2 inhibition. Transactivation of EGFR by SRC has been reported (27) (43), but the mechanism and relationship with ligand or stress induced trafficking remains to be established. Thus, further studies are warranted to define an effect of CFTR deficiency on SRC trafficking and activity towards its substrates.

Extracellular REDOX potential regulates EGFR/ADAM17-dependent AREG shedding from CFBE cells.

ADAM17 activity is dependent on the interaction between a protein (thiol) disulfide isomerase (PDI) with the extracellular membrane proximal domain (MPD) of ADAM17,

which maintains ADAM17 in an inactive, "closed" conformation. This thiol isomerase is active in the endoplasmic reticulum, presumably involved in protein maturation, but has also been observed in the extracellular space (18). It is inactivated when the extracellular environment is oxidized, which results in an active "open" conformation of ADAM17 (81) (18). In addition to this direct effect of oxidation on ADAM17 activity, reactive oxygen species (ROS) may affect ADAM17 indirectly through the activation of ROS sensitive molecules in the ADAM17 signaling pathway, like SRC (63) (84) and EGFR (54). Therefore, we speculated that a change in redox homeostasis is involved in enhanced EGFR/ADAM17 dependent AREG shedding in CFTR deficient cells.

Indeed, the impermeant natural thiol antioxidant Glutathione (GSH) (29) strongly inhibited EGFR/ADAM17-mediated shedding of AREG in CFBE cells (Fig. 8), but not the cell permeant GSH ester, which is cleaved by intracellular phosphodiesterases increasing the intracellular GSH concentration (Fig. 8). N-acetyl cysteine (NAC), a ROS scavenger and reducing agent, and the NOX inhibitor diphenyleneiodonium (DPI) reduced AREG release to a lesser extent. These data suggest that EGFR/ADAM17-mediated AREG release is primarily sensitive to the extracellular redox potential through the extracellular PDI-MPD domain interaction (18), whereas the EGFR and SRC redox sensitive cysteine residues that are localized intracellularly (54) play a limited role. This would explain why high extracellular GSH effectively blocked AREG shedding, likely by inhibiting ADAM activity at the PDM domain, but did not reduce EGFR phosphorylation (Scholte et al, data not shown).

Abnormal extracellular redox potential in iCFTR- ALI contributes to enhanced EGFR/ADAM activity.

The redox sensitivity of ADAM17 could explain its enhanced activity in CFTR deficient cells. CFTR is involved in the maintenance of intra and extracellular redox potential in airways through its role in the transport of GSH (30) (89). In CFTR-KO mice reduced levels of GSH were reported in the luminal fluid in comparison to wild type mice, whereas total GSH levels in the lung tissue did not differ (78). Furthermore, in wild type mice, *P aeruginosa* infection increases GSH in the epithelial lining fluid, however, this infection induced-increase is absent in CFTR−/− mice (14). Similarly, GSH levels were reduced in lung lavage fluids of CF patients compared to healthy subjects (58). This is attributed to defective GSH transport in CFTR deficient epithelial cells (26), likely contributing to enhanced downstream inflammatory signaling in CF lung disease (32) (89). Importantly, treatment of CF patients with GSH reduced inflammatory markers in BALF (32). The decreased GSH in the epithelial lining fluid may contribute to the poor response to infections and aberrant activation of inflammatory molecules sensitive to oxidation (25).

Consistent with the role of CFTR in GSH transport we established enhanced oxidation of ASL in iCFTR- cells compared to iCFTR+, monitored by Grx1-roGFP2 anchored to the apical membrane (Grx1-roGFP2-GPI) (Fig. 7). In contrast, the ratiometric measurement of cytosolic roGFP-Grx-1 fluorescence did not reveal significant differences in the intracellular redox potential between iCFTR- cells and iCFTR+ cells (31) (49) (Fig. 7D,E). We propose that this oxidized extracellular redox potential, which is likely due to reduced GSH transport in iCFTR- cells, activates ADAM17 shedding activity through the redox sensitive extracellular MPD. How this GSH redox signal is transferred to the active ADAM17 pool remains to be established in future studies. ADAM17 and its substrates actively recycle through the

plasma membrane, exposing the MPD and protease sensitive domains to the extracellular environment. Though ADAM17 reportedly has a preferentially basolateral sorting route, we cannot exclude that it passes the apical membrane, in particular in CFTR deficient cells that have improper trafficking of membrane proteins, as shown by our (Fig 5) and published data (45). Furthermore, we cannot exclude that the apical GSH pool affects the lateral and basal domains of the epithelial plasma membrane, in particular in CFTR deficient cells that have instable tight junctions (42). Further studies with modified targeted redox probes in polarized are required to establish this. Finally, we cannot exclude that extracellular GSH affects the intravesicular compartments, in which ADAM17 is active. We have shown previously (69) that CS induced interactions between ADAM17 and its substrates occur intracellularly in polarized airway cells. GSH can enter the cell through endocytosis/pinocytosis, trans-cytosis, and in the shape of glutathione-protein conjugates (65), which could allow an interaction with the ADAM17 ectodomain even when that is not apically localized. Further studies in advanced cell culture models are required to establish relative ADAM17 and GSH localization in CF airway epithelia.

Taken together, We propose that supplementation with extracellular GSH severely inhibits ADAM17 related AREG shedding through reduction and inactivation of its extracellular domain (18). Conversely, an oxidized extracellular redox potential (Fig 6), due to insufficient GSH secretion in CFTR- cells (4) and consistent with a lower ASL GSH concentration in the ASL of CF patients and CF cell models in comparison to normal controls (26) (58) may activate ADAM17, resulting in enhanced AREG release (Fig 1). This may involve the feedback loop action of the EGFR/ADAM17 axis and enhanced EGFR activity (Fig 5).

Summary and conclusions

Our data provide more insight in the role of CFTR deficiency in regulating the EGFR/ADAM17 axis, which is likely involved in the triggering and resolution of CF lung inflammation. Activity of the EGFR/ADAM17 shedding system is higher in differentiated CFTR deficient CFBE cells at air-liquid interface compared to CFTR expressing cells. This is related to stronger EGFR activation and abnormal trafficking in CFTR deficient cells, likely involving a paracrine feedback loop through enhanced ADAM17 dependent AREG shedding. The extracellular REDOX potential, which as we show strongly affects ADAM17 shedding activity, was changed to a more oxidized state in CFTR deficient cells, likely due to reduced GSH secretion. We propose that abnormal extracellular redox potential in CFTR deficient epithelial cells enhances the basal activity of the EGFR/ADAM17 axis. Further studies are required to establish the link between extracellular redox potential and EGFR/ADAM activity in primary cells and airway in situ. Based on our results we propose that Intervention in this pathway may be beneficial to CF patients.

MATERIALS AND METHODS

Compounds and antibodies

TMI-2 (PF-5480090) and TMI-1 (85) were obtained from Wyeth inc. (Philadelphia, Pennsylvania, USA). AG1478 (1 µM), diphenyleneiodonium chloride (DPI), genistein, reduced glutathione (GSH), herbimycin A**,** hydroxyl peroxide (H2O2), N-acetylcystein (NAC), orthovanadate, tert-butyl-hydroxyperoxide (t-BHP) were purchased from Sigma-Aldrich. EGF, PP2, PP3, UO126 were purchased from R&D. Rat monoclonal anti-EGFR antibodies specific for extracellular domain used in dilution 1:200 for IF and 1:1000 for PM, were purchased from Abcam.

CFBE cell culture.

The generation of the human immortalized CFBE41o– cell line (abbreviated as CFBE) harboring the inducible expression of WT- or G551D CFTR has been described before (77). The CFBE cells were propagated on plastic dishes coated with 3 mg/ml collagen (Pure Col, Advanced Biomatrix), 1 mg/ml human fibronection (BD Biosciences) with and 100 µg/ml bovine serum albumin (Sigma-Aldrich) in LHC-basal medium (Biosource). Cells were cultured in MEM (Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco), 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, Gibco), 200mM L-glutamine (Gibco). Cells containing the transactivator alone (TetON) were maintained in medium containing 200 µg/ml G418 (Invitrogen), and cells harboring inducible CFTR expression were maintained in presence of 200 µg/ml G418 (Invitrogen) and 3 µg/ml Puromycin (Invitrogen). When cells reached 80% confluence, they were transferred on the 1.12 cm^2 Transwell filters with 0.4µm pore size (Corning) at a density of $1x10^5$ cells per filter and cultured with 0.5 ml and 1.5 ml medium in the apical and basolateral chamber, respectively. 24 h after seeding (day 1) CFTR expression was induced with 500ng/ml doxycycline. At day 4 the medium was changed to OptiMEM (+/- doxycycline) supplemented with 2 mM L-glutamine (Gibco). At day 5 compound treatments were performed in OptiMEM and samples were collected at different time points after ALI exposure: 4h (0-4h), 8h (4-8h), 24h (8-24h). At each investigated time point after sample collection, whole medium was changed.

Generation of Redox Sensor Cells

The ratiometric redox sensor Grx1-roGFP (31) was shuttled into the lentiviral vectors pLVX-IRES-Hyg (clontech) for stable expression in different cellular compartments. The basic variant is expressed in the cytosol as described by Gutsher et al. Another variant was equipped with a N-terminal signal-sequence and a C-terminal glycosylphosphatidylinositol (GPI) anchor consensus sequence (SS-Grx1-roGFP-GPI) to determine the redox potential of the ASL. CFBE cells with inducible expression of WT-CFTR were transduced with these lentiviral particles followed by selection with 200 µg/ml hygromycin B.

Measurement of redox state in CFBE redox sensor cells

The initial characterization of the redox senor cells was performed in a fluorescence plate reader. For this purpose CFBE cells expressing the redox sensor variants were seeded into black 96 wells and kept for ≥4 days post confluent. Fluorescent intensities were measured

at 520 nm (±20 nm) using TECAN plate reader after excitation with 405 nm and 488 nm, respectively. Higher 405/488-ratio value represents a higher oxidative state (49).
To determine the redox state in differentiated CFBE under ALI CFBE redox sensor cells, grown on Snapwell inserts (Corning) were used for ratiometric confocal imaging. Generally a Z-stack of 20-30 optical xy-sections was acquired (0.7-1-µm apart) with LSM700 microscope (Carl Zeiss, Jena, Germany) equipped with a water immersion objective lens (40x, numerical aperture, 0.8; achroplan; Zeiss). For immersion of the objective lens, cells were covered with non-toxic the hydrophobic, Fluorinert™ FC-70 fluid (boiling point, 215°C; refractive index, 1.303; Hampton Research) that allows normal gas exchange. During the experiment cells were maintained at 37°C, with 5% CO_2 in a humidified top-stage incubator mounted on the microscope. The biosensor was excited sequentially with 405 nm and 488 nm lasers and emitted intensities were collected from 520 nm. CFBE cells without Grx1-roGFP2 were used as a background control. Ratios of intensities were calculated from 5 cells on a single apical (GPI) or intracellular z-slice that was selected manually from the Z-stack images for each cell. Three microscopic areas were recorded on each filter. The redox probe maximal and minimal oxidation emission ratios were determined in some experiments by basal addition of H_2O_2 (100 µm) and 500 µM DTT or 50 mM MESNA, respectively.

AREG ELISA

Samples for AREG ELISA were collected form the basolateral and apical medium of differentiated CFBE41o$^-$ conditioned for the indicated times. AREG levels were determined by ELISA (Duoset human AREG, DY262, R&D Systems) following the manufactures instructions. The final detection was performed with a SuperSignal ELISA Femto luminescence substrate (Pierce) on a VICTOR Light plate reader (PerkinElmer). Data were corrected for the dilution factor and insert size. The amount of the shed AREG was expressed as pg/filter per hour. Measurement of the metabolic activity of the cells with alamar blue (Invitrogen) showed that the induced CFTR expression had no effect on the cell number or viability of the CFBE cells (data not shown).

EGFR plasma membrane density measurements (PMD)

The PM density of EGFR was determined by cell-surface ELISA (50). CFBE cells for PM density measurements were grown on 1.12 cm^2 Transwell filters with 0.4µm pore size (Corning), as described above. At the indicated time points, the cells were incubated in reagent diluent (1% BSA in PBS containing 0.1 mM $CaCl_2$ and 1mM $MgCl_2$(PBSCM) for 30 min at RT. EGFR localized at the apical or basolateral membrane was detected by incubation with rat monoclonal anti-EGFR antibody specific for the extracellular domain (1:500, ab231, Abcam) followed by extensive washing and subsequent incubation with anti-rat-HRP antibody (1:1000, 112-035-167, Jackson ImmunoResearch), both for 60 min at RT. Excess antibody was removed by extensive washing with PBSCM, and specific binding was determined with Amplex Red (Invitrogen) HRP substrate. The fluorescence intensity was measured at 544 nm excitation and 590 nm emission wavelengths using an Infinite M1000 (Tecan) fluorescence plate reader, and values determined from cells without primary antibody labeling were subtracted as background.

mRNA isolation and qPCR

The mRNA isolation and q-PCR was performed as described previously (77). The primers are listed in Table 1.

Table 1: qPCR primers used in this study

Gene symbol	Direction	Sequence
AREG	forward	GTGGTGCTGTCGCTCTTGATA
AREG	reverse	ACTCACAGGGGAAATCTCACT
ADAM17	forward	TCTCCTATTCCTGACCAGCG
ADAM17	reverse	GTCTGAGAGCAAAGAATCAAGC
EGFR	forward	GGGCTCTGGAGGAAAAGAAA
EGFR	reverse	AAATTCCCAAGGACCACCTC
GAPDH	forward	CATGAGAAGTATGACAACAGCCT
GAPDH	reverse	AGTCCTTCCACGATACCAAAGT

Immunofluorescence (IF)

Cells for PM measurements were grown under ALI on the 1.12cm^2 Transwell filters with 0.4µm pore size (Corning), as described above. At the investigated time points, cells were fixed with 4% paraformaldehyde (Sigma Aldrich) for 10 min at RT, followed by permeabilization with 4% paraformaldehyde and 0,5% Triton-X (Sigma Aldrich) in PBS for 5 min at RT. This was followed by incubation with 100 mM glycine pH 7.5 for 10 min and blocking with 1% BSA (in PBSCM) for 45 min, incubation for 1h with rat monoclonal anti-EGFR antibodies specific for the extracellular domain (1:200, ab231, Abcam), and subsequent incubation with the secondary fluorescent antibody Alexa Fluor 488 (1:1000, A11006, Invitrogen) for 1h at RT in darkness. Antibodies were diluted in 0.5% BSA in PBSCM. Nuclei were stained with 300 ng/ml DAPI for 10 min in darkness. Washing steps were performed with PBSCM. Cut out filters were mounted with mounting medium (100 mM Tris/HCl pH 8.5, 10% mowiol, 25% glycine, 150 mM 1,4-Diazabicyclo[2.2.2]octane) on glass cover slides. 20-30 z-optical sections were acquired using a confocal LSM-780 microscope equipped with a Plan-Apochromat 63X/1.40 oil differential interference contrast objective (Carl Zeiss), reconstituted using the Zen 2012 software package and representative xz-sections are shown.

Western blotting

105 iCFTR CFBE cells were seeded per insert (1.12cm² PET filters, pore size 0.4µm,Corning) with (iCFTR+) or without (iCFTR-) doxycycline induction. The cells were cultured as

described above in CFBE cell culture section. Four independent experiments were performed with four (3) or three (1) inserts per condition each. At 8h ALI, medium was collected for AREG ELISA (R&D Systems Europe, Abingdon, UK), whereas cell samples were transferred to 100µl RIPA buffer (50 mM Tris HCl, 150 mM NaCl, 1% Triton-X, 0,5% Sodium deoxycholate, 0,1% SDS) containing phosphatase (PhosStop, Sigma-Aldrich) and protease inhibitors (Complete Mini, Roche). Protein concentration was determined with BCA assay (Pierce Rockford, IL). The lysates were centrifuged at 15,000 rpm for 15 min at 4 °C. The clear supernatants were immediately transferred to SDS loading buffer (5µl 4x sample buffer, 1µl 0.1M DTT, 15µl protein sample) and stored at -20°C until use. 7% gel electrophoresis was performed with iCFTR+ and iCFTR- samples loaded in parallel on the same gel and proteins were transferred to PVDF membranes (0.45 micron filter, Merck Millipore). The membranes were blocked with 3% BSA and 0,05% Tween20 in PBS, and then stained for EGFR EP38Y (1:5000, Abcam), and a fluorescent second antibody (1:10.000, 926-32213, Li-Cor). EGFR-P was detected using antibody Tyr1173 (1:200, Santa Cruz) and a HRP labeled second antibody (1:10.000, P0448, Dako). The proteins were detected using Amersham ECL prime Western blotting detection reagent (GE Healthcare Europe GmbH) and scanned with Alliance (luminescence) or Odyssey (fluorescence) scanners. In separate experiments equal amounts of protein from iCFTR- and iCFTR+ cells cultured in parallel were loaded on the same blot. The density of each band was quantitated with ImageJ software. Acquired data for EGFR-P and EGFR were normalized to the average iCFTR- data for each individual blot and presented as percentage of the average of iCFTR- values in Figure 5.

ADAM17 substrate assay

The ADAM17 enzyme activity in polarized CFBE grown on Transwell filters and exposed to ALI for 24 h, was measured by supplementing the basolateral medium with 10 µM fluorogenic TACE substrate IV (Millipore). Following 1 h incubation the medium was collected and the fluorescence was determined at 320 nm excitation and 420 nm emission wavelengths using an Infinite M1000 (Tecan) fluorescence plate reader. Values determined from filters without cells were subtracted as background control.

Statistical analysis

Statistical analyses were performed with GraphPad Prism Software. The statistical test used for the specific experiment is indicated underneath each figure. Each experiment was performed in triplicate, meaning that samples were collected from three different wells, grown simultaneously, averaged and considered as one experiment in analysis (indicated by n). Graphs present the averages of n experiments with SEM values. Differences at P-value >0.05 were considered to be not statistically significant (ns>0.05), P-values <0.05 were considered to be statistically significant and indicated as *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

Acknowledgements

Wyeth Research, Cambridge MA 02140 USA kindly provided the ADAM inhibitors TMI-1 and TMI-2. We are deeply indebted to the late Dr Dieter Gruenert, who generated, characterized and kindly provided the original CFBE41o–cell line.

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Funding

We thank the Lung Foundation Netherlands (#3.3.10.027) and the Dutch Cystic Fibrosis Foundation NCFS (MV HIT-CF1-C7) for supporting this study. We also acknowledge EMBO (ASTF525–2014) for supporting M.S short-term fellowship at McGill University in Gergely L. Lukacs laboratory with Guido Veit's supervision.

Competing interest

BS obtained a research grant from Lexicon inc (CA, MA USA) for work not related to this study. All other authors declare no conflict of interest.

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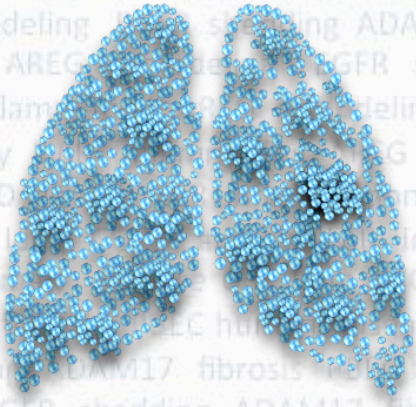
CHAPTER 4



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(published – Am J Physiol Lung Cell Mol Physiol)

Veltman M, Stolarczyk M, Radzioch D, Wojewodka G, De Sanctis JB, Dik WA, Dzyubachyk O, Oravecz T, de Kleer I, Scholte BJ

Am J Physiol Lung Cell Mol Physiol 311: L1000–L1014, 2016.
First published September 23, 2016; doi:10.1152/ajplung.00298.2016.

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Correction of lung inflammation in a F508del CFTR murine cystic fibrosis model by the sphingosine-1-phosphate lyase inhibitor LX2931

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Submitted 14 July 2016; accepted in final form 19 September 2016

Veltman M, Stolarczyk M, Radzioch D, Wojewodka G, De Sanctis JB, Dik WA, Dzyubachyk O, Oravecz T, de Kleer I, Scholte BJ. Correction of lung inflammation in a F508del CFTR murine cystic fibrosis model by the sphingosine-1-phosphate lyase inhibitor LX2931. *Am J Physiol Lung Cell Mol Physiol* 311: L1000–L1014, 2016. First published September 23, 2016; doi:10.1152/ajplung.00298.2016.—Progressive lung disease with early onset is the main cause of mortality and morbidity in cystic fibrosis patients. Here we report a reduction of sphingosine-1-phosphate (S1P) in the lung of unchallenged *Cftr*^{tm1EUR} F508del CFTR mutant mice. This correlates with enhanced infiltration by inducible nitric oxide synthase (iNOS)-expressing granulocytes, B cells, and T cells. Furthermore, the ratio of macrophage-derived dendritic cells (MoDC) to conventional dendritic cells (cDC) is higher in mutant mouse lung, consistent with unprovoked inflammation. Oral application of a S1P lyase inhibitor (LX2931) increases S1P levels in mutant mouse tissues. This normalizes the lung MoDC/cDC ratio and reduces B and T cell counts. Lung granulocytes are enhanced, but iNOS expression is reduced in this population. Although lung LyC6+ monocytes are enhanced by LX2931, they apparently do not differentiate to MoDC and macrophages. After challenge with bacterial toxins (LPS-fMLP) we observe enhanced levels of proinflammatory cytokines TNF-α, KC, IFNγ, and IL-12 and the inducible mucin MUC5AC in mutant mouse lung, evidence of deficient resolution of inflammation. LX2931 does not prevent transient inflammation or goblet cell hyperplasia after challenge, but it reduces MUC5AC and proinflammatory cytokine levels toward normal values. We conclude that lung pathology in homozygous mice expressing murine F508del CFTR, which represents the most frequent mutation in CF patients, is characterized by abnormal behavior of infiltrating myeloid cells and delayed resolution of induced inflammation. This phenotype can be partially corrected by a S1P lyase inhibitor, providing a rationale for therapeutic targeting of the S1P signaling pathway in CF patients.

cystic fibrosis; mouse model; dendritic cells; macrophages; granulocytes

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CYSTIC FIBROSIS (CF) is the most common recessive congenital disease in Caucasians, affecting ~75,000 patients worldwide. It is caused by a deficiency of the CF transmembrane conductance regulator (CFTR) involved in transcellular ion and fluid transport and characterized by severe progressive pancreatic, intestinal, and lung pathology. Progressive distal airway disease, causing severe morbidity, is already observed in a majority of CF infants younger than 5 years (63, 68). This is accompanied by airway remodeling, chronic inflammation and bacterial infections, frequent exacerbations, and eventually fatal loss of lung function. The molecular mechanisms involved in the development of CF lung disease are not completely understood despite intensive research in the past two decades. Experiments in cellular and animal models have suggested that CFTR dysfunction affects a network of interrelated regulatory signaling molecules, including growth factor shedding and cytokine secretion, which regulates cell fate decisions, inflammation, and tissue remodeling in the affected tissues. An effective therapy for a majority of CF patients is not available, despite recent progress with CFTR-targeted pharmaceutics. Therefore additional or alternative intervention with anti-inflammatory drugs should be considered (76).

Abnormal bioactive lipid metabolism in CF lung disease. Previous studies in CF humans and CF mutant mice have highlighted the involvement of various receptor-modulating bioactive lipids in the development of CF lung pathology. In particular the sphingosine pathway (77, 84) and phospholipase A-dependent signaling pathways (18) are affected and are likely involved in CF pathology through regulation of inflammation, airway plugging, and tissue remodeling. Several pharmaceutical interventions in these pathways were effective in animal models, and clinical trials are underway, highlighting the potential of anti-inflammatory treatment in addition to CFTR targeted therapeutics (7, 18, 22, 24, 76, 86).

The role of S1P in CF lung disease. In the present study we focus on sphingosine-1-phosphate (S1P), a receptor-activating lipid that is involved in several cellular processes that are crucial for the development of lung disease. S1P is produced by intracellular kinases (S1PK1 and S1PK2) from sphin-

gosines. S1P lyase (SPL) removes intracellular S1P from the sphingolipid pool and is considered an important drug target for cancer, inflammatory, and autoimmune disease (31, 32). The food colorant 2-acetyl-4-tetrahydroxy butylimidazole induces lymphopenia and effectively reduces SPL activity in mice (65). The orally available chemical derivative SPL inhibitor LX2931 (LX3305) reduced pathology in a mouse model of rheumatoid arthritis (5), was well tolerated, and showed clinical benefit in a phase II clinical trial (37).

S1P is transported to the extracellular compartment by ABC transporters and the major superfamily member Spinster 2 (19), which allows autocrine and paracrine signaling to a family of differentially expressed extracellular receptors ($S1P_{1-5}$), followed by activation of intracellular signaling pathways. S1P also acts on intracellular targets (1, 49, 60) and is involved in NFkB activation (4, 49). In cross talk with other signaling pathways, notably ceramides (77), S1P has important effects on cell migration, growth, and differentiation and plays a role in tissue repair. The importance of S1P metabolism in inflammatory lung disease and fibrosis has been documented in several publications in murine (50), rat (40), and canine models of acute lung injury (71). In a recent report Xu et al. (85) reported reduced S1P levels in bronchoalveolar lavage fluid (BALF) of CFTR knockout (KO) mice, correlating with abnormal dendritic cell infiltration.

In the present study we have confirmed and substantially extended these results using a unique mouse model (*Cftr^{tm1EUR}*) for the most common mutation in CF patients (F508del CFTR), generated in our laboratory (21). These mice display electrophysiological abnormalities and intestinal disease typical of CF (82). In the lung, enhanced lung mucin secretion (82), abnormal lung inflammation and fibrotic response to bleomycin (27, 45), excessive response to LPS and *Pseudomonas* infection (48), and naphthalene injury (11) were observed. Furthermore, lung epithelial cells in *Cftr^{tm1EUR}* mice show deficient autophagocytosis and accumulation of aggresomes, reportedly mediated by excessive production of reactive oxygen (42), which could affect the activity of key enzymes in S1P metabolism or intracellular S1P receptor systems.

Our data show that abnormal myeloid infiltration and delayed resolution of induced inflammation in *Cftr^{tm1EUR}* mice correlate with reduced lung tissue S1P levels, which can be partially corrected by chronic treatment with the SPL inhibitor LX2931.

METHODS

Animal studies. Breeding, maintenance, and genotyping of *Cftr^{tm1EUR}* F508del CFTR mice (F12 C57Bl/6 backcross) was performed as described previously (21, 81, 82). Mice were kept in sterile ventilated cages, with sterile normal chow and water ad libitum. Adult males and females were used for intervention studies. All experiments were performed with approval (DEC 138-11-09) of the Independent Committee on Ethical Use of Experimental Animals, Rotterdam, according to national and European Union guidelines. Oral LX2931 treatment was performed by adding 500 mg/l LX2931, obtained from Lexicon Pharmaceuticals, to the drinking water for the period indicated.

Salivary gland activity measurement (8) was performed as described in Ref. 82. Briefly, in mice under ketamine/xylasine/diazepam anesthesia, basal cholinergic secretion activity is inhibited by subcutaneous injection of atropine to the cheek (50 μl, 1 mM), and saliva is collected at 3-min intervals in 2 × 20-mm filter paper, measured by weighing the filter before and after collection. Adrenergic (CFTR-dependent) secretion is induced by subcutaneous injection of 50 μl, 1 mM atropine 50 μl, 100 μM isoproterenol and followed for at least four further 3-min intervals.

Gated microCT lung scanning was performed on a Quantum FX CT scanner (Perkin Elmer), under isoflurane anesthesia. CT scans were viewed and analyzed with Caliper Analyze software. Scoring of CT scans was performed in a five-grade binning mode by measuring the relative surface of the lung volume occupied by high-density areas, indicating focal inflammation as illustrated in Fig. 8.

Histology and quantitative analysis. Animals were terminated with a lethal dose of ketamine/xylazine/diazepam, blood was removed from the circulation by ventricle perfusion with PBS. Lungs lobes and other tissues were removed immediately and either fixed in 4% paraformaldehyde/PBS and processed for standard paraffin histology or snap frozen in liquid nitrogen for analysis. Hematoxylin-eosin, periodic acid-Schiff (PAS)/Alcian blue (AB), and immunohistology staining were performed using standard procedures. Quantification of alveolar space sizes and septum thickness was performed using a modification of the method described in Ramnath et al. (58). After illumination correction, the final air space/septum masks are obtained from the binarized channel images, and air spaces smaller than 300 pixels (31 μm^2) are removed. For measuring the septum thickness, the smallest distance between pairs of neighboring air spaces was used. The mean value of the aggregated vector of the pairwise distances defines a single septum thickness measure for the entire image. For defining the neighborhood relation between the air spaces, skeletonization of the identified septum region is performed. Consequently, the entire image domain is subdivided into connected regions separated by the obtained skeleton. Since each of the obtained regions contains only one air space, both the air space and the region that contains it are assigned the same unique label. Two air spaces are considered as neighboring if the regions containing them are separated by a single skeleton edge.

Cytokine analysis by sequential ELISA was performed essentially as described by Van den Berg et al. (79). We analyzed cytokines in total lung lysates made from the unperfused upper right lung lobe. The total lung lysates were obtained by homogenizing the lung lobe, two times in the TissueLyser II (Qiagen) for 90 s at 30 Hz, in ice-cold PBS with protease inhibitor cocktail (Complete Mini, Roche), and stored at −80°C for further analysis. Cytokines were analyzed in total lung lysates by Sequential Enzyme-Linked Immuno Sorbent Assay (ELISA) (R&D Systems Europe, Abingdon, UK), and 96-well microtiter plates (Nunc Immunoplate, Neptune, NJ) were coated with anti-cytokine antibody (Duoset, R&D Systems, Minneapolis, MN) diluted 1,000× in PBS (50 μl/well) overnight rotating at 300 rpm at 4°C. Plates were washed with PBS 0.05% Tween 20, incubated for 1 h with blocking solutions: Blocker Blotto in TBS (eotaxin, MCP-1), Blocker Casein in PBS (KC, IL-6, IL-10, TNF-SR2, TNF-SR1, IL-1ra, IL-6R, IL-P70, MIP-2, IFNγ), and Super Blocking Buffer in PBS (TNF-α, IL-1β) (150 μl/well, Pierce, Rockford, IL) at room temperature. After washing, the lung lysate samples diluted in PBS with 0.05% Tween 20 and standards (Duoset, R&D Systems) were incubated for 2 h at room temperature on a plate shaker at 300 rpm. Then the samples were removed and stored in a 96-deep-well polypropylene microtiter plate for further sequential use. After transfer, the plates were washed and the polyclonal biotinylated detection antibody in dilution buffer was added (Duoset, R&D Systems) (50 μl/well, 300 rpm). The plates were washed and incubated for 30 min with streptavidin-conjugated horseradish peroxidase (Bio-Rad), diluted 1:10,000 in PBS with 0.1% BSA and 0.05% Tween 20 (50 μl/well, 300 rpm), followed by 10–40 min incubation with the TMB peroxidase substrate system (KPL, Gaithersburg, MD) (1:1, 100 μl/well). The reaction was stopped with 1.0 M sulfuric acid (100 μl/well), and absorbance was measured using a plate reader (Bio-kinetics, BioTek) at 450 nm. Data are expressed as picograms

per milligram protein. Protein concentration in lung tissue samples were determined by BCA kit (Pierce).

S1P and sphingosine measurements. Lipids extracted from the unperfused middle lung lobe of *Cftr*^{tm1EUR} mutant animals and littermate controls aged 33–35 wk kept on normal chow were purified similarly as described previously (9). Briefly, tissues are spiked with internal standards, homogenized in organic solvent [chloroform:methanol 2:1 (vol/vol) and butylated hydroxyl anisole]. One volume of cold water is added to the mixture and samples are mixed for 90 min at 4°C. The organic phase is collected, to which diethyl ether is added to remove protein contamination. The organic phase is dried and resuspended in 100 μl of chloroform, dried and resuspended in petroleum ether, purified by thin-layer chromatography (TLC), and detected by iodine. After scraping from the TLC plate, the amount of recovered lipids per sample was normalized according to the protein content in the initial samples measured via the bicinchoninic assay and then by normalization to same amount of inorganic phosphate in each of the analyzed samples. Final amounts were expressed in picomoles per nanomole of total phospholipid phosphate present in each analyzed sample. Contents of S1P and sphingosine in the samples were analyzed on a Thermo-Finnigan Trace DSQ fast-scanning single-quadrupole mass spectrometer using electron impact ionization. The instrument was tuned and calibrated for mass resolution and mass accuracy on a daily basis. Accurate mass determination and MS/MS fragmentation (LC/MS and LC/MS/MS): the LC/MS portion of the platform was based on a Waters ACQUITY UPLC and a Thermo-Finnigan LTQ-FT mass spectrometer, which had a linear ion-trap front end and a Fourier transform ion cyclotron resonance mass spectrometer back end. For ions with counts greater than 2 million, an accurate mass measurement could be performed. Accurate mass measurements could be made on the parent ion as well as fragments. The typical mass error was less than 5 ppm. Ions with less than 2 million counts require a greater amount of effort to characterize. Fragmentation spectra (MS/MS) were typically generated in a data-dependent manner. S1P measurements in unperfused lung tissues from LX2931-treated mutant mice were performed by LC-MS at Lexicon Pharmaceuticals laboratories, using procedures described previously (5).

Flow cytometry. For flow cytometry we obtained anti-mouse Ly6C-FITC (clone AL-21), SiglecF-PE (clone E50-2440), Ly6G-PE (clone 1A8), CD103-PE (clone E50-2440), CD19-APC_Cy7 (clone ID3), and CD64−AF647 (clone X54-5/7.1) from Becton Dickinson (BD). Anti-mouse CD11b− Horizon450 (clone M1/70), CD11c− PE_Cy7 (clone N418), CD3-APC_Cy7 (clone I45-2c11) and MHCII-APC_Cy7 (clone I3/2.3), and inducible nitric oxide synthase (NOS2/iNOS) PE-cyanine7 (clone CXNFT) were obtained from eBioscience. Anti-mouse CD45-PE_TxRed (clone M5-114.5) was obtained from BioLegend, and arginase-1-FITC (polyclonal sheep IgG) from R&D Systems. The life-dead marker Aqua-AmCyan was obtained from Invitrogen. Lungs were digested using RPMI containing Liberase TM (Roche) and 10 U DNAse (Roche). Cell suspensions were lysed for 4 min at room temperature in 1 ml osmotic lysis buffer. All staining reactions were performed at 4°C for 30 to 45 min. Cells were incubated with 2.4G2 Fc receptor antibody to reduce nonspecific binding, together with monoclonal antibodies. To facilitate intracellular staining of arginase-1 and iNOS, cells were fixed and permeabilized using the Fixation/Permeabilization Solution Kit of BD Biosciences. Cell data acquisition took place on a LSRII flow cytometer (BD). Gating strategy of CD45+ cells was as follows: granulocytes (CD11b+Ly6G+Ly6C−), macrophages (CD11c+CD11b−MHCII− SiglecF+); Ly6C^{high} monocytes (CD11c−MHCII−CD11b+LyC6high+); B cells (CD19+ MHCII+ CD11c−); T cells (CD3+ MHCII−). Dendritic cells (DC, CD11c+MHCII+); conventional DC (cDC: CD11c+ MHCII+ CD11b+ CD64−); monocyte-derived DC (MoDC: CD11c+ MHCII+ CD11b+CD64+); CD103+ DC (CD11c+ MHCII+ CD11b−).

RESULTS

Reduced sphingosine-1-P in Cftr^{tm1EUR} *mouse lung.* We analyzed lipid profiles by gas chromatography-mass spectrometry (GC-MS) in whole lung tissue from adult male mutant C57Bl/6 *Cftr*^{tm1EUR} F508del CFTR mice. Lipids extracted from the unperfused middle lung lobe of mutant animals ($n = 10$) and littermate controls ($n = 8$) aged 33–35 wk, kept on normal chow, were analyzed in parallel as described in METHODS. We observed a reduction of total sphingosine species and in particular a threefold reduction of S1P in the lungs of unchallenged adult *Cftr*^{tm1EUR} mutant mice compared with wild-type (Fig. 1).

Abnormal infiltration of myeloid cells and DC imbalance in F508del CFTR mouse lung. The reduction of lung S1P homozygous mutants in this strain correlates with enhanced mucus production, low-level basal lung inflammation, and enhanced response to bleomycin, lung injury, and *Pseudomonas aeruginosa* infection (27, 42, 45, 48, 82), suggesting a causal relationship between abnormal S1P tissue levels and CF lung pathology. Therefore, we investigated the effect of the SPL inhibitor LX2931 on basal and induced inflammation in this model. First, we established the basal myeloid cell profile in total lung by FACS analysis. The total number of CD45$^+$ cells in F508del mutant mice is significantly higher than in wild-type littermates (Fig. 2*A*). This can be attributed mainly to a significant increase in total granulocytes (Fig. 2*B*) and to a lesser extent alveolar macrophages (Fig. 2*C*) and Ly6C^{high} monocytes (Fig. 2*D*). The total number of B cells and T cells is increased in mutant mice compared with wild-type, adding substantially to the total number of CD45+ cells (Fig. 2, *E* and *F*). The total number of DCs is higher on average but not significantly different from normal (Fig. 3*A*). Importantly, however, we show here for the first time that the percentage of CD11b+ cDC is reduced in F508del CFTR mutant mouse lung compared with normal (Fig. 3*B*), whereas we observe a significant increase in the percentage CD64+ MoDC, also known

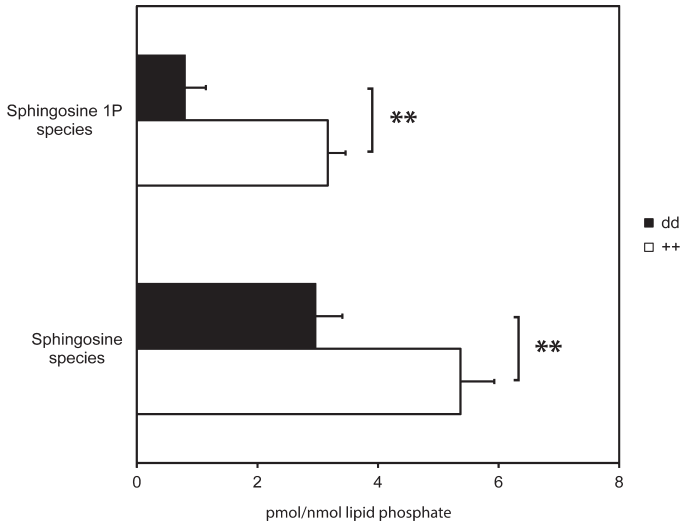

Fig. 1. Sphingosine and sphingosine-1-phosphate (Sphingosine 1P) in F508del CFTR mouse lung. Lipids were extracted from total unperfused middle lung lobes from homozygous mutant mice (dd, $n = 10$) and wild-type littermates (WT, $n = 8$) aged 33–35 wk, kept on normal chow, and analyzed by LC-MS as described in METHODS. Data are shown as pmol/nmol total lipid phosphate. Average ± SD; $^{**}P < 0.01$, unpaired *t*-test.

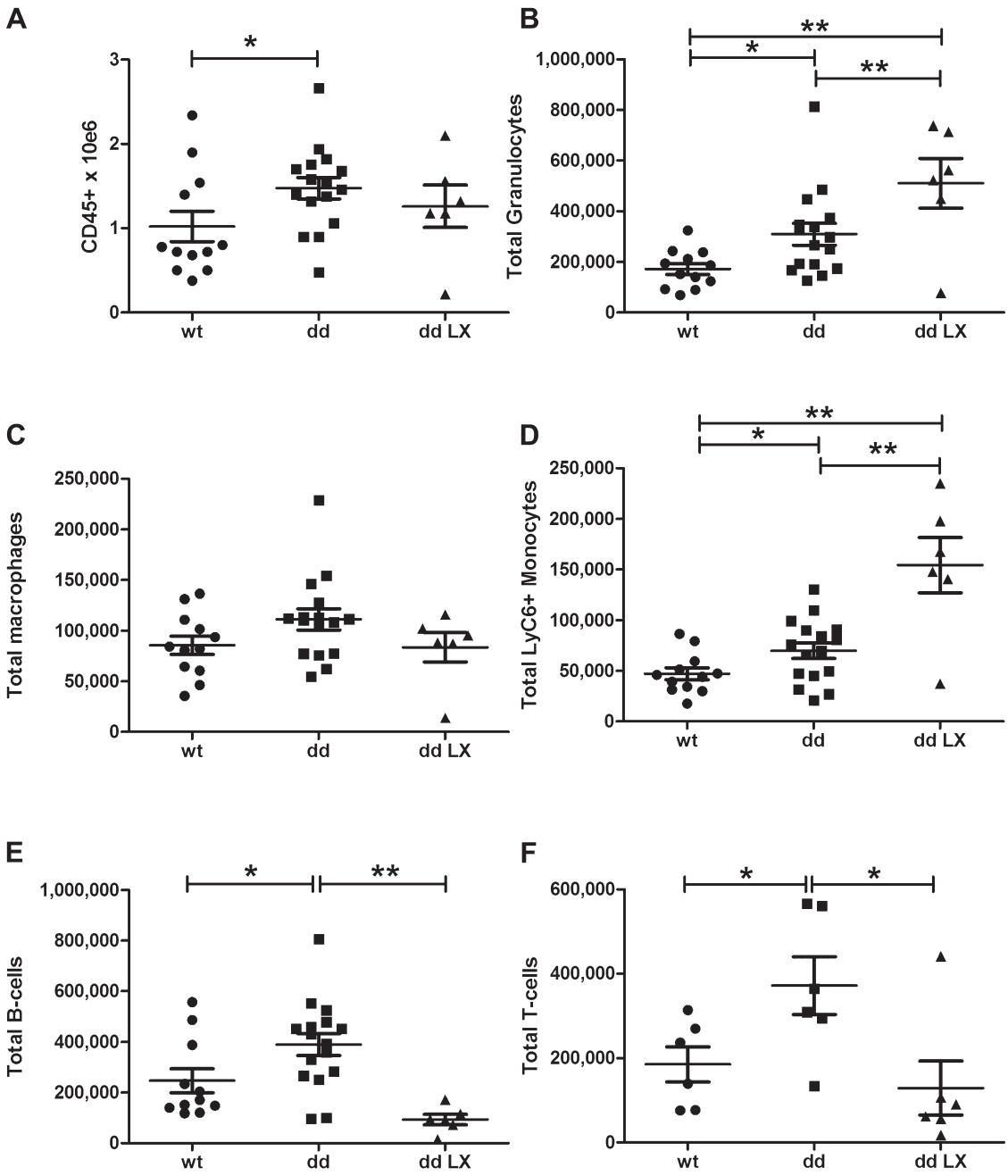

Fig. 2. Abnormal myeloid infiltration in F508del CFTR mutant mouse lung. Total lung from wild-type (WT; $n = 12$), mutant (dd; $n = 15$), and mutant mice treated with SP-1 lyase inhibitor LX2931 in drinking water (dd LX; $n = 6$) were analyzed by FACS to quantitate myeloid subpopulations as described in METHODS. Total cell counts per lung (average ± SE) are shown for myeloid cells (CD45+; *A*), granulocytes (CD11b+Ly6G+ Ly6C−; *B*), macrophages (CD11c+CD11b−MHCII− SiglecF+; *C*) LyC6^{high} monocytes (CD11c− MHCII− CD11b+ LyC6+; *D*), B cells (CD19+ MHCII+ CD11c−; *E*), and T cells (CD3+ MHCII−; *F*). $^{**}P < 0.01$, $^{*}P < 0.05$; 1-way ANOVA.

as inflammatory DC (Fig. 3*C*). In contrast to Xu et al. (85) in CFTR KO mice, we did not observe reduced CD103+ DC in our F508del CFTR mutant mice, either total numbers (not shown) or as a percentage of DC (Fig. 3*D*).

We conclude that unchallenged adult mutant *Cftr^{tm1EUR}* mice under our conditions show enhanced myeloid cell infiltration and an increased MoDC/cDC ratio, compared with wild-type littermates, consistent with sterile lung inflammation, which correlates with reduced S1P levels in the lung.

An S1P lyase inhibitor corrects abnormal MoDC/cDC ratio in F508del CFTR mutant. To establish the role of S1P in this hyperinflammatory phenotype of mutant mice, and the therapeutic potential of pharmaceutical intervention in this pathway, we treated adult F508del CFTR mutant mice with the SPL inhibitor LX2931 in drinking water for 2 wk. As expected (5), this treatment caused a near-hundredfold increase in tissue S1P levels in total lung tissue measured by LC-MS [0.3 vs. 55 ng/mg, homozygous mutant (dd) vs. dd treated with LX2931,

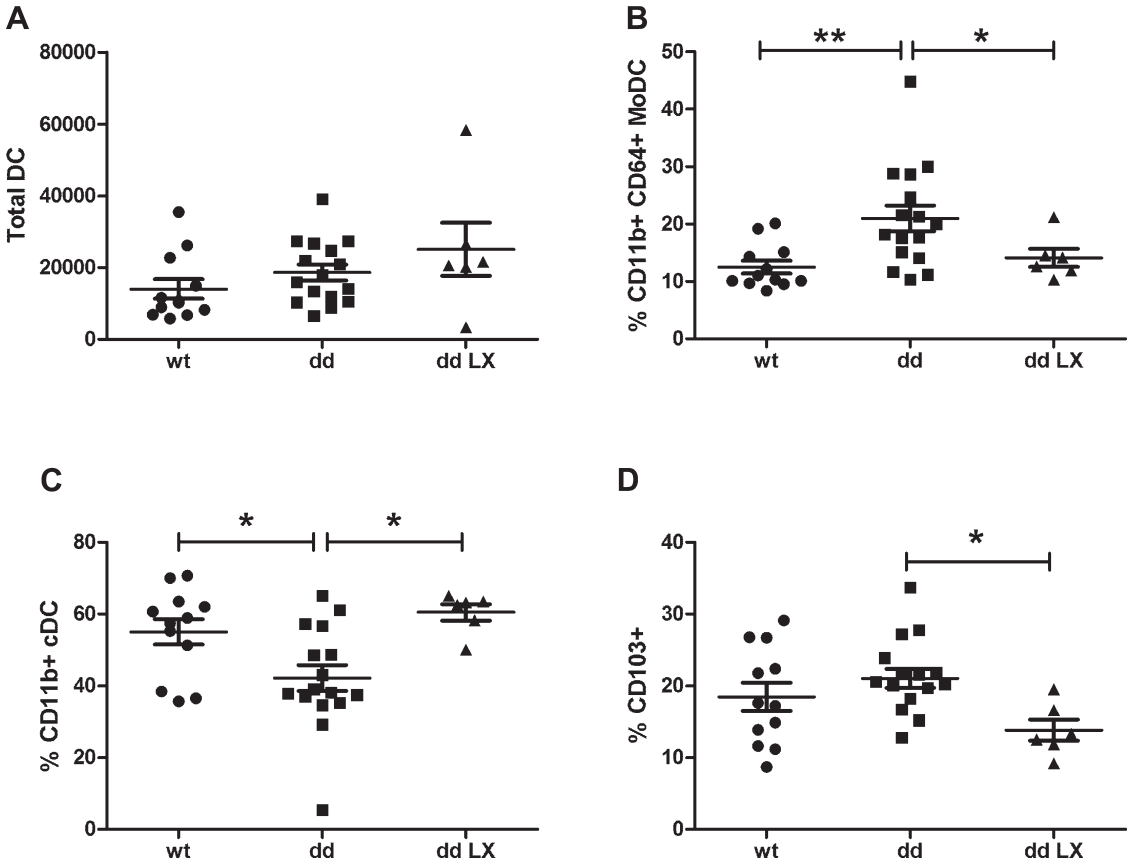

Fig. 3. Abnormal dendritic cell population in F508del CFTR mutant mouse lung. Lung homogenates from wild-type (WT; $n = 12$), mutant (dd; $n = 15$), and mutant mice treated with SP-1 lyase inhibitor LX2931 in drinking water (dd LX; $n = 6$) as described in Fig. 2 were analyzed for markers of dendritic cells (DC). *A*: total DC per lung (CD11c+MHCII+). *B*: percentage monocyte-derived DC of total DC (MoDC, CD11c+ MHCII+ CD11b+ CD64+). *C*: percentage conventional DC (cDC, CD11c+ MHCII+ CD11b+ CD64−). *D*: percentage CD103+ DC (CD103+ CD11C+ MHCII+ CD11b−). Average ± SE, $^{**}P < 0.01$; $^{*}P < 0.05$, 1-way ANOVA.

$n = 10$ in each group, $P < 0.001$]. Total white blood cell counts were reduced by LX2931 treatment in mutant mice (4.36 ± 0.45 vs. $2.63 \pm 0.3 \times 10^9$/l, dd vs. dd treated with LX2931, $n = 10$ in each group, $P < 0.01$), no morbidity or mortality was observed in any of the LX2931-treated mutant animals. These data are comparable to those previously reported in wild-type mice with this compound (5). White and red blood cells in untreated mutant mice were not significantly different compared with normal littermates (3.9 ± 0.5, $n = 10$). Red blood cells in LX2931-treated mutant mice were comparable with normal and untreated mutant.

FACS analysis of myeloid cells from the lungs of LX2931-treated mutant animals in parallel with untreated animals revealed a complex response. First, we observe no effect on total CD45+ (Fig. 2*A*) or total DC cell counts (Fig. 3*A*) in the lung. However, we see an increase in cDC and a concomitant reduction of MoDC. Thus LX2931 corrects the abnormal MoDC/cDC composition in mutant mice to wild-type levels (Fig. 3, *B* and *C*). Furthermore, LX treatment reduced the percentage of CD103+ DC (Fig. 3*D*), which are considered important regulators of the airway immune response (16, 34).

Remarkably, cells gated as granulocytes and Ly6C+ monocytes are significantly increased by LX2931 (Fig. 2, *B* and *D*). Consistent with this increase, the myeloid attractant MCP-1 was moderately enhanced in lung tissue of mutant mice by LX2931 treatment, but not MIP-2, eotaxin, IFNγ, and IL12p70 (Fig. 4).

The LX2931-mediated influx of granulocytes and Ly6C+ monocytes may represent myeloid-derived suppressor cells (MDSC), defined as CD11b+ Gr1/Ly6G+ myeloid cells. These cells are considered anti-inflammatory by suppressing NK cell activity and T cell proliferation (59, 66). In a recent study, Liu et al. (39) have shown that MDSC recruitment to the concanavalin A-induced inflamed liver in mice is enhanced by the S1P receptor modulator FTY720, reducing hepatic injury. Therefore, we stained in FACS analysis CD45+ cells from total lungs of F508del CFTR mutant mice, untreated and treated with LX2931 ($n = 6$ in each group) for the MDSC markers arginase (ARG1) and iNOS/NOS2. However, we did not observe staining for ARG1 in any of the myeloid subpopulations, including Ly6C+ monocytes, ruling out significant recruitment of MDSC in response to LX2931 (data not shown).

Interestingly, we observed a distinct iNOS+ granulocyte population in unchallenged mutant mice (Fig. 5), which was not detected in normal mice ($n = 3$, not shown). This confirms basal lung inflammation in mutant mice. This iNOS+ granulocyte population in mutant mice was significantly reduced by LX2931 treatment (Fig. 5), suggesting a reduction of lung inflammation.

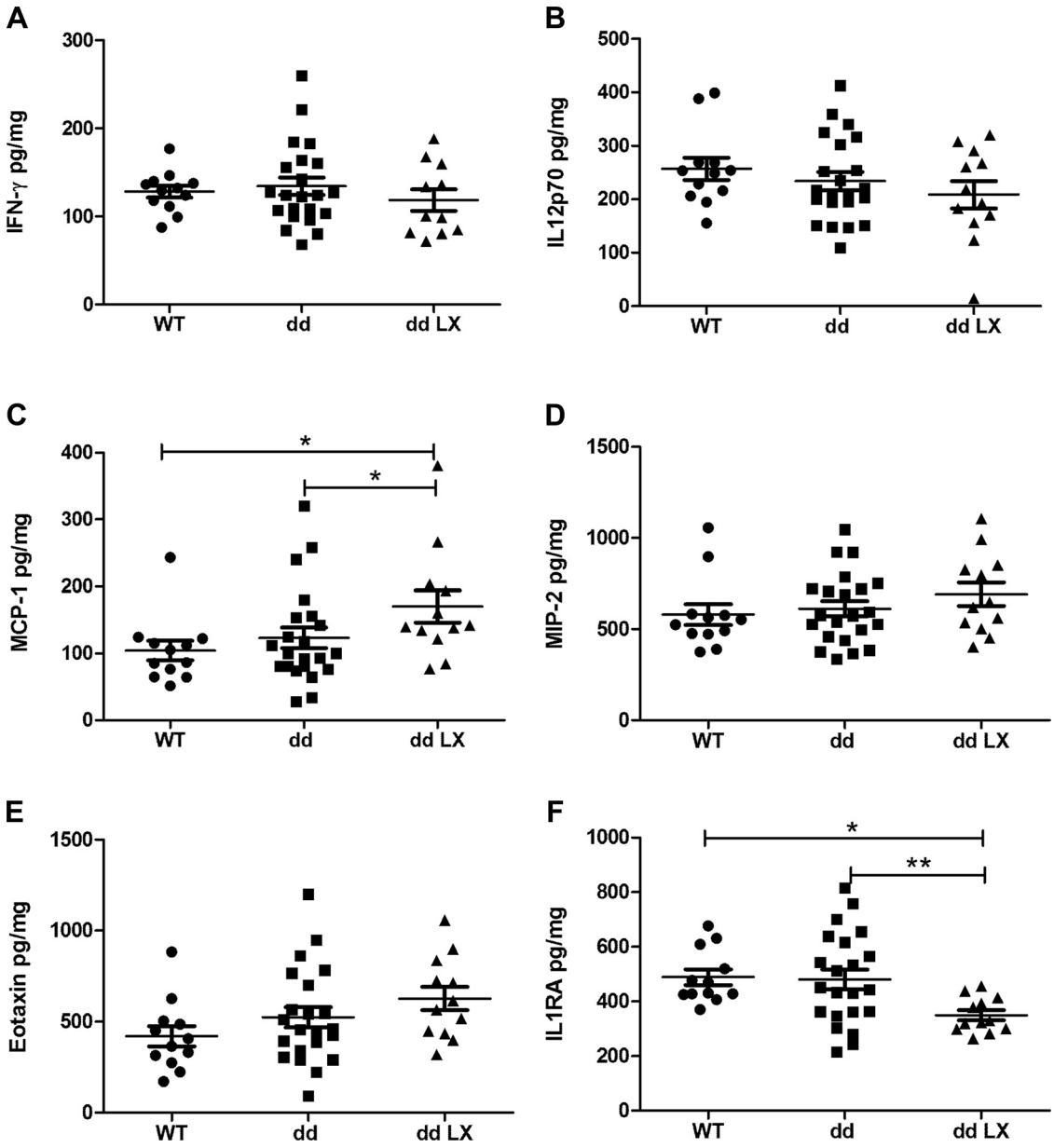

Fig. 4. Total lung cytokines in unchallenged F508del mutant and LX2931-treated mice. Total lung extracts of untreated adult mutant mice (dd) or treated with LX2931 (dd LX), compared with wild-type littermates (WT), were analyzed in parallel by sequential ELISA as described in METHODS. Data are expressed as pg/mg protein. Average ± SE, $^{**}P < 0.01$; $^{*}P < 0.05$, 1-way ANOVA.

Furthermore, LX2931 treatment caused a strong reduction of the enhanced B cells and T cells counts in mutant lung, even below values observed in normal mice (Fig. 2, *E* and *F*). The relevance of these results for CF pathology will be discussed below.

S1P lyase inhibitor does not recover F508del CFTR epithelial fluid transport activity. It has been postulated that inflammation and associated cellular stress reduces the efficiency of F508del CFTR trafficking or conversely that inefficient folding of mutant CFTR causes oxidative stress and defective membrane trafficking of murine and human F508del CFTR (14, 43). S1P has also been linked to inflammation, autophagy, and oxidative stress (26, 55). Therefore, we considered that modulation of S1P signaling could have an effect on F508del CFTR activity. We have investigated this in a separate experiment using several independent assays.

Consistent with previous data, adult (80–115 days) untreated male and female mutant mice were on average 10% underweight (Table 1) compared with age- and sex-matched wild-type littermates, primarily due to enterocyte CFTR deficiency (82). Two weeks of LX2931 treatment does not significantly affect the increase in body weight compared with untreated mutant mice. This confirms that LX2931 treatment does not negatively affect the health of the mice, and also that no major rescue of intestinal CFTR activity occurs under our conditions. Isoproterenol-induced salivary gland fluid secretion activity is severely affected in F508del CFTR mutant mice (82). LX2931-treated mutant mice on average did not show a

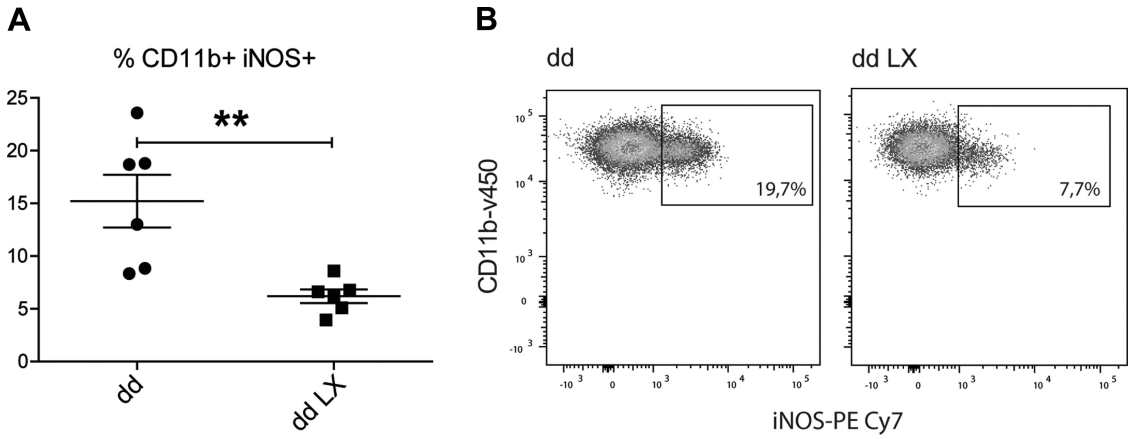

Fig. 5. SP-1 lyase inhibitor (LX2931) reduces iNOS expression in granulocytes from F508del CFTR mouse lung. In a separate experiment, lungs from unchallenged adult F508del CFTR mutant mice ($n = 6$) were analyzed by FACS, as in Figs. 2 and 3. *A*: percentage of granulocytes gated as iNOS+ were threefold higher in untreated mutant mice compared with mutant mice treated with LX2931 (**$P < 0.01$, unpaired *t*-test). *B*: representative scatter graphs of untreated mutant (dd) and LX2931-treated mutant (dd LX) show the distinct iNOS+ granulocyte subpopulation, which is absent in normal littermates ($n = 3$) and isotype control (not shown).

significant increase in isoproterenol-induced fluid secretion (Fig. 6) Furthermore, we report here for the first time frequent plugging of ducts in the parotic salivary gland with mucous material in F508del CFTR homozygous mice, which is virtually absent in wild-type mice. This is not observed in the submandibular and sublingual glands. Treatment with LX2931 for 3 wk does not significantly affect parotic duct plugging in mutant mice (Fig. 7).

We conclude that increasing S1P tissue levels by LX2931 treatment does not significantly affect F508del CFTR channel activity and associated fluid transport in secretory epithelia in this model. Changes affecting the behavior of myeloid cells cannot be excluded, however (10, 35), and are the subject of current studies.

S1P lyase inhibitor does not interfere with bacterial toxin-induced myeloid infiltration in F508del CFTR mice. To establish whether LX2931 has an effect on bacterial toxin-induced inflammation in mutant mice, we treated mutant and wild-type mice intranasally with bacterial liposaccharides (LPS) in combination with the modified peptide fMLP, that is required for full polymorphonuclear leukocytes activation and neutrophil elastase secretion (13).

Mutant mice, both untreated and LX2931 treated, respond to LPS/fMLP with a substantial weight loss (10–20%) 48 h after challenge, whereas wild-type mice suffered only a minor change (Table 1). Two of 13 untreated mutant animals did not survive the first week after treatment; no mortality was observed in the LX2931-treated group. One week after challenge, recovery of body weight is not complete in untreated and LX2931-treated mutant animals, in contrast to normal (Table 1). We conclude that F508del CFTR mutant mice are more susceptible to LPS/fMLP induced weight loss than wild-type, in agreement with previous findings using LPS only and *Pseudomonas aeruginosa* challenge (48). Furthermore, LX2931 treatment does not prevent this aspect of acute LPS-induced pathology.

Transient lung inflammation was monitored by microCT scanning before, 48 h after and 1 wk after challenge (Fig. 8). In all LPS/fMLP-treated animals we observed high-density areas covering 0–40% of the left and right lung lobes, indicating large areas of intense focal inflammation. In unchallenged mutant and wild-type animals this was not observed (not shown). Using a five-grade scoring scheme, microCT scores were high in all groups at 48 h (Fig. 8). One week after challenge, no remaining foci were observed in the wild-type group, whereas in a minority of the of the untreated and LX2931-treated mutant animals low but detectable focal microCT scores were still present. Thus by this method we find

Table 1. *Effect of LPS/fMLP and LX2931 on mouse body weight*

	N	Age, days (SD)	Initial weight, g (SD)	% Weight change 2 wk	% Weight change LPS 48 h	% Weight change LPS 7 days
dd female	7	81.4 (23.5)	18.3* (2.3)	4.2 (6.6)	−8.0* (8.9)	−0.6 (3.2)
dd male	6	112.5 (46.3)	22.3* (1.2)	9.4 (11.0)	−13.2* (8.2)	−8.2* (2.1)
dd female LX	5	90.2 (25.8)	17.8* (3.7)	15.9 (7.6)	−19.1* (2.7)	−12.9* (8.2)
dd male LX	7	90.2 (25.8)	22.8* (1.9)	9.8 (4.4)	−11.2* (5.3)	−12.9* (8.2)
WT female	12	95.0 (19.5)	20.9 (0.9)	4.3 (4.7)	0.5 (6.4)	0.1 (8.7)
WT male	8	115.6 (47.4)	25.8 (2.0)	5.0 (4.8)	−0.6 (6.3)	0.7 (4.5)

Initial body weight of age- and sex-matched F508del mutant (dd) and normal littermates (WT) were monitored in parallel. Data are presented are presented as average (± SD). A subset of the dd mice were treated with LX2931 in the drinking water, as described in METHODS. After 2 wk the percentage change in body weight was recorded (% weight change 2 wk). Subsequently all mice were treated intranasally with LPS/fMLP, and the change in body weight was determined after 48 h and 1 wk. Untreated male and female mutant mice are ~10% underweight compared with age- and sex-matched littermates, consistent with previous data, primarily due to intestinal disease. Two-week LX2831 treatment does not significantly affect average body weight of dd mice. LPS/fMLP challenge causes a reduction of body weight in mutant but not in normal animals after 48 h, but no mortality (*$P < 0.01$ compared with sex-matched WT, ANOVA-Tukey). One week after challenge, we observe an incomplete recovery of body weight in mutant mice. Treatment with LX2931 does not significantly affect this response. This suggests that LX2831 does not substantially correct mutant CFTR activity, nor does it have adverse effects during basal of challenge conditions.

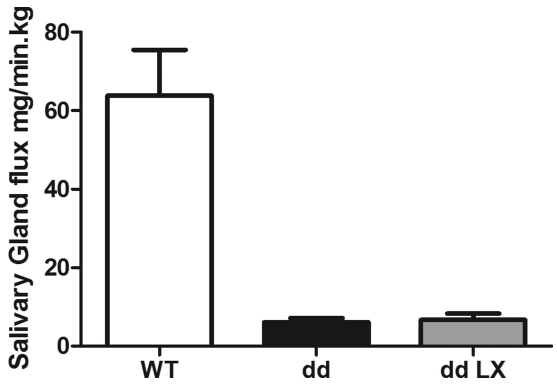

Fig. 6. Salivary gland activity in LX2931-treated F508del mutant mice is not affected. LX2931-treated mutant mice (dd LX) did not show a significant increase in cAMP-induced salivary gland activity ($mg \cdot min^{-1} \cdot kg^{-1}$) compared with untreated mutant (dd), which is severely affected compared with wild-type littermates (WT) in F508del CFTR mice (1-way ANOVA, Tukey's test) (82). Isoproterenol-induced salivary gland fluid secretion was measured in parallel in adult mutant mice that were untreated (dd, $n = 6$) or treated with LX2931 (dd LX $n = 7$), compared with wild-type littermates (WT, $n = 4$). Data are expressed as $mg \cdot min^{-1} \cdot kg^{-1}$ body wt (average ± SE).

no evidence for a reduction of focal myeloid infiltration by LX2931 in mutant animals either in acute phase or during resolution.

Whereas focal infiltration was transient, an increase in thickness and apparent density of the septa of the alveolar region is observed in the microCT scans of all experimental groups 1 wk after challenge (not shown). This is confirmed by quantitative histology of all mice 2 wk after challenge. Inflammatory scores for septal infiltration, focal inflammation, and luminal monocytes were high compared with unchallenged in all experimental groups. In quantitative analysis of paraffin sections, septum thickness is also increased by LPS/fMLP challenge in mutant and wild-type mice compared with untreated (Fig. 9). Remarkably, this parameter is significantly higher in LX2931-treated mutant mice compared with untreated mutant, suggesting an increase in myeloid infiltration compared with untreated mutant 2 wk after LPS challenge, consistent with the microCT data (Fig. 8).

LPS/fMLP induced Muc5AC expression is reduced by LX2931. Unchallenged untreated or LX2931-treated mutant and wild-type mice do not show goblet cells in the bronchi by PAS/AB staining of paraffin sections. In contrast, distinct bronchial goblet cell hyperplasia is observed 2 wk after LPS/fMLP challenge in all experimental groups to a comparable extent (Fig. 10, *A* and *B*). LX2931 treatment did not significantly reduce bronchial goblet cell numbers. Importantly, we observed a higher expression of the inflammation-responsive mucin mMUC5AC in mutant mice compared with wild-type in total lung extracts from LPS/fMLP-challenged mice (Fig. 10*C*). In LX2931-treated mutant mice a significant reduction toward wild-type values was observed. This suggests that LX2931 does not interfere with inflammation-induced goblet cell hyperplasia in this model but does reduce the expression of inducible gel-forming mucins, which is of considerable importance for CF pathology.

High LPS/fMLP induced cytokine levels in mutant mice restored by LX2391. We measured a panel of cytokines known to be involved in the resolution of inflammation by sequential ELISA in total lung homogenates of mutant and wild-type mice, 2 wk after challenge (Fig. 11). In F508del CFTR mutant mice we report here for the first time a twofold IFNγ and fourfold interleukin 12 (IL-12p70) level compared with wild-type 2 wk after LPS/fMLP challenge (Fig. 11, *A* and *B*). Also,

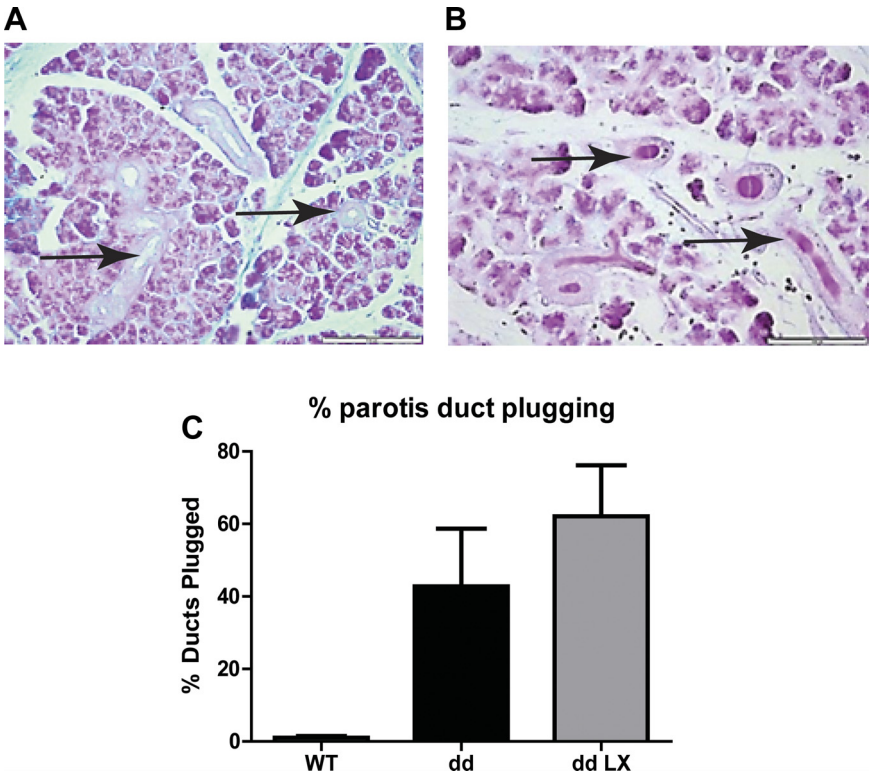

Fig. 7. LX2931 does not rescue parotic duct plugging in F508del CFTR. Paraffin sections of parotic glands were stained for mucus by PAS stain, from adult mutant mice that were untreated (dd, $n = 7$) or treated with LX2931 as described (dd, LX; $n = 9$), compared with wild-type littermates (WT, $n = 5$). In normal mice major ducts (arrows) were free of mucous material (*A*), whereas in F508del CFTR mice variable but frequent plugging with PAS positive material was observed (arrows) (*B*). This plugging was not observed in the submandibular and sublingual glands of mutant mice (not shown). *C*: multiple (3–6) nonsequential low sections of each mouse were analyzed and the percentage of plugged vs. open ducts was calculated. The graph shows the average per experimental group ± SE. Plugging is significantly higher in mutant mice compared with WT (Kruskal-Wallis/Dunn's test). Treatment with LX2931 has no significant effect on parotic duct plugging.

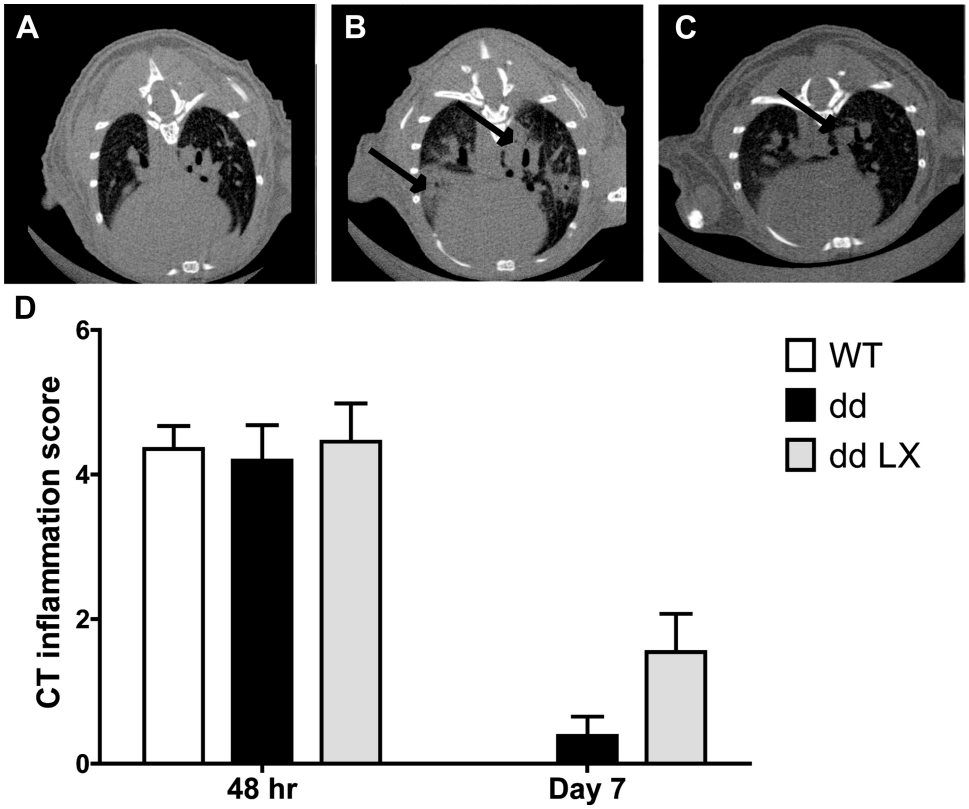

Fig. 8. Focal inflammation induced by bacterial toxins analyzed by microCT. Gated lung microcomputed tomography (microCT) scans on a Quantum FX CT scanner (Perkin Elmer) under isoflurane anesthesia was performed on all animals treated with LPS/fMLP as described in METHODS. CT scans were viewed and analyzed using Caliper Analyze software. Scoring of CT scans was performed in a 5-grade binning mode by measuring the relative surface of the lung volume occupied by high-density areas (arrows), as in the representative figures obtained with a F508del CFTR mutant mouse before (*A*), 48 h after (*B*), and 1 wk after (*C*) intranasal LPS/fMLP challenge of each animal. *D*: quantitative scoring of focal inflammation. Wild-type (WT, $n = 17$), homozygous F508del CFTR mutant (dd, $n = 14$), and homozygous mutant orally treated with LX2931 (dd LX, $n = 11$) for 3 wk prior to challenge and 2 wk after challenge (average score ± SE). Before-challenge scores were zero in all animals (not shown). At 14 days lungs were collected for quantitative histology and ELISA (Figs. 9–11).

TNFα, IL-10, and KC showed increased levels in mutant mice after challenge (Fig. 11, *C–E*).

Importantly, the high IFNγ, IL12P70, KC, and IL-10 levels 2 wk after challenge are corrected toward normal values by LX2391 treatment (Fig. 11). TNFα shows a similar trend, but this does not reach statistical significance in this experiment. A notable exception to this pattern is the increase of the monocyte attractant MCP-1 (CCL2) induced by LX2931 (Fig. 11*F*), which is increased by LX2931 treatment. This is consistent with the enhanced myeloid infiltration after LPS/fMLP challenge (Figs. 8 and 9).

We conclude that resolution of LPS/fMLP-induced inflammation is delayed in F508del mutant mice compared with normal. Furthermore, LX2391 treatment corrects the enhanced expression of several, but not all proinflammatory cytokines under these conditions.

DISCUSSION

Abnormal dendritic cell ratio and myeloid infiltration in CF mouse lung. Unchallenged *Cftr*^{tm1EUR} F508del CFTR mice kept under pathogen-free conditions display low-level lung inflammation (36, 43, 82). In this model we show here an enhanced level of myeloid cell (CD45+) infiltration, mainly granulocytes and B and T cells (Fig. 2), and low levels of cDC, whereas MoDC are increased (Fig. 3, *B* and *C*). MoDC arise from Ly6cHi monocytes at inflammatory sites (56) and play a proinflammatory role via the production of chemokines with chemotactic activity for effector T cells, monocytes and granulocytes (53). Therefore, the presence of high MoDC numbers in the lung may perpetuate inflammation in these mutant mice.

In addition, we observed a distinct iNOS-expressing granulocyte population that was not present in wild-type mice (Fig. 5). iNOS expression is associated with inflammation and tissue damage. This suggests that the iNOS+ granulocyte population in mutant mice is an aspect of basal inflammation caused by CFTR deficiency. A third subset, CD103+ DC, involved in antiviral responses (17), is not significantly different from normal in F508del CFTR mice (Fig. 3, *A* and *D*). Xu et al. (85) reported an increase in the percentage of CD11c+ CD11b+ DC in CFTR KO mice, which does not discriminate between CD64− cDC and CD64+ MoDC as in our approach. In contrast to our results (Fig. 3*D*), they reported a reduced percentage of CD103+ DC (CD11C+ MHCII+ CD11b−). The apparent discrepancy could be related to strain or husbandry differences. However, a direct comparison of these data sets is difficult, since Xu et al. do not report absolute counts and the use of a marker to exclude dead cells from analysis.

In CF infants with early-stage lung disease, CT scans show bronchial thickening and distal air-trapping even in the absence of apparent bacterial infection (41, 68), suggesting unprovoked inflammation in CF lung. In newborn CF piglets, however, a CF-like lung pathology develops within months without enhanced neutrophil infiltration prior to infection, arguing against significant sterile inflammation in newborn CF pig lung (2, 6, 69). However, no detailed quantitative analysis of tissue DC and other myeloid populations by FACS was reported in early stages of disease in either CF pig or human. Therefore, abnormalities similar to what we report here in adult mutant mice could be involved in the development of chronic CF lung disease in human.

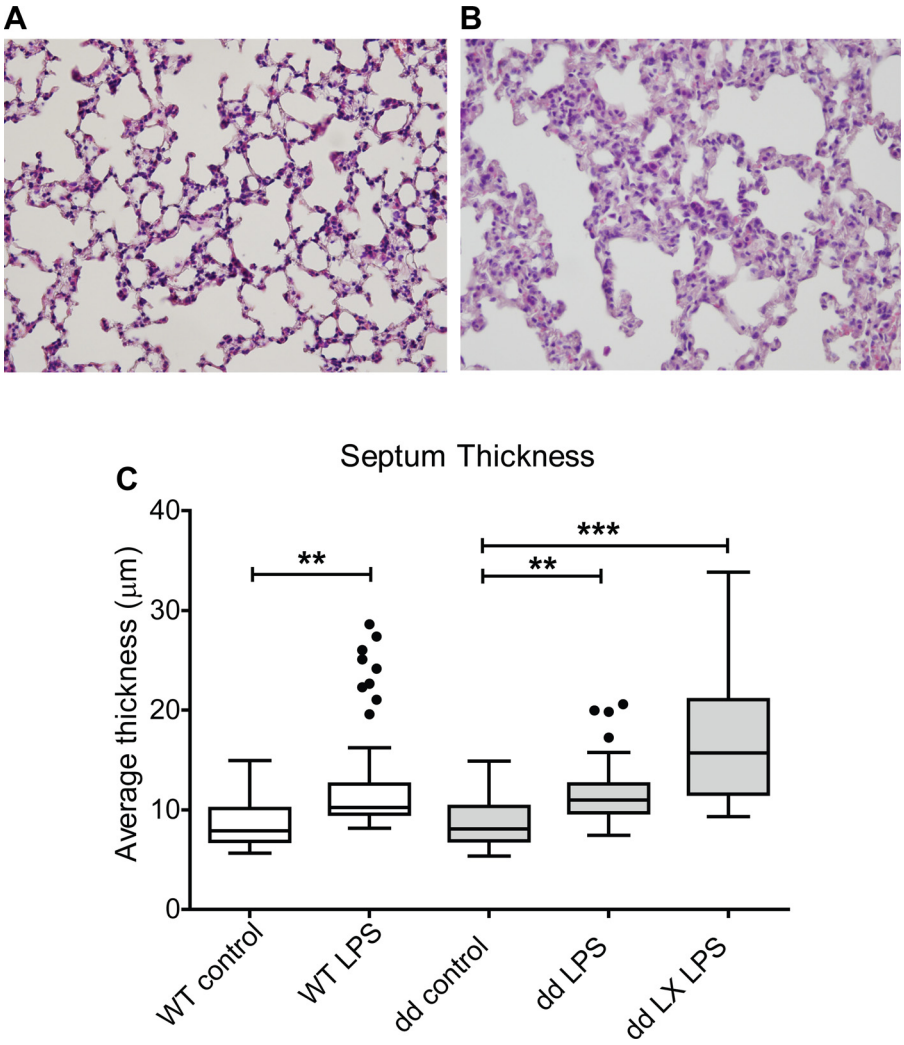

Fig. 9. Quantitative analysis of myeloid infiltration after LPS/fMLP challenge. Representative hematoxylin-eosin stain of normal mouse lungs before (*A*) and 2 wk after (*B*) LPS/fMLP challenge show sustained septum thickening, associated with appearance of ARG1-positive interstitial and luminal myeloid cells in all experimental groups (not shown). *C*: quantitative analysis of septum thickness, as described in METHODS, of LPS/fMLP treated and untreated mice as in Fig. 5. A significant increase in septum thickness was observed 2 wk after LPS/fMLP challenge in normal (WT), mutant (dd), and most strongly in mutant mice treated with LX2931 (dd LX) ($n = 9$ mice per experimental group, 3–4 nonadjacent sections per mouse were analyzed). $^{**}P < 0.01$, $^{***}P < 0.001$, 1-way ANOVA.

Sustained response to LPS/fMLP challenge in F508del mutant mice. Enhanced levels of proinflammatory cytokines, including TNF-α and KC 2 wk after LPS/fMLP exposure, show defective resolution of inflammation in F508del CFTR mutant mice (Fig. 11). This is consistent with earlier reports of enhanced and sustained lung pathology after naphthalene-injury (11), bleomycin (27), LPS, and *Pseudomonas aeruginosa* challenge (48) in this model.

Our present data show enhanced levels of both IFNγ and the active form of IL-12 (IL-12p70) in the lung of LPS/fMLP challenged mutant mice, 2 wk after exposure. IFNγ overexpression causes enhanced macrophage and neutrophil infiltration in mice (30). Th1-dependent IFNγ production is stimulated by IL-12, which in turn is secreted by triggered mature DC (30). Thus it seems likely that the high numbers of lung MoDCs in F508del CFTR mutant mice contribute to the delayed resolution of induced inflammation through activation of the IL12/IFNγ axis. Consistent with our finding, IFNγ is increased in BALF of young CF patients compared with non-CF (74).

IL-10 levels were high in mutant mice 2 wk after LPS/fMLP challenge compared with wild-type, which is also testimony to an abnormal resolution of inflammation. IL-10 is often considered an anti-inflammatory signal and therefore benign. However, this concept has been challenged in the context of chronic lung inflammation. Chronic overexpression of IL-10 causes a strong fibrotic response, likely through upregulation of MCP-1 (CCL2), enhanced myeloid infiltration, and a bias toward M2 macrophage differentiation (70). Proinflammatory macrophage polarization and accumulation were also observed in *Cftr*^{tm1EUR} (FVB) mice (45). Furthermore, a stronger fibrotic response to bleomycin-induced inflammation was reported in this strain, with enhanced myeloid infiltration and MCP-1 signaling (27). Both in the *Cftr*^{tm1EUR} and the IL-10 overexpression models isolated myofibrocytes displayed a profibrotic phenotype compared with control, suggesting that chronic lung inflammation in CF lung changes the epigenetic program of subepithelial connective tissue, involving the IL-10/MCP-1 axis.

Inflammation in Cftr^{tm1EUR} *mice correlates with low lung S1P levels.* The effect of CFTR deficiency on myeloid infiltration and DC imbalance (Figs. 2 and 3) and resolution of inflammation (Fig. 10 and 11) is not readily explained at the molecular level. The myeloid attractants MCP-1, MIP-2, and eotaxin were not significantly increased in unchallenged mutant mice under our basal conditions (Fig. 4). However, CFTR deficiency affects other processes that control myeloid cell behavior in the lung, in particular the production of bioactive

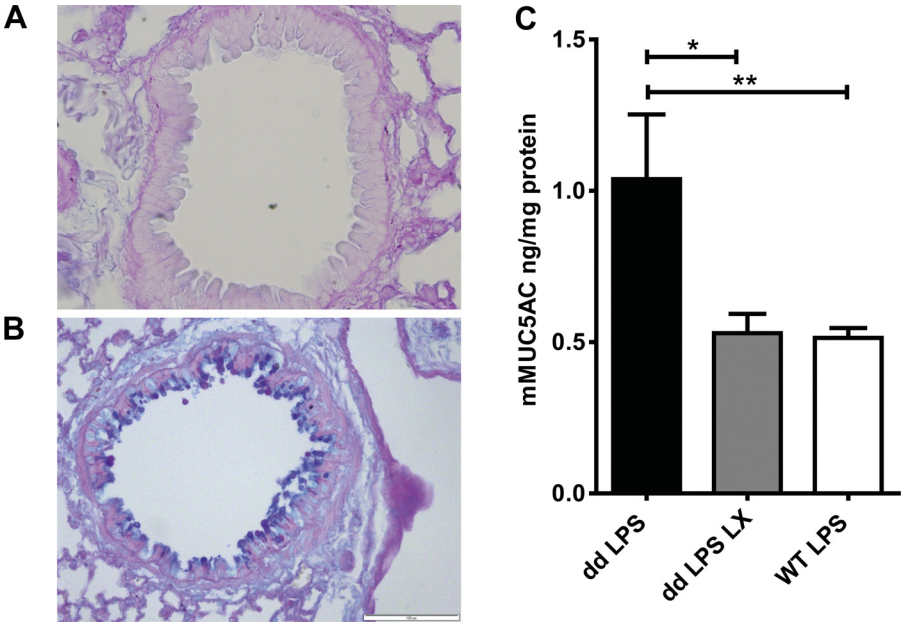

Fig. 10. Enhanced MUC5AC production in F508del CFTR mutant mouse lung after LPS/fMLP challenge is reduced by SP-1 lyase inhibitor (LX2931). Representative micrographs (hematoxylin-eosin/PAS staining) of unchallenged F508del CFTR mutant mouse (*A*) 2 wk after LPS/fMLP challenge (*B*) (bar: 100 μm). Mutant ($n = 20$) and normal mouse lung ($n = 20$) show very few goblet cells in bronchi. Goblet cell hyperplasia is observed 2 wk after LPS/fMLP challenge in bronchi of all normal ($n = 17$) and mutant ($n = 14$) animals, including animals treated with LX2931 ($n = 9$). *C*: total lung extracts of LPS/fMLP-treated mutant mice (dd) and wild-type littermates (WT) were analyzed in parallel by ELISA for murine MUC5AC 2 wk after challenge. Mutant mice produced more mMUC5AC antigen than normal, which was reduced to normal levels by LX2931 treatment (dd LPS LX) as in Fig. 5 ($n = 10$). $^{**}P < 0.01$; $^{*}P < 0.05$, 1-way ANOVA.

lipids, as exemplified in our study. CFTR deficiency in epithelial cells reportedly causes destabilization of membrane protein scaffolds involving lysolipid receptors (25), ion transport systems (12, 20, 67) and tight junctions (64), activation of cellular phospholipase A (18), ceramidases (22), and enhanced oxidative stress associated with abnormal autophagy (43). Furthermore, CF lung DCs, macrophages, and neutrophils show phenotypic abnormalities (29, 54, 61, 78, 86). Any of these processes may affect inflammatory signaling in CF lungs. Here we report low sphingosines and S1P in total lung tissue of unchallenged *Cftr*^{tm1EUR} F508del CFTR mice that are kept on normal chow (Fig. 1). This confirms and extends the data of Xu et al. (85), who reported low S1P in BALF of a C57/Bl6 CFTR KO (*Cftr*^{tm1UNC}) strain. Low sphingosine levels have been reported in another CF mouse model related to enhanced susceptibility to bacterial infections (51, 72), which we also observe in our model (48).

Abnormalities of sphingolipid metabolism in CF mutant mice and patients have been extensively studied, focusing on the role of acid sphingomyelinase (SMase) in ceramide synthesis (23, 73, 83). In the *Cftr*^{tm1EUR} model, acid and alkaline sphingomyelinase (SMase), and neutral ceramidase activities are normally expressed in mutant mouse lung (47). Enzymes directly involved in S1P metabolism, in particular sphingosine kinase SPHK (33) and SPL (31), do not emerge from differential lung transcriptome analysis in our mouse model (Scholte BJ, unpublished observations). However, further studies are required to establish the relationship between CFTR deficiency and the activity and tissue distribution of key enzymes and transporters that determine the intracellular and extracellular S1P gradients that orchestrate S1P receptor activity.

Treatment with a SLP inhibitor corrects inflammation in unchallenged Cftr^{tm1eur} *mice.* Oral treatment of adult F508del homozygous mice with the SPL inhibitor LX2931 strongly enhanced S1P lung tissue levels, as expected (5). No serious adverse effects were noticed during the 3 wk of the experiment. The relative abundance of the proinflammatory MoDC and reduced levels of cDC in mutant mouse lung are corrected by LX2931 treatment (Fig. 3). This suggests that the abnormal S1P level is a causative factor in CF lung inflammation in this model. We also observe a significant reduction of CD103+ DC in LX2931-treated mutant mice compared with untreated. Together, these data suggest that LX2931 shifts the mutant mouse lung DC population toward a lower state of inflammation.

LX2931 increases lung granulocyte and LyC6+ monocyte infiltration in unchallenged mutant mice (Fig. 2), consistent with an increased level of MCP-1 in LX2931-treated mutant mice (Fig. 4). These LX2931 induced LyC6+ monocytes do not have a suppressor cell (MDSC) phenotype, lacking the typical ARG1^{+} iNOS^{+} FACS stain. Importantly, however, they do not differentiate to proinflammatory MoDCs and macrophages, as they would under inflammatory conditions, since their total numbers are not affected (Fig. 2). Furthermore, the infiltrating granulocytes show reduced iNOS expression in LX2931-treated mutant mice (Fig. 5). Together, this suggests that LX2931 allows monocyte and granulocyte infiltration to the lung but induces a block in differentiation toward proinflammatory phenotypes.

Since our primary purpose was to establish the role of S1P in CF lung inflammation we did not test the effect of LX2931 on wild-type cells from our colony. In an SPL KO mouse model, alveolar myeloid infiltration is reported, comparable to our observations in mutant mice treated with LX2931 (3, 80) (Fig. 2, *B* and *D*). However, this was not observed in wild-type mice treated with LX2931 (T. Oravecz, unpublished data) or the parent compound THI (5), or in a strain expressing reduced levels (17%) of SPL activity (80). As our data indicate, CF mouse lungs may offer a different signaling environment compared with wild-type mice, resulting in enhanced infiltration of myeloid cells during LX2931 treatment, that do not differentiate to proinflammatory phenotypes.

Total B cell and T cell counts, which are increased in mutant mouse lungs, are strongly reduced by LX2931 treatment below wild-type levels (Fig. 2, *E* and *F*). The role of T and B cells in CF lung pathology is not well understood. Infiltrating B cells form lymphoid aggregates in CF lungs during end stage disease

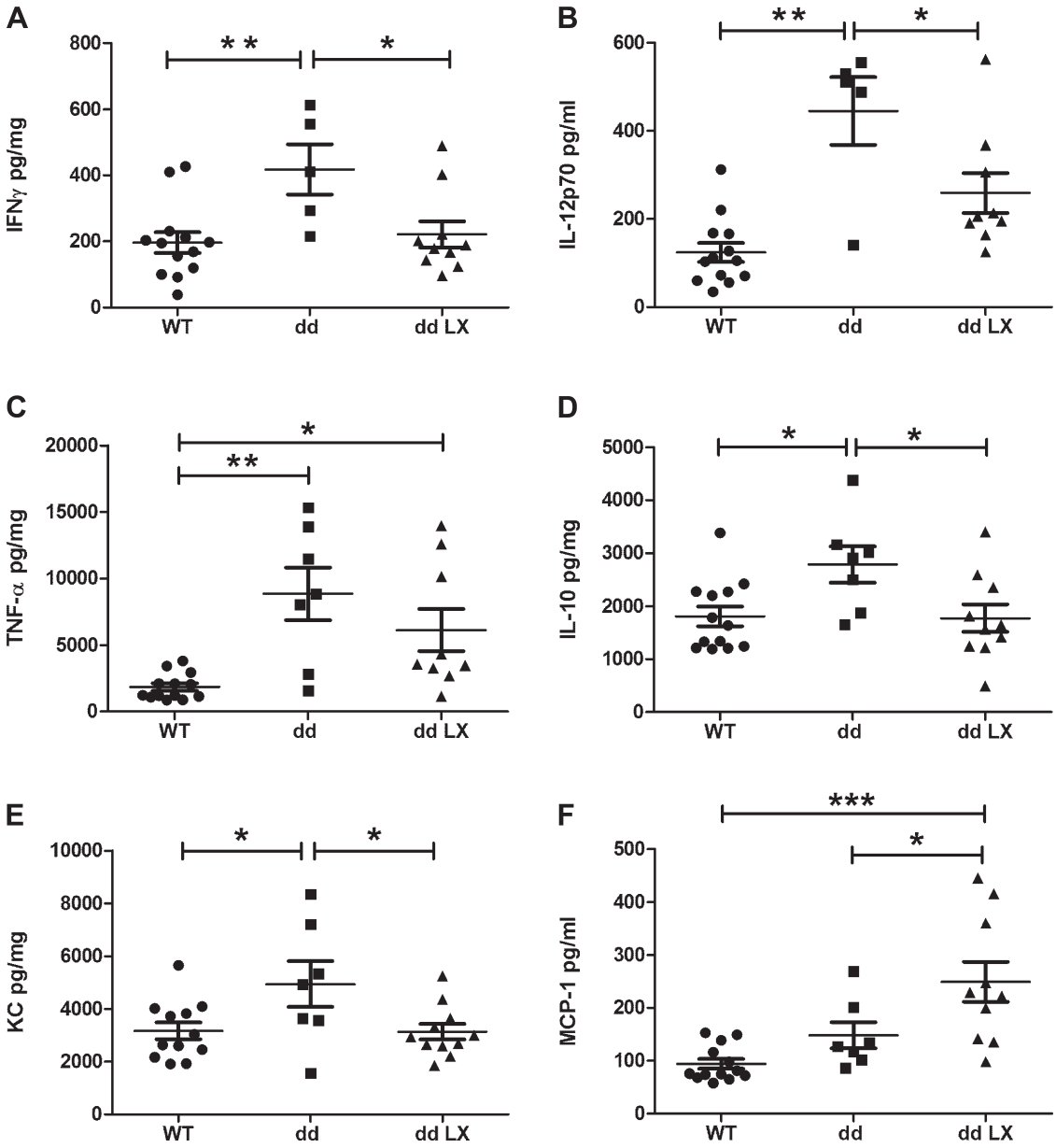

Fig. 11. Abnormal cytokine levels in F508del CFTR mouse lung after LPS/fMLP challenge partially corrected by SP-1 lyase inhibitor (LX2931). Total lung extracts of wild-type (WT), F508del CFTR (dd), and mutant treated with LX2931 (dd LX) were analyzed by sequential ELISA 2 wk after LPS/fMLP challenge for IFNγ (*A*), IL12p70 (*B*), TNF-α (*C*), IL-10 (*D*), KC (*E*), and MCP-1 (*F*). Results from individual mice are shown as pg/mg total protein. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$, 1-way ANOVA.

(28). B cell differentiation factor (BAFF) is high in BALF from young CF patients compared with healthy controls, suggesting involvement in early CF lung disease (46). B cell depletion is considered a potential treatment in chronic inflammatory disorders (38) and may be beneficial to CF patients as well. T cell subpopulation (Th17/Th2) imbalance was reported in CF lung (74). However, subtype-specific quantitative data on T cell infiltration in early human CF or CF pig lung have not been reported to our knowledge. Clearly, further studies are required to establish the role of T and B cell subtypes in CF pathology and the potential use of pharmaceutical intervention.

LX2931 reduces LPS/fMLP induced inflammatory response in mutant mice. LX2931 treatment does not prevent the LPS/fMLP induced acute myeloid infiltration (Fig. 8) or subsequent hyperplasia of goblet cells in mutant mice but reduces the enhanced production of mMUC5AC to normal levels (Fig. 10). This is highly relevant to CF since CFTR deficiency in humans causes abnormal mucus viscosity and clearance, which contributes to the progression of CF lung disease (52, 57, 63, 68). Whether LX2931 can prevent or reduce distal airway plugging in CF infants by reducing inflammation and associated mucin secretion remains to be established.

MCP1 levels are enhanced in LX2931-treated mutant animals after challenge (Fig. 11*F*), which is likely related to the increased myeloid infiltration under these conditions (Fig. 9). However, since LX2931 prevents proinflammatory differentiation of infiltrating cells this does not lead to more severe pathology (Table 1, Fig. 10). In contrast, the proinflammatory

IL8 orthologue KC, IL-10, as well as IFNγ and IL-12 are significantly reduced by LX2931 treatment after LPS/fMLP challenge (Fig. 11, *A* and *B*). This suggests that LX2931 treatment attenuates the IL12/IFN inflammation cascade, possibly through an effect on the resident DC population (Fig. 3).

The molecular mechanism of action of LX2931. Our data do not support a correction of mutant CFTR activity by LX2931, since it does not correct the low body weight (Table 1), CFTR-dependent salivary gland fluid transport activity (Fig. 6), or parotic duct plugging in CF mutant animals (Fig. 7). Chronic treatment with the SPL inhibitor THI, a parent compound to LX2931, strongly increased S1P tissue levels in medullary structural cells and also severely reduced S1P1 receptor expression on infiltrating thymocytes, probably by ligand-induced receptor inactivation (44). By analogy, it is highly likely that chronic LX2931 treatment inactivates S1P1 receptors by substantially increasing S1P levels in susceptible tissues. This may subsequently affect trafficking and differentiation of lymphoid cells.

Summary and perspective. Our results reveal for the first time by FACS analysis and sequential ELISA that unchallenged F508del CFTR mutant mouse lung displays abnormal myeloid infiltration and a delayed resolution of LPS/fMLP induced inflammation. This allows us to extend previous studies with experimental therapeutics in this model, targeting F508del mutant CFTR (15, 62, 75, 81) or using anti-inflammatory drugs (18, 43, 45). Our data and recent reports in the literature cited above imply that CF lung pathology should be considered a disease of myeloid cells as well as epithelial cells and can be at least partially corrected using drugs targeting affected signaling pathways (76).

Treatment with the SPL inhibitor LX2931 successfully reverses the low lung tissue S1P levels of F508del CFTR mutant mice and partially corrects the excessive basal and induced inflammation responses in these mice. This would support the hypothesis that pharmaceutic intervention in this pathway could be advantageous to the patients. Since the compound was shown to be safe in animals (Ref. 5; this study) and in humans (37), an intervention in CF patients could be considered.

ACKNOWLEDGMENTS

We gratefully thank J.C.P.A. (Conny) Van Holten-Neelen, Erasmus MC Immunology Dept., for invaluable assistance with the sequential ELISA assay.

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GRANTS

Part of this research was funded by grants from the Dutch CF Foundation (NCFS HIT-CF C6/7) and the Dutch Lung Fund (3.3.10.027). Part of this study was funded by a research grant from Lexicon Pharmaceuticals Inc.

DISCLOSURES

T. Oravecz was employed by Lexicon Pharmaceuticals Inc. at the time of the studies.

AUTHOR CONTRIBUTIONS

M.V., M.S., D.R., T.O., and B.J.S. conceived and designed research; M.V., G.W., J.B.D.S., W.A.D., T.O., I.D.K., and B.J.S. performed experiments; M.V., M.S., D.R., G.W., J.B.D.S., W.A.D., O.D., T.O., I.D.K., and B.J.S. analyzed data; M.V., M.S., D.R., G.W., J.B.D.S., W.A.D., O.D., T.O., I.D.K., and B.J.S. interpreted results of experiments; M.V., O.D., I.D.K., and B.J.S. prepared figures; M.V., T.O., I.D.K., and B.J.S. drafted manuscript; M.V., M.S., D.R., G.W., J.B.D.S., W.A.D., O.D., T.O., I.D.K., and B.J.S. edited and revised manuscript; M.V., M.S., D.R., G.W., J.B.D.S., W.A.D., O.D., T.O., I.D.K., and B.J.S. approved final version of manuscript.

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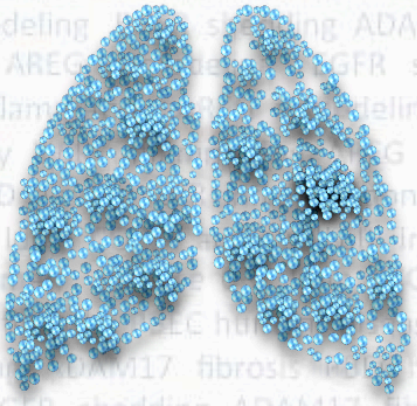
CHAPTER 5

General discussion



Is abnormal activation of EGFR-ADAM17 axis in COPD and CF a relevant target for therapeutic intervention?

Stolarczyk M, Scholte BJ

GENERAL DISCUSSION AND FUTURE PERSPECTIVES

General introduction

Airway epithelium, apart from providing a structural barrier against microbes and inhaled particles (mucociliary clearance), also plays an active role in a first line of inflammatory responses [1], and thus emerged as a therapeutic target in chronic lung disease [2]. Airway epithelial cells respond dynamically to bacterial and viral infections [3] [4] [5] or inhaled noxious particles by transducing inflammatory signals [6] [7]. They produce cytokines, growth factors and other inflammatory mediators to recruit a range of inflammatory cells including neutrophils and macrophages [1] [8]. Importantly, airway epithelial cells also cross-talk with the underlying fibroblasts/myoblasts [9] [10]. This trans-signaling, when exaggerated by stress factors and bacterial infections during the course of disease development, leads to structural changes of the tissue structure termed lung tissue remodeling, a feature of both CF and COPD pathology [11] [12] [13].

A disintegrin and metalloprotease 17 (ADAM17), a sheddase, formerly known as TACE, that releases a broad spectrum of soluble biologically active substrates from airway epithelial cells is recognized as an important transducer of airway epithelial cis- and trans- signaling [14]. The growth factor AREG [15] and cytokine receptor IL6R [16], both shed by ADAM17, activate EGFR and gp130 respectively, and have been linked to lung pathology [17] [18] [19], but were never directly investigated in the context of COPD and CF lung malfunction. Therefore, the main aim of this thesis was to elucidate whether ADAM17 responds differently to external stimuli like cigarette smoke (CS) in COPD airway epithelial cells and whether CFTR deficiency activates ADAM17 as a cell autonomous defect. During the course of our studies it emerged that EGFR is an equally potent regulator of IL6R and AREG release and mRNA expression as ADAM17, indicating the role of the EGFR-ADAM17 axis in COPD and CF pathology (Chapter 2 and 3). It is known that both EGFR and ADAM17 play an essential role in lung tissue inflammation and regeneration [20] [21]. However, so far the role of ADAM17 and EGFR has only been broadly studied in carcinogenesis and little is known about the organization and function of this signaling network in differentiated CF and COPD airway epithelial cells.

In view of the comparable pathological events in CF and COPD [22] [23] [24] (Chapter 1) and the recently observed downregulation of CFTR by CS exposure [25] [26], we proposed that the molecular mechanism underlying the development of these diseases is similar and may involve abnormal activation of the EGFR-ADAM17 axis. Our studies and the literature cited show that ADAM17-mediated release of bioactive substrates is a tightly regulated, multistep, spatiotemporal process that likely requires activation by upstream kinase signaling (Chapter 2 and 3) [27], trafficking of ADAM17 towards the substrate in the intracellular compartment (Chapter 2) [28], thiol-isomerase and redox regulated disulfide bridge rearrangements (Chapter 3) [29], and dissociation of the inhibitory protein TIMP-3 [30]. EGFR/ADAM17 signaling cross-talks with many regulatory pathways like TLR5, G-coupled receptors, p38 MAPK, ERK1/2 (Chapter 2 and 3) [31] [32] [33] [34] [35]. In this thesis we addressed some of the issues of ADAM17 activation and the role of EGFR in this

signaling cascade in order to define the druggable targets in the ADAM17/EGFR axis in CF and COPD pathology (Chapter 1, Figure 7).

A model of lung pathology

Although mouse models can contribute considerably to our understanding of CF (Chapter 4, Appendix) and COPD lung pathology [36], we have focused on differentiated at air-liquid interface human airway epithelial cells in culture. Firstly, because it has been reported that ADAM17 activity towards its substrates is species specific, as exemplified by IL-6R [37] [38] [10]. Additionally, in humans and pigs, the loss of CFTR impairs respiratory host defenses due to acidification of ASL causing airway infection, but in mice this acidification is not observed [39]. CF mice do not develop CF type obstructive distal lung disease [40], however, they do show evidence of chronic lung disease characterized by inflammation and MUC5AC hypersecretion with abnormal myeloid infiltration and exaggerated responses to bacterial challenge (Chapter 4, Appendix). To provide clinically relevant data focusing on the contribution of airway epithelial cells to the signaling cascade, we investigated our hypothesis in human bronchial epithelial cells cultured at the air-liquid interface.

We examined the effect of cigarette smoke (CS) on the ADAM17/EGFR axis in well-differentiated bronchial epithelial cells from COPD patients under air-liquid interface (ALI) (ALI-COPD HBEC) (Chapter 2). This gave us the opportunity to compare airway epithelial cells from COPD with non-COPD subjects. Apart from that we exposed the cells to whole cigarette smoke, instead of cigarette smoke extract (CSE) or noxious particles, that cannot be considered as the substitute of whole cigarette smoke [41] (Chapter 2). Although we were able to obtain significant differences, we noticed that primary human bronchial cells from different donors respond with high variability to external stimuli (Chapter 2). This can be considered as a disadvantage of the primary culture model in the event of studying the effect of CFTR deficiency on downstream pathways. Apart from that, cell material from the CF patient population allows us only limited access to end-stage disease after lung transplant. An alternative would be epithelial cell culture from nasal brushes, however, this does not allow expansion of large amounts of cells for culture, and this system differs from the distal airways and is also characterized by a large donor and subculture variation [42]. Thus, in further studies to define the effect of CFTR deficiency on ADAM17/EGFR axis activity, we used a well-characterized airway epithelial cell line CFBE41o- differentiated under air-liquid interface with inducible CFTR expression (ALI-CFBE41o-). This model allowed us not only to overcome donor and culture variation (Chapter 3), but also the use of CFTR inhibitors or siRNA to obtain CFTR deficient cells is not preferred due to reported off target effects [43] [44]. In future studies in our laboratory induced pluripotent stem cells (iPS) from CF patients and parallel lines corrected by gene editing will be used. This will allow us to study CFTR deficiency in a single genetic background in differentiated epithelial lineages.

ADAM17 is the main sheddase of IL6R and AREG in human bronchial epithelial cells

Previous studies have shown that external stress factors (like cigarette smoke extract [7] [45] [46] [47] [48] [49], bacterial or viral infections [3] [4], cytokine or growth factor treatment [50] [51]) abnormally activate ADAM17 and EGFR in different airway epithelial

cell models, mainly in submerged cell lines. As an extension of previous finding, the studies described in this thesis show the mechanism of ADAM17 activation in CF and COPD lung disease in an advanced model of lung airway epithelial cells. By using the selective ADAM17 inhibitor TMI-2 (Wyeth), we showed that ADAM17 is the main sheddase responsible for AREG and IL6R release from differentiated primary airway epithelial cells (Chapter 2) and AREG from ALI-CFBE cell line (soluble IL6R has not been detected in supernatants from ALI-CFBE41o- in our assay) (Chapter 3).

ADAM17 establishes a signaling axis with EGFR, regulating protein and mRNA levels of ADAM17 substrates.

To further investigate the signaling cascade leading to release of ADAM17 substrates, we tested several kinases that have been potential regulators of ADAM17 signaling cascade, like SRC [52] or ERK1/2/MAPK [35] [33], but EGFR emerged as the most potent regulator of AREG and IL6R release, with a strength equal to ADAM17 (Chapter 2). These data provided the proof of existence of a EGFR-ADAM17 positive feedback loop [53] [52] [54], also in differentiated airway epithelial cells (Chapter 1, Figure 4). Importantly, for the first time we present evidence that both EGFR and ADAM17 regulate not only protein release, but also mRNA expression of IL6R and AREG. The involvement of EGFR in mRNA regulation of other signaling molecules such as IL-8 has been already shown [7] [49]. Here we observed that also a selective ADAM17 inhibitor blocks IL8 mRNA production in ALI-HBEC through the feedback loop action of the system (Chapter 2). Thus, our results highlight that the EGFR-ADAM17 axis is a potent regulator of both ADAM17 substrate shedding and EGFR targets at the protein and mRNA level in airway epithelial cells, likely playing a crucial role in inflammation and tissue repair.

Airway epithelial cells from COPD patients respond more strongly to cigarette smoke than normal

In COPD ALI-HBEC we showed that under basal conditions release of ADAM17 substrates does not depend on COPD disease status, but upon CS exposure both AREG and IL-6R release is stronger in COPD cells. The difference between COPD and non-COPD in CS-induced ADAM17 activity is not caused by changes in the relative mRNA levels of AREG or IL-6R in COPD ALI-HBEC. Thus, we concluded that COPD lung disease makes airway epithelial cells more sensitive to external stress factors. Since our studies show induction of ADAM17 activity upon CS exposure, and previous studies of Amatngalim et al showed that CS also induces phosphorylation of EGFR [7], it is likely that the difference between COPD and non-COPD is due to post-transcriptional regulation of ADAM17 and EGFR activity (Chapter 2). This may include the level of ADAM17 and EGFR phosphorylation (Chapter 2 and 3) or structural changes (disulfide bridges) (Chapter 3). Epigenetic changes, involving DNA methylation and histone modifications, causing various levels of metaplasia, may also play a role. DNA methylation is affected in COPD lung tissue and mice exposed to CS [55], and is likely also a factor in CF lung disease development, though this has not been studied in detail. It has been proposed that both CF and COPD patients have altered microRNA (miRNA) expression [56] [57] [58] [59], which may affect ADAM17 [60] [61] [62] and EGFR [63] [64] activity. Of interest to our purpose is the fact that both miRNA and epigenetics offer new possibilities of therapeutic intervention.

CFTR dysfunction hyperactivates the ADAM17/EGFR axis and is a possible link between pathological events observed in COPD and CF

To further establish the importance of the EGFR-ADAM17 axis in lung pathology, we showed that CFTR deficient airway epithelial cells have induced ADAM17 activity and EGFR phosphorylation (Y1173), compared to CFTR expressing cells (Chapter 3). These data suggest that CFTR deficient cells have an intrinsic defect in the ADAM17/EGFR signaling pathway. Kim et al reported that CFTR inhibitor treatment increases EGFR and ADAM17 related inflammatory responses in submerged immortalized cells [65]. However, we used differentiated cells under ALI conditions with inducible CFTR expression, overcoming the off-target effects of CFTR inhibitors [43] [44] and donor variation observed in ALI-HBEC (Chapter 2). Importantly, enhanced ADAM17 shedding activity and EGFR phosphorylation in CFTR deficient cells is not caused by a difference in induced mRNA levels of ADAM17 or EGFR, suggesting that dysfunctional CFTR affects ADAM17/EGFR signaling post-transcriptionally. Moreover, the difference was only observed during ALI culture, and not in submerged conditions, suggesting that CFTR deficient cells in comparison to cells with induced CFTR activity adapt abnormally to the ALI environment, affecting the ADAM17/EGFR axis activity. This also highlights that 3D ALI culture, but not submerged cultures, resembles the in vivo conditions more precisely.

Interestingly, CS recently has been shown to reduce CFTR function, observed as CFTR internalization [25], reduced CFTR activity measured by nasal potential differences (NPD) in patients and ASL dehydration in airway epithelial cells [26], mimicking this aspect of CF pathology [23] [24] [66]. Recent studies show that treatment with cigarette smoke extract (CSE) transiently also reduced CFTR gating activity, due to formation of acrolein adducts, and that short term (1 h) application of VX-770 partially restores CFTR function [67]. Thus, the CS induced ADAM17/EGFR axis activity (Chapter 2) and the CFTR-dependent AREG release in CFBE cells (Chapter 3) suggest that CFTR dysfunction is a common cause of enhanced activity of the EGFR/ADAM17 axis observed in CF and COPD lung disease.

We measured CFTR related short-circuit current (Isc) 24h after CS exposure, when we observed induced shedding activity, but at this time point we did not observe abnormal CFTR function (data not shown). However, CFTR internalizes quickly after CS exposure and complete recovery takes approximately 24h [25] [68]. Therefore, further experiments with shorter time points are required to establish the relationship between CFTR activity and shedding in our CS exposure system.

Solone et al showed that the CFTR potentiator Ivacaftor (VX-770) activated CFTR-dependent chloride transport in non-CF patients, increased ASL height and mucociliary transport, suggesting this may be of therapeutic importance for COPD patients [26]. However, in our culture model we showed that long-term treatment (24h) of ALI-HBEC with VX-770 enhances CS triggered IL6R and AREG secretion significantly, in comparison to CS exposure alone (M Stolarczyk, 3 independent experiments in triplicate $P<0.05$; data not shown). In the light of conflicting data showing that chronic treatment with VX-770 either reduces wild-type CFTR function [69], improve CFTR function [26] or has no effect on CFTR activity [70], it

is difficult to establish whether the increase in shedding is related to CFTR inactivation or to an off target effect of VX-770 on the ADAM/EGFR axis. It is clear, however, our present data do not support the option to treat COPD or smoking patients with VX-770 chronically, as a therapy. In the future new generation potentiators, which are currently developed by several teams, could be further investigated in our system.

One should consider here the long-term and short-term effects of CS exposure. COPD patients still have COPD pathology long after they stop smoking and their CFTR levels are restored. This is probably related to long-term epigenetic changes and metaplasia in COPD caused by frequent CS exposure, as also suggested by our experiments (chapter 2), and not with the acute effects of CS. Long-term suppression of CFTR activity in CF patients and associated pathology would likely have similar effects. This may mean that fully developed CF lung disease cannot be treated with CFTR targeted agents only, but requires additional treatment to counter these effects.

EGFR/ADAM17 axis is regulated by CFTR related oxidative stress

The extracellular membrane proximal domain of ADAM17 [29] [71] and the intracellular domain of EGFR [72] are both activated in an oxidized environment, in particular by rearrangements of intramolecular disulfide bridges by thiol-isomerases (PDI) that are not only involved in ER protein maturation, but also act on the extracellular membrane [29] [71]. ADAM17 can also be activated by upstream signaling kinases sensitive to ROS, for instance SRC [73]. In line with these observations, we showed in Chapter 3 that AREG shedding mediated by ADAM17 is sensitive to ROS scavengers (NAC, DPI) and it is most strongly inhibited by addition of an impermeant natural thiol antioxidant glutathione (GSH) [74]. These data suggested that the ADAM17/EGFR axis is sensitive to the extracellular redox potential. By expression of roGFP-Grx-1 fused to GPI [75] [76], which enables ratiometric measurements of the apical extracellular redox potential, we detected enhanced oxidation in the apical lining fluid of CFTR deficient CFBE41o- cells. In contrast, we reported unchanged cytoplasmic redox potential in CFTR deficient CFBE41o- cells expressing roGFP-Grx1. Thus, we propose that induced ADAM17 dependent AREG shedding in CFTR deficient cells (Chapter 3) is caused by an abnormal ratio of GSH/GSSG in the extracellular environment. However, at this stage it is still not clear whether this hyperoxidation occurs only in ASL or also in other compartments, such as the lateral and intra-vesicular space.

CFTR was previously reported to regulate the extracellular redox potential through GSH transport in the airways [77] [78], so it is likely that the abnormal transport of GSH causes ASL oxidation. In line with our speculations, in lung lavage fluids of CF patients the levels of GSH were reduced in comparison to healthy controls [79]. Furthermore, we observe that several lipid markers of oxidative stress in lung lavages (BALF) from juvenile CF patients, in particular isoprostanes which are peroxidation products of unsaturated fatty acids, correlate with the severity of CF lung disease (Scholte, Stick et al, in preparation). Current longitudinal studies by our group of bronchial lavage fluids from CF infants will focus on the relationship between oxidative stress signaling and the development of CF lung disease. Also in our F508del CFTR mouse model enhanced inflammation (Chapter 4, Appendix) correlates with lipid markers of oxidative stress (Scholte, Radzioch, in preparation). Importantly, GSH administration in CF patients reduced inflammatory markers in BALF [80].

The impaired GSH transport in CF may enhance downstream signaling [77] [80] and affect activity of ADAM17, which is sensitive to redox potential through its extracellular domain [29]. Since the extracellular domain of ADAM17 is not primarily exposed to the extracellular apical membrane, it is also plausible that the GSH/GSSG ratio, which is an indicator of oxidative stress, may be altered in the ER compartment [81], where it may affect protein disulfide bridge formation [82] in the ADAM17 molecule. However, redox potentials at the cell surface and in the ER were not different in CF nasal epithelial cells in comparison to CFTR corrected cells, as measured with roGFP1 [83]. But as indicated previously, nasal epithelial cells and bronchial epithelial cells may differ in their properties.

Interestingly, it has been proposed that GSH/GSSG balance may affect the optimal redox potential for PDI to catalyze disulfide bond formation not only at the external surface of the plasma membrane, but also in the ER [82] [83] [84]. PDI has been shown to be affected by GSH/GSSG balance, so it is likely that CFTR related ER oxidative stress [85] may interfere with ER GSH/GSSG balance and thus directly activate PDI/ADAM17 signaling cascade inducing EGFR/ADAM17 axis activity in CF bronchial epithelial cells. Importantly, PDI may also be a therapeutic target, as shown in the context of carcinogenesis [86], so further studies of membrane trafficking are of interest to establish the exact localization and mechanism how PDI and abnormal GSH levels affect ADAM17 activity in CFTR deficient bronchial epithelial cells.

Does ADAM17 shed its substrates at the cell membrane or in the intracellular vesicular compartment?

Most of the reports assume that ADAM17 cleaves it substrates at the extracellular membrane, but this has not been confirmed yet. Lorenzen et al showed that an ADAM17 construct containing the unmodified intracellular part was expressed at the cell surface to a lesser extent than the GPI anchored variant [87]. In accordance with this, ADAMs and its substrates have been localized in various subcellular compartments like lysosomes [88], endosomes [89] [90] and exosomes [91], thus it is difficult to identify an exact site of ADAM17 mediated shedding [28]. We show for the first time by proximity ligation assay (PLA) that upon CS exposure ADAM17 and ADAM17-P appears in close proximity with its substrates AREG and IL-6R in the intracellular compartment in ALI-HBEC, whereas under basal condition ADAM17 or ADAM17-P-substrate complexes were infrequent (Chapter 2). However, our data do not establish whether ADAM17 sheds its substrate in the intracellular vesicles followed by secretion, or whether the ADAM17/substrate complexes in these vesicles need to be transported to the membrane first in order to deliver ADAM17 and its substrate for cleavage. In both cases a vesicle trafficking and membrane fusion event is involved, which may be subject to regulation.

In line with our observation, Soond et al showed that expression of constitutively active MEK-1 and ERK in HeLa cells relocates ADAM17 fused to GFP (ADAM17-GFP) from the ER to intracellular vesicles that are distinct from the ER [35]. Also the phosphorylated form of ADAM17 at position T735 fused to GFP (ADAM17-T735-GFP) co-localized with the ER-Golgi transport marker Sec- 23/COPII and trans-Golgi network protein TGN-46 [35]. Furthermore, Lorenzen et al based on the co-immunoprecipitation of both immature and mature ADAM17 with its substrate suggested that ADAM17 and its substrate meet in the ER/Golgi

pathway, and that the pro-domain does not interfere with the enzyme-substrate interaction [87]. All these data may explain the CS triggered ADAM17-substrate interaction in the intracellular vesicular compartment observed by us (Chapter 2). However, they do not suffice to clarify the molecular regulation of ADAM17-mediated shedding.

According to some reports PKC activation by PMA induces ADAM17 dependent shedding, without increasing ADAM17 at the extracellular membrane [71] [92], whereas other studies did show that PMA stimulation increases expression of ADAM17 at the cell surface and decreases the presence of L-selectin at the cell surface, indicating that the cleavage of ADAM17 substrates occurs at the cell membrane [93]. Further studies by Xu et al. suggested that ADAM17 is localized at the cell surface as a dimer and associates with tissue inhibitor of metalloproteinase-3 (TIMP-3) [34]. Upon activation with PMA or anisomycin, the amount of ADAM17 at the cell surface increases, followed by dissociation of ADAM17 from TIMP-3, and ADAM17 dimers convert to monomers. According to the authors, these monomers correspond to the active form of ADAM17 and are predominantly found in cytoplasm [30], suggesting that the monomers are internalized and shedding may occur intracellularly. Also recent reports suggest that ADAM-mediated cleavage occurs from an intracellular vesicular pool in several cell types [88] [94]. In line with these studies, Doedens et al proposed that endocytosis from the cell surface is a pre-requisite for ADAM17 catalytic activity [95]. As an extension of these studies, [90], two proteins that regulate ADAM17-dependent shedding of EGFR ligands, annexins [96] and a phospho-furin acidic cluster sorting protein 2 (PACS-2) [90] have also been shown by proximity ligation assay (PLA) to be in close proximity to ADAM17 in the intracellular vesicular compartment, again suggesting that the shedding process may occur from intracellular compartment. However, all these data do not provide the complete picture of ADAM17 activation and localization. Thus, in light of other and our findings it is important to address where PDI leads to conversion of ADAM17 conformation, does it affect the interaction of ADAM17 with TIMP3 and what is the role of GSH in these events. These questions should be addressed in general terms as well as in CFTR experimental systems.

Taken together, our data showed that upon activation, ADAM17 or its phosphorylated form interact with the substrate in an intracellular vesicular compartment (Chapter 2). However, further studies focusing on dynamic trafficking events and protein-protein interactions during the activation of the ADAM17 system are required to reveal whether induced ADAM17 proteolytic activity occurs in the intracellular vesicles or requires membrane trafficking. These questions need to be addressed to allow the design of a next generation of drugs targeting ADAM17 activity, that avoids the disadvantages of current systemic ADAM inhibitors [97].

Is dynamic trafficking of EGFR and ADAM17 an additional regulatory mechanism of EGFR/ADAM17 axis activation?

- ADAM17-substrate complexes appear in the intracellular compartment, whereas EGFR internalizes after CS exposure

We showed that not only the localization of ADAM17 and it substrates changes upon activation by CS, but also EGFR undergoes internalization from the basolateral membrane into an intracellular compartment in CS treated ALI-HBEC (Chapter 2, Figure S2), associated with enhanced EGFR phosphorylation [7]. However, we have not established yet whether EGFR-P co-localizes with ADAM17-P-substrate complexes in the intracellular compartment, where they potentially may activate each other. The co-localization of EGFR and ADAM17-substrate complexes and their role in shedding in human airway epithelial cells upon activation is an important research question that should be further investigated. Additionally, the multifunctional sorting protein PACS-2 involved in retrograde trafficking of membrane proteins from endosomes and Golgi not only regulates ADAM17 trafficking toward an active pool and thus shedding of EGFR substrates, but also affects EGFR phosphorylation [90], giving another indication for the importance of co-trafficking as a potential regulatory mechanism of the ADAM17/EGFR axis.

Up till now, EGFR signaling has been believed to occur at the plasma membrane, however, upon activation by ligand binding, EGFR is rapidly internalized into endosomes [98]. Also EGFR ligands undergo intracellular trafficking [9]. Different stress factors lead to accumulation of EGFR in non-degradative endosomes [99] [100] [101] [102], and this endocytic trafficking has been correlated with active signal transduction [103] [104] [105] [91] [106] [107] [108]. We observed that CS exposure causes EGFR internalization (Chapter 2), which may prolong EGFR signaling from an intracellular vesicular pool. Interestingly, inhibition of EGFR with tyrosine kinase inhibitors (AG1478 and BIBW2948BS) prevents EGF-induced EGFR internalization [109]. These data have been confirmed in ex-vivo EGF-stimulated airway biopsies form patients who inhaled EGFR inhibitor (BIBW2948 BS) prior to the experiment, which caused diminished EGFR internalization [109]. Thus, our and published data highlight the importance of investigating further the role of EGFR trafficking upon exposure to external stimuli, like CS or in CFTR deficiency. Apart from that, the secretion of EGFR and its ligands into exosomes has been reported [9] [110]. Exosomes containing EGFR or its ligands can be taken up by epithelial, endothelial, and fibroblast cells [91] [110], thus it would be interesting to investigating their role in the trans-signaling.

- EGFR appears in the apical compartment in CFTR deficient cells

The protein trafficking pathways together with tight junctions establish the apical-basolateral polarity in epithelial cells. EGFR and its ligands are preferentially delivered to the basolateral cell surface and they all have a conserved basolateral sorting motif [9] [111]. Overexpressed EGFR in polarized epithelial kidney cells additionally appears at the apical cell membrane and as a consequence of this mistrafficking, EGFR is less sensitive to downregulation and undergoes less efficient endocytosis, which may result in prolonged EGFR activity [112]. We observed that upon induction of CFTR in CFBE41o-ALI cells EGFR is expressed mainly at the basolateral membrane, but in CFTR deficient cells it appears more frequently at the apical side (Chapter 3). Thus, we speculate that this apically expressed receptor may be less sensitive to downregulation [112] and more susceptible to external stimuli.

There are several possible scenarios that may explain abnormal EGFR trafficking in CFTR deficiency. Firstly, EGFR trafficking is highly pH sensitive. In acidic endosomes TGF-α dissociates from EGFR which results in recycling of unbound EGFR to the plasma membrane [113] and its exposure to other ligands [113] [111], whereas in an acidic environment binding of betacellulin to EGFR is highly resistant [113]. This suggests that ligand binding and EGFR behavior depends on acidification of its cellular environment. In CF human and CF pig the airway surface liquid (ASL) is acidified [114] [39]. The pH in the lateral compartment, where most EGFR appears to be localized, has not been investigated yet. Also, it is still contested whether endosomal and lysosomal pH differs in primary human airway epithelial cells from CF versus non-CF patients [115] [116] [117]. Therefore, further investigations are required to establish whether vesicular or apical pH affects EGFR trafficking in CFTR deficiency.

Another possible link between CFTR deficiency and abnormal EGFR activity is its effect on common factors regulating EGFR and CFTR trafficking. For instance, Wiskott-Aldrich Syndrome protein N-WASP when inhibited accelerates CFTR internalization in HT29 intestinal adenocarcinoma cells [118], whereas in murine and HeLa cells it impairs EGF-mediated endocytosis of EGFR [119] [120]. Some studies also suggest a role of SRC family kinases in EGFR trafficking [102] [121], which also phosphorylate CFTR [122] [123] [124] [125]. Targeting these molecules in CFTR deficient tissues may normalize EGFR trafficking and thus downregulate EGFR signaling. Our observation that EGFR is mislocalized in CF cells (Chapter 3) may be related to the finding that CFTR depletion by siRNA delays EGFR degradation in 16HBE14o- cells. This delayed EGFR degradation can be abrogated by application of the ROS scavenger cystamine or BECN-1 expression that encodes an autophagy activator protein, Beclin-1. [126]. These data are part of a series of studies by Maiuri et al, which implicate CFTR deficiency in ROS related abnormalities in aggresome formation, defective autophagy and inflammatory signaling [127] [128] [129] [130] [131]. Thus, abnormal trafficking of EGFR and other elements of the EGFR/ADAM17 axis observed in CFTR deficiency is potentially linked to ROS induced defective autophagy [128].

Crosstalk between different pro-inflammatory pathways in CF lung inflammation.

The data presented in Chapter 4 and the Appendix, show that not only the EGFR/ADAM17 axis, but multiple pro-inflammatory and tissue remodeling signaling pathways are involved in CF lung disease, in accordance with abundant evidence presented by other authors. The molecular link between CFTR deficiency, the EGFR/ADAM17 axis and these parallel signaling pathways is not immediately obvious and requires further studies.

Multiple bioactive lipids are affected in CF, in particular the sphingosine pathway, probably signaling through the complex S1P receptor system (Chapter 4). Extracellular S1P induces ADAM17 activation via p38MAPK in an immortalized cell model [132]. Enhanced inflammatory responses mediated by interleukin-1-beta (IL-1β) in F508del CFTR mutant mouse lung provides another node in the signaling web relating CFTR deficiency with inflammation (Appendix). The IL-1β signaling pathways cross-talks with the EGFR/ADAM17 signaling cascade, in airway epithelial cell lines IL-1R activates ADAM17/EGFR/IL8 signaling [65]. Interestingly, in human chondrocytes extracellular S1P signaling down regulates IL-1β-

induced phosphorylation of p38MAPK [133] suggesting cross-talk between S1P, IL-1β and the ADAM17/EGFR pathway. However, how these signaling pathways interact in CF lungs in situ remains to be established.

Current studies in our laboratory also show a change in ceramide species ratio in bronchial lavage fluid (BALF) from preschool CF children, in F508del mutant mouse lung, and in primary bronchial cells from CFTR deficient piglets in ALI culture (Scholte et al in preparation). A CFTR dependent change in the ceramide membrane pool likely plays an important role in membrane protein trafficking and receptor signaling, in particular of EGFR during oxidative stress [134].

Moreover, we observe enhanced lysolecithin (LPC) and lysophosphatidic acid (LPA) concentrations in CF infant BALF, positively correlating with CF lung disease (Scholte et al, in preparation). This is probably related to enhanced activity of phospholipase A activity observed previously in CFTR deficient epithelia [150]. Preliminary studies in our laboratory also show that LPA receptor agonists strongly activate the EGFR/ADAM17 activity and secretion of pro-inflammatory cytokines in human bronchial cells, evidence of strong crosstalk between these pathways, likely contributing to chronic inflammation in the CF lung.

Taken together, these data provide a strong rationale for cross-talk between CFTR related oxidative stress, lysolipid and sphingosine metabolism, IL-1β and the ADAM17/EGFR pathway in CFTR deficiency. At this time we cannot establish whether there is a simple linear cascade of events, or a more diffuse imbalance of multiple related signaling pathways caused by the CFTR primary defect leading to pro-inflammatory and tissue remodeling signaling. Therefore, this should be further investigated in ALI-HBEC or even better in 3D organotypic culture (lung on a chip),to mimic the conditions *in vivo,* in order to better understand the activation of EGFR/ADAM signaling pathway in CF and COPD lung disease.

Clinical relevance

Our studies show that lung disease in CF and COPD is characterized by exaggerated ADAM17/EGFR signaling, which is a proven druggable target. In a clinically relevant model, air-liquid interface culture of primary epithelial cells, we show that cells from COPD patients respond more pronouncedly to external triggers like CS exposure, and that these responses involve the ADAM17/EGFR axis (Chapter 2). In CFBE41o- cells differentiated under ALI, we showed that the ADAM17/EGFR axis is activated as a cell autonomous defect caused by CFTR deficiency (Chapter 3). CFBE cells give a representation of the in vivo situation, however, the data should be verified in differentiated CF primary human airway epithelial cells and more advanced systems.

Considering the involvement of ADAM17 and EGFR in many pathological events observed in CF and COPD [21] [20], like inflammatory responses [135] [136], regeneration [137] [138] and mucus hypersecretion [139] [140], intervention in this signaling pathway could be beneficial to alleviate these symptoms. Importantly, both ADAM17 and EGFR not only mediate inflammatory processes, but also are linked with protective responses to injury

(wound healing), so complete blocking of EGFR or ADAM17 may have serious effects on the anti-inflammatory and pro-regenerative responses. Finding the balance between removing excessive inflammation while keeping the protective responses to infection and injury would be the main goal in CF and COPD therapy. Several inhibitors or antibodies against EGFR [109] [141] and ADAM17 [142] are currently in clinical trials showing positive results mainly in cancer treatment. Initial experiments in our laboratory testing systemic application of the ADAM inhibitors TMI1 and TMI2 (Wyeth) in our mouse model of CF showed only a partial reduction of pro-inflammatory cytokine signaling after a challenge with either naphthalene, causing airway injury, or intranasal LPS/fMLP causing acute neutrophilemia (Scholte et al, unpublished). Further studies should try to find the optimal dose range of available compounds, but also define alternative druggable targets in the ADAM17/EGFR pathway. Furthermore localized delivery methods, avoiding the disadvantages of systemic inhibitors that were initially developed for cancer treatment should be considered. Novel developments include the use of single chain humanized antibodies, and technology that allows targeted delivery of nano-carriers to inflamed tissue.

Our studies also highlight the involvement of oxidative stress (ROS/GSH) as one of the important regulators of EGFR/ADAM axis activity, which offers opportunities for treatment of both CF and COPD. Thus so far such attempts have met with limited success, due to the limitations of the available experimental treatments. Further development of such interventions would be warranted.

Conclusions and future direction

The results provided in this thesis help to explain the activation of the ADAM17/EGFR axis in CF and COPD lung disease. However, to define the druggable targets in this signaling cascade future studies should identify the molecular events leading to activation of ADAM17/EGFR axis in more detail.

Here we showed for the first time that EGFR is a potent regulator of ADAM17 activity and that they both regulate IL-6R and AREG protein release and mRNA synthesis (Chapter 2). As an extension of our results (Chapter 2), studies by Amatngalim et al show the importance of EGFR/ERK1/2/MAPK pathway in CS induced regulation of IL-8 mRNA levels [7]. This supports the hypothesis that EGFR establishes a positive feedback loop with ADAM17 (EGFR/ADAM17 axis). Furthermore, our studies point towards SRC as the druggable candidate in the ADAM17/EXFR axis. It not only affects ADAM17 activity (Chapter 3) [52], but also CFTR can be phosphorylated by tyrosine kinases from the SRC family (Fyn, Lyn, Fgr, Src) in vitro [122] [123] [124] [125]. However, we have not observed an induced SRC phosphorylation level in CFTR deficient compared to CFTR expressing CFBE41o- cells (data not shown). Current studies are aimed at SRC regulation in primary differentiated cells in response to oxidative stress.

Since ROS activates ADAM17, EGFR and SRC directly or indirectly [29] [52] [72], and is thought to be induced in CFTR deficiency [128], we questioned whether it is a link between CFTR deficiency and ADAM17 activity. Our data show that airway epithelial cells are oxidized extracellularly, but not in the cytosol. Thus, we propose that extracellular

GSH/GSSG imbalance induces CFTR-mediated EGFR/ADAM17 signaling, contributing to inflammation and tissue remodeling. However, in the light of findings cited above, also a GSH/GSSG imbalance in the ER lumen may affect ADAM17 activity, by PDI-mediated conformational changes of ADAM17 [29] [84]. In line with these speculations, upon CS exposure ADAM17 appears in close proximity with its substrates in the intracellular compartment in ALI-HBEC (Chapter 2). Thus CFTR dependent GSH/GSSH imbalance in ER, may directly affect PDI-mediated ADAM17 activation, bypassing the ADAM17/EGFR axis. Our data show that extracellular GSH completely inhibits ADAM17 dependent AREG shedding, but not EGFR phosphorylation in CFBE cells (Chapter 3). However, further studies should reveal if CFTR deficiency and extracellular GSH affect PDI dependent ADAM17 mediated shedding in the ER lumen or in a related subcellular compartment.,

Based on our data and recent reports, we also speculate that trafficking of EGFR and ADAM17 may be one of the regulatory mechanisms activating EGFR/ADAM17 axis in CF and COPD pathology. Upon CS exposure ADAM17-substrate PLA signal appears in the intracellular compartment in ALI-HBEC (Chapter 2), where also EGFR internalizes. In line with our findings, the presence of EGFR in endosomes and exosomes has been described, similar to ADAM17 and its substrates, however, the mechanism of the EGFR/ADAM17 signaling via a vesicular compartment is still poorly understood. Also apical mislocalization of EGFR induced by CFTR deficiency in CFBE41o- cells (Chapter 3) raises the question how CFTR deficiency affects the EGFR and ADAM17 localization and thus whether it has consequence for activation of the EGFR/ADAM17 axis in CF pathology. Thus, validation of the CFTR-dependent EGFR trafficking to the apical membrane in primary CF bronchial epithelial cells would be important. So far several potentially common regulators of EGFR and CFTR endocytic trafficking have been identified, like an autophagy activator Becline-1 [128] [143] [144], [145], Transglutaminase 2 (TG2) [127] [128] [146] [147] [148], p62 [128] [126] and Rab5 [126] [149], providing potential druggable targets for mistrafficked EGFR in CFTR deficiency, which should be tested in primary CF airway epithelial cells.

Taken together, we highlight the need to further investigate the spatiotemporal regulation of the EGFR/ADAM17 axis, localize the place of shedding of the ADAM17 substrates, the role of redox potential (measured as GSH/GSSG ratio) and the druggable intermediates in the ADAM17/EGFR pathway (membrane/intracellular) in CFTR deficiency and upon exposure to stress factors like CS. This is also crucial in order to define the methods of potential drug delivery to the airways (permeable/impermeable agents).

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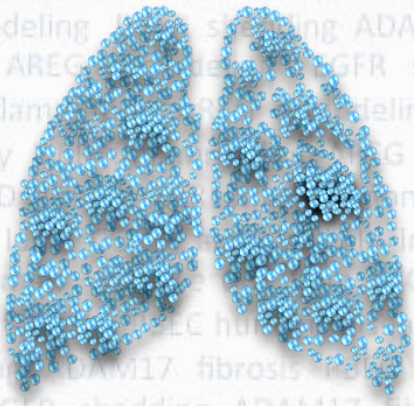
APPENDIX



Role of IL-1β in experimental cystic fibrosis upon *P.aeruginosa* infection

(published – PLoS One)

Palomo J, Marchiol T, Piotet J, Fauconnier L, Robinet M, Reverchon F, Le Bert M, Togbe D, Buijs-Offerman R, Stolarczyk M, Quesniaux VF, Scholte BJ, Ryffel B

PLOS ONE

RESEARCH ARTICLE

Role of IL-1β in Experimental Cystic Fibrosis upon *P. aeruginosa* Infection

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OPEN ACCESS

Citation: Palomo J, Marchiol T, Piotet J, Fauconnier L, Robinet M, et al. (2014) Role of IL-1β in Experimental Cystic Fibrosis upon *P. aeruginosa* Infection. PLoS ONE 9(12): e114884. doi:10.1371/journal.pone.0114884

Editor: Min Wu, University of North Dakota, United States of America

Received: October 3, 2014

Accepted: November 14, 2014

Published: December 12, 2014

Data Availability: The authors confirm that all data underlying the findings are fully available without restriction. All relevant data are within the paper.

Funding: This study was part of the European project n°10IM1016 financed by the French association "Vaincre La Mucoviscidose", supported by the ERASMUS Medical Center of Rotterdam (The Netherlands), and by Région Centre (Respig project) and CNRS of Orléans (France). RBO was supported by the Dutch CF foundation NCFS; MS was supported by the Dutch Lung foundation (Longfonds 3.3.10.027). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors confirm that co-author Bernhard Ryffel is a PLOS ONE Editorial Board member. And the authors confirm that they have declared that no competing interests exist. T. Marchiol, L. Fauconnier, M. Robinet and D. Togbe are employed by the commercial company "Artimmune", but this does not alter the authors' adherence to PLOS ONE policies on sharing data and materials.

Abstract

Cystic fibrosis is associated with increased inflammatory responses to pathogen challenge. Here we revisited the role of IL-1β in lung pathology using the experimental F508del-CFTR murine model on C57BL/6 genetic background (*Cftr*^{tm1eur} or d/d), on double deficient for d/d and type 1 interleukin-1 receptor (d/d X IL-1R1$^{-/-}$), and antibody neutralization. At steady state, young adult d/d mice did not show any signs of spontaneous lung inflammation. However, IL-1R1 deficiency conferred partial protection to repeated *P. aeruginosa* endotoxins/LPS lung instillation in d/d mice, as 50% of d/d mice succumbed to inflammation, whereas all d/d x IL-1R1$^{-/-}$ double mutants survived with lower initial weight loss and less pulmonary collagen and mucus production, suggesting that the absence of IL-1R1 signaling is protective in d/d mice in LPS-induced lung damage. Using *P. aeruginosa* acute lung infection we found heightened neutrophil recruitment in d/d mice with higher epithelial damage, increased bacterial load in BALF, and augmented IL-1β and TNF-α in parenchyma as compared to WT mice. Thus, F508del-CFTR mice show enhanced IL-1β signaling in response to *P. aeruginosa*. IL-1β antibody neutralization had no effect on lung homeostasis in either d/d or WT mice, however *P. aeruginosa* induced lung inflammation and bacterial load were diminished by IL-1β antibody neutralization. In conclusion, enhanced susceptibility to *P. aeruginosa* in d/d mice correlates with an excessive inflammation and with increased IL-1β production and reduced bacterial clearance. Further, we show that neutralization of IL-1β in d/d mice through the double mutation d/d x IL-1R1$^{-/-}$ and in WT via antibody neutralization attenuates inflammation. This supports the notion that intervention in the IL-1R1/IL-1β pathway may be detrimental in CF patients.

Introduction

Cystic fibrosis (CF) is an autosomal recessive genetic disease that affects 1 newborn out of 3,500 in the USA (WHO), and 1 per 2,000–3,000 in Europe, with an average 40-year life expectancy. This pathology is caused by mutations within the gene encoding the CFTR (*Cystic Fibrosis Transmembrane Conductance Regulator*) chloride channel [1]. In humans, the most common mutation (F508del) found in patients is a deletion of phenylalanine 508 (F508del) in the CFTR chloride channel [2], the modified protein is not efficiently folded and is rapidly degraded [3]. CF affects secretory epithelia from different organs, leading to gastro-intestinal tract pathology, with a reduced pancreatic digestive enzyme production [4] and chronic intestinal malabsorption [5]. CF mortality and morbidity are mainly due to respiratory disease, characterized by the production of abnormally viscous mucus, plugging of distal airways, and increased susceptibility to chronic infections with opportunistic bacteria, excessive lung inflammation, bronchiectasis and fibrosis leading to progressive loss of lung function [6]. The exact molecular link between CFTR mutations and hypersensitivity to infections remains unclear and controversial. In CF patients, *S. aureus* predominates in the lung of children and teenagers, while *P. aeruginosa* prevails in adults [7]. Exopolysaccharide-enriched biofilms produced by *P. aeruginosa* increase the mucus viscosity, resistance to antibiotics and host immune effectors. Chronic bacterial infections are common in CF patients and facilitates lung inflammation, mucous obstruction and tissue remodeling, resulting in fatal loss of function [1]. CF lungs display excessive inflammatory response, especially with increased neutrophil recruitment [8], the mechanism of this phenomenon is not adequately explained. However, intervention in this process likely will benefit CF patients.

The role of the pro-inflammatory signaling cytokine Interleukin 1β (IL-1β) in CF lung disease has been reported before. *P. aeruginosa* induces IL-1β or IL-18 production through NLRC4 inflammasome activation [9, 10]. *P. aeruginosa* flagellin and highly acylated LPS is recognized by TLR5 [11] and TLR4 [12] respectively. Human polymorphisms observed in the *IL1B* gene were associated with CF disease [13]. CFTR deficient mice were found to be more susceptible to acute [14, 15] and chronic [16] *P. aeruginosa* infection and display an exacerbated inflammatory response to LPS and *P. aeruginosa* activated alveolar macrophages from F508del mutant mice have enhanced expression of IL-1β [17, 18]. Huaux et al recently showed a deregulated inflammatory and fibrotic response in F508del mutant mice to bleomycin, which is IL-1R1 signaling dependent [19, 20].

Here we revisited the role of IL-1β in the resolution of *P. aeruginosa* infection, in a murine model based on mice carrying the most common CF mutation F508del CFTR [21–23]. In this study, we show that excessive activation of IL-1β correlates with increased bacterial load, inflammation and lung damage in F508del CFTR mice. Further, we show that IL-1β antibody neutralization attenuates the inflammatory response to *P. aeruginosa* infection.

Materials and Methods

Mice

Mice were on C57BL/6(J) background, wild type (WT), or homozygotes for F508del CFTR mutation in the murine *Cftr* gene (*Cftr*^{tm1eur} or d/d) [21–23], and deficient for type 1 interleukin 1 receptor (d/d X IL-1R1$^{-/-}$) [24]. Mice, obtained from Erasmus MC Rotterdam [21] were bred at TAAM – UPS 44, Orléans (Institut Transgenose) and manipulated in an SPF zone. This study was carried out in strict accordance with the recommendations of CNRS, and in the Guide "Animaleries de laboratoire''. The protocol was approved by the Committee on the Ethics of CNRS of Orléans (Permit Number: CLE CCO 2012-042). All animals are under daily inspection by the animal facility staff and the experimenters. All efforts were made to minimize suffering.

IL-1β antibody administration

Mice were treated intraperitoneally, once a week for 8 weeks, with anti-IL-1β antibody (Dr. Hermann Gram, Novartis Pharma, Basel) [25], 10 mg/kg. Control mice received 150 µL PBS. Mice were euthanatized 1 week after the last treatment, by carbon dioxide inhalation (80–90% in a dedicated inhalation chamber).

Repeated LPS induced lung inflammation (4 weeks)

Mice were treated intranasally with 80 µg of *P. aeruginosa* endotoxins/LPS [Sigma Chemical Co., St. Louis, MO] in 40 µL PBS, under isoflurane anesthesia, once a week for 4 weeks. Control mice were untreated. Mice were euthanatized 24 h after the last challenge, by carbon dioxide inhalation (80–90% in a dedicated inhalation chamber). All animals are under daily inspection by the experimenters. Humane endpoints were used during this survival study: in case that mice unexpectedly present obvious health problems (over 20% weight loss, signs of suffering, difficulties in their movements and uptake of water and food, apathy) were sacrificed, by carbon dioxide inhalation (80–90% in a dedicated inhalation chamber).

Pseudomonas aeruginosa infection

Mice were infected with freshly prepared inoculum of *P. aeruginosa* strain 2310.55 of serotype IATS O11. An overnight culture in 10 mL BHI medium was prepared, starting from the frozen stock at 37℃ and shaken at 150 rpm. Of this culture, 2.5 mL was taken to start a fresh 10 mL BHI culture. The culture was stopped when an OD of about 0.4 was reached (corresponding to a bacterial titer of about 2×10^8 bacteria/ml). Mice were anaesthetized with a low dose of intravenous ketamine/xylazine (1.25 mg/ml/0.5 mg/mL) and 40 µl of the bacterial solution or the corresponding vehicle solution (isotonic saline) was applied intranasally using an ultrafine pipette tip. Mice were monitored until they woke up and evaluated 6 h and 24 h after infection.

IL-1β antibody administration to infected mice

Mice were treated intraperitoneally, with anti-IL-1β antibody (Novartis Pharma, Basel) [25], 200 μg/mice, 15 h and 1 h before *P. aeruginosa* infection. Control mice received 150 μL PBS. Mice were euthanatized 20 h after infection, by carbon dioxide inhalation (80–90% in a dedicated inhalation chamber).

Bacterial load in lung

Lung total weights were recorded after sacrifice and expressed as a percentage of the body weight. Lung homogenates were prepared in 1.5 ml of isotonic saline solution using a Dispomix tissue homogenizer (Medic Tools). Tenfold serial dilutions of homogenate were plated on BHI agar (BHI 37g/l, Agar 15g/l) plates (Biovalley). Plates were incubated at 37℃ and 5% CO_2 and the numbers of CFU were enumerated after 24 h.

Bronchoalveolar lavage (BAL)

After CO_2 inhalation deep euthanasia, bronchoalveolar lavage fluid (BALF) was collected by cannulating the trachea and washing the lung with 1 mL saline at room temperature. The lavage fluid was centrifuged at 2,000 rpm for 10 min at 4℃ and the supernatant was stored at −80℃ for analysis. The cell pellet was resuspended in PBS, counted in a haemocytometer chamber and cytospin preparations were made using a Shandon cytocentrifuge (1000 rpm for 10 min). The cells were stained with Diff-Quick (Dade Behring, Marburg, Germany) and counted for neutrophils, macrophages, lymphocytes and eosinophils.

Total RNA extraction and RT-q-PCR from lung

Total mRNA was isolated from homogenized lung using TRI-Reagent (Sigma), purified by RNeasy Mini Kit (Qiagen, Valencia, CA), and quantified by NanoDrop (Nd-1000). Reverse transcription was performed in with SuperScriptIII Kit according manufacturer's instructions (Invitrogen). cDNA was subjected to quantitative real-time PCR using primers for *Il1b, Il6* or *Ccl2* (Qiagen) and GoTaq qPCR-Master Mix (Promega). *GAPDH* and *18S* expression was used for normalization. Raw data were analyzed using the Relative Expression Software Tool (REST, http://www.rest.de.com/).

Cytokine and chemokine measurement

TNF-α, IL-6, IL-1β and keratinocyte-derived chemokine (KC or CXCL-1) concentrations in BALF and lung homogenates were measured by ELISA (Duoset Kit; R&D Systems) according to the manufacturer's instructions.

MPO activity in lung

Lungs were homogenized in Dispomix [MedicTools. Zug, CH] with 1.5 mL NaCl 0.9%, and centrifuged. The supernatant was discarded. The pellet was homogenized in 1 mL PBS - HTAB 0.5% - EDTA 1 mM. After centrifugation, 20 µL supernatant were added with: 1 mL HBSS, 200 µL of PBS - HTAB 0.5% - EDTA 1 mM buffer, 100 µL o-Dianisidine (1.25 mg/mL) and 100 µL H_2O_2 0.048%. The reaction was stopped with 100 µL NaN_3 1%. MPO (myeloperoxidase) activity was determined as absorbance at 460 nm against medium.

Protein assay

Total protein concentration was analyzed in BALF, using Bradford assay with BIORAD DC Protein assay kit (Biorad), according to the manufacturer's instructions.

Histology

Lungs were fixed in 4% buffered formalin, dehydrated in ethanol and embedded in paraffin. Sections (3 µm) were stained with haematoxylin and eosin (H&E), CAB (collagen staining) or PAS (mucus staining) and all lung sections were evaluated by two independent observers for pathological changes, collagen deposition, mucus production and cellular recruitment.

Statistical analysis

Statistical evaluation of differences between the experimental groups was determined by using Mann-Whitney non-parametric test, or Log Rank test for survival. All tests were performed with GraphPad Prism [GraphPad Software Inc., San Diego, CA, USA; www.graphpad.com]. A *P*-value <0.05 was considered significant and symbolized with *; ** for $p<0.01$ and *** for $p<0.001$. A *P*-value>0.05 was considered not significant and symbolized with ns.

Results

1. IL-1β signaling enhances lung inflammation in F508del CFTR mutants in response to LPS

Since LPS, *P. aeruginosa* [14–16] and bleomycin induced injury and inflammation [26] are increased in F508del CFTR (d/d mice), and since the IL-1R1 pathway is involved [20, 27] and required for the development of bleomycin-induced fibrosis [20], we studied the role of the IL-1β pathway in the response to inflammation and lung damage in F508del CFTR mice. In order to induce chronic lung damage, repeated *P. aeruginosa* LPS challenges were performed in d/d and in double mutant d/d x IL-1R1$^{-/-}$ mice, and compared to untreated WT littermate controls. Lung analysis was performed at day 29, 24 h after the last LPS challenge. Survival upon repeated LPS challenge was reduced to 50% in the d/d mice, while

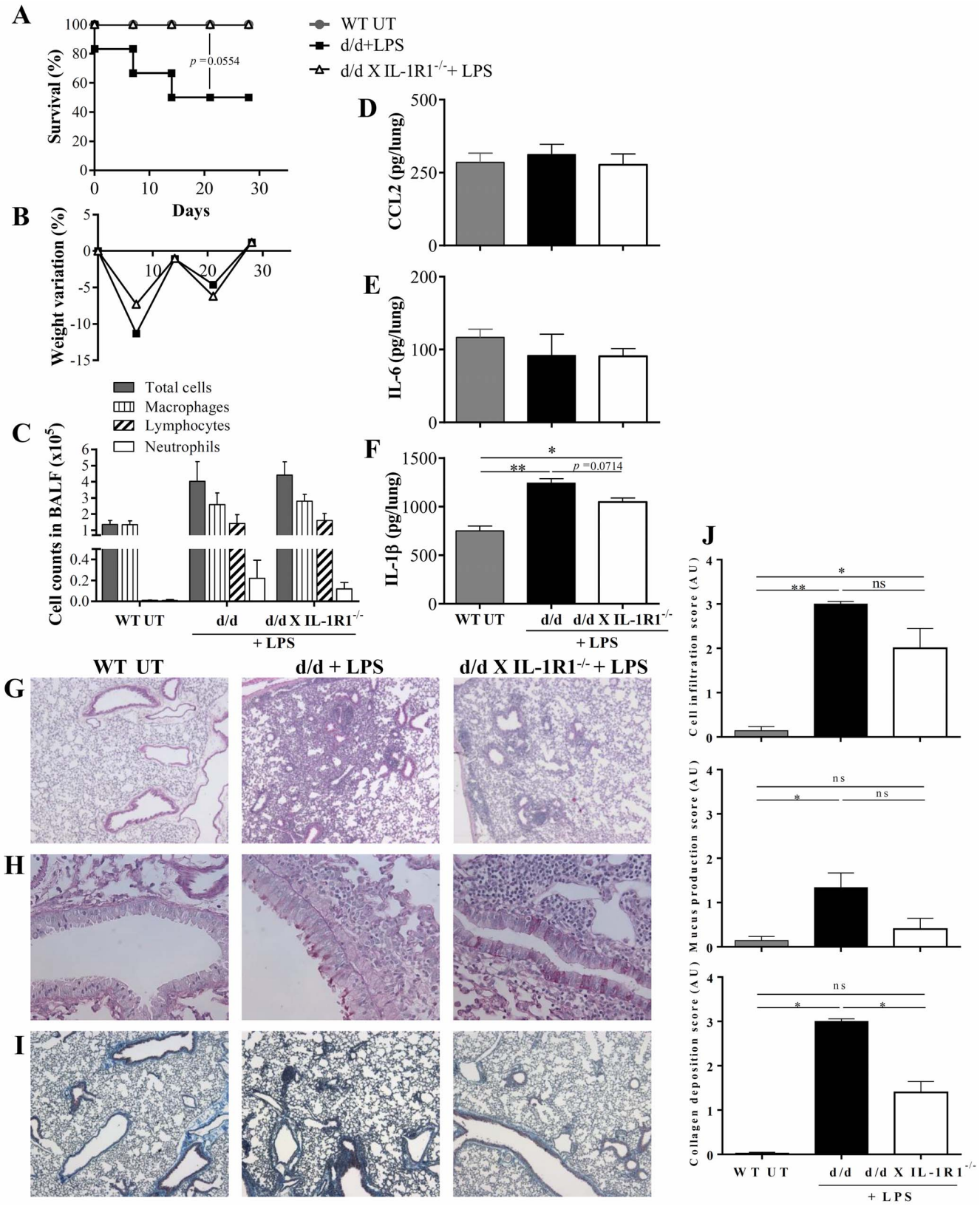
A
Survival (%)
Days
p =0.0554
WT UT
d/d+LPS
d/d X IL-1R1-/-+ LPS
B
Weight variation (%)
C
Cell counts in BALF (x10^5)
Total cells
Macrophages
Lymphocytes
Neutrophils
WT UT
d/d
d/d X IL-1R1-/-
+ LPS
D
CCL2 (pg/lung)
E
IL-6 (pg/lung)
F
IL-1β (pg/lung)
p =0.0714
J
Cell infiltration score (AU)
Mucus production score (AU)
Collagen deposition score (AU)
ns
G
H
I
WT UT
d/d + LPS
d/d X IL-1R1-/- + LPS

Fig. 1. IL-1β participates in pathologic inflammation in F508del CFTR mutants, in response to *P. aeruginosa* LPS. d/d and double KO d/d x IL-1R1$^{-/-}$ mice were treated intranasally with 80 μg of *P. aeruginosa* endotoxins/LPS in 40 μL PBS, once a week for 5 weeks. Untreated WT mice (UT) were used as control. Survival is presented in (A) and body weight variation 24 h after the last LPS challenge in (B). Absolute numbers of cells, (macrophages, lymphocytes and neutrophils) were measured in BALF 24 h after the last LPS challenge (C). CCL2 (D), IL-6 (E) and IL-1β (F) were measured in lung homogenate. Cell infiltration was observed on H&E staining (G), mucus production on PAS staining (H) and collagen deposition on CAB staining (I). Histopathological scores are shown in (J). n=6–7 initially, n=3 for d/d+LPS mice 24 h after last challenge instillation. Values are in mean +/− SEM; * for $p<0.05$ and ns for non-significant.

doi:10.1371/journal.pone.0114884.g001

100% of double KO d/d x IL-1R1$^{-/-}$ mice survived (Fig. 1.A). The results suggest that in F508del CFTR mutant, the active IL-1β pathway is deleterious in response to repeated inflammatory injury. Reduced survival of d/d mice was apparently associated with higher loss of body weight at day 7, as compared to d/d x IL-1R1$^{-/-}$ mice (Fig. 1.B). Nevertheless, all surviving mice recovered their initial weight after 4 weeks, indicating tolerance to repeated LPS challenges. LPS-induced inflammation was confirmed by increased total cell number in BALF (macrophages and lymphocytes) 24 h after the last challenge but no significant difference was observed between d/d and d/d X IL-1R1$^{-/-}$ mice at this time point (Fig. 1.C). Similarly, *P. aeruginosa* LPS also induced a recruitment of neutrophils in BALF of both d/d and d/d X IL-1R1$^{-/-}$ mice (Fig. 1.C). No increase compared to untreated WT mice of the CCL2 chemokine and IL-6 could be detected in lung homogenate after repeated LPS challenges (Fig. 1.D and E). These results also suggest tolerance since LPS is known to induce these cytokines after single challenge [28]. Single acute LPS challenge is also known to induce IL-1β [29]. Indeed, the pulmonary IL-1β production was still augmented after repeated LPS challenges in d/d mice, but to a lesser extend in d/d x IL-1R1$^{-/-}$ mice (Fig. 1.F). Semi-quantitative analysis of the lung histology indicated a distinct increase of cell infiltration, mucus production and collagen deposition in d/d mice upon repeated LPS challenges as compared to untreated control mice. Numerous inflammation parameters were reduced in d/d x IL-1R1$^{-/-}$ mice compared to d/d, but did not reach statistical significance likely due to the low number of surviving d/d mice (Fig. 1.A and F-J). In conclusion, the present data support the notion that in response to LPS challenge, IL-1β participates in inflammation and fibrosis in F508del CFTR mutants, and that genetic ablation of IL-1R1 signaling has a protective effect.

2. Increased inflammatory response after *P. aeruginosa* injection in cystic fibrosis lung

To further support this hypothesis in another model of CF pathology we used *P. aeruginosa* infection, a common lung infection occurring in CF patients. We established the model in our facility previously and showed that antibody neutralization of LPS inhibited acute and fatal *P. aeruginosa* induced pneumonia [30]. First, a *P. aeruginosa* (PA011) infection was performed in WT and d/d mice, WT mice administered saline were used as control. Twenty hours after *P. aeruginosa* infection, all mice developed severe pathology, as indicated by weight loss (Fig. 2.A). Lung inflammation, analyzed by the myeloperoxidase (MPO) activity, total cell and neutrophil counts in BALF was significantly increased in all

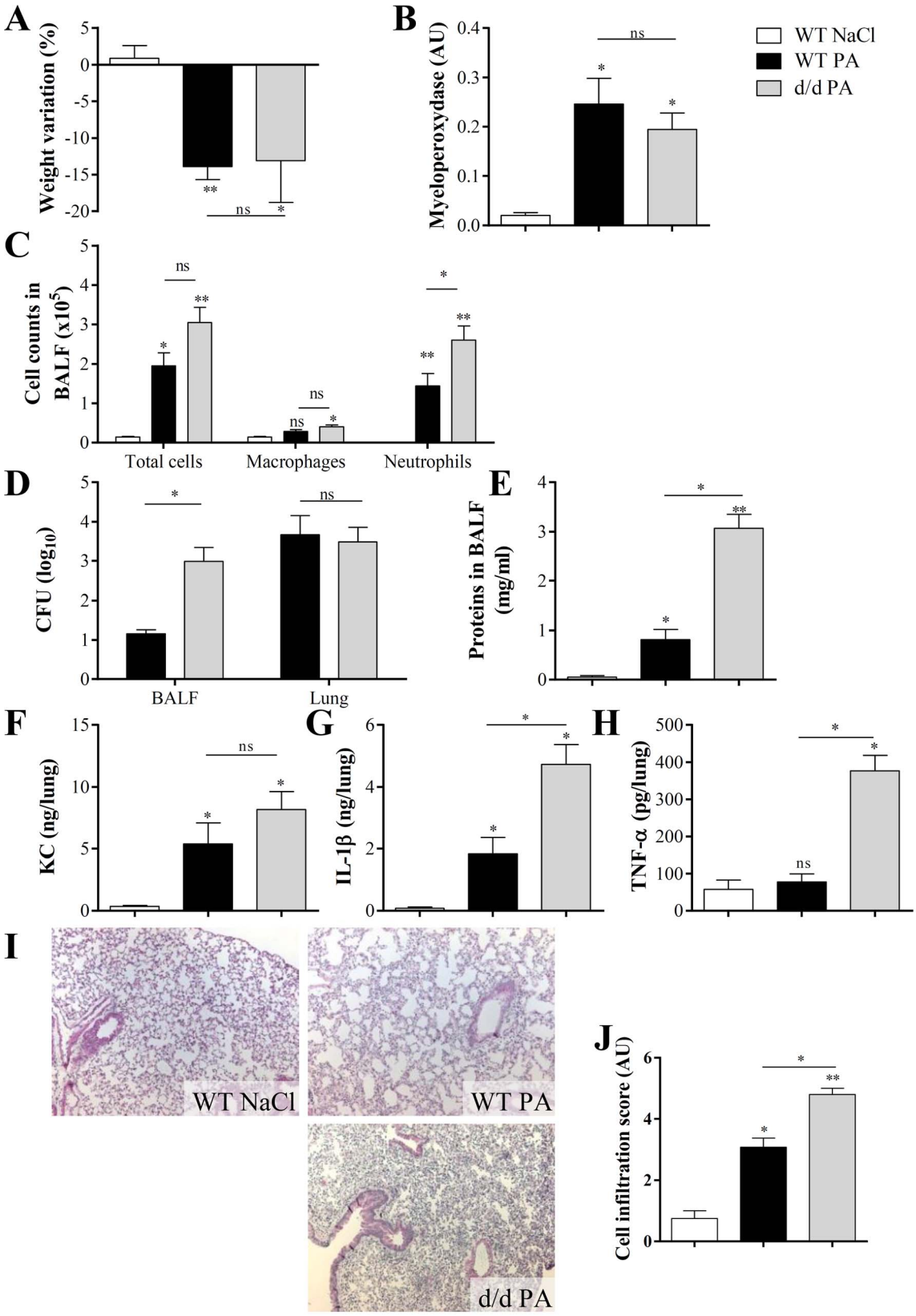
A
Weight variation (%)
B
Myeloperoxydase (AU)
WT NaCl
WT PA
d/d PA
C
Cell counts in BALF (x10^5)
Total cells
Macrophages
Neutrophils
D
CFU (log10)
BALF
Lung
E
Proteins in BALF (mg/ml)
F
KC (ng/lung)
G
IL-1β (ng/lung)
H
TNF-α (pg/lung)
I
WT NaCl
WT PA
d/d PA
J
Cell infiltration score (AU)

Fig. 2. *P. aeruginosa* infection induces an increased inflammation in d/d mice. WT and d/d mice received 2×10^5 CFU of *P. aeruginosa* PA011 intranasally, and WT mice treated with NaCl 0.9% were used as control. Body weight variation 20 h after infection is shown in (A). Myeloperoxidase activity was quantified (B) and absolute numbers of cells; (macrophages and neutrophils) were measured in BALF (C). Bacterial load (total CFU) was determined in BALF and in lung homogenate (D). Total protein concentration was evaluated in BALF (E) and KC (F), IL-1β (G) and TNF-α (H) were measured in lung homogenate. Cell infiltration was observed on H&E stained slide (I) and scored (J). (n=5–6) Values are in mean +/− SEM; * for $p<0.05$; ** for $p<0.01$ and *** for $p<0.001$. ns for non-significant.

doi:10.1371/journal.pone.0114884.g002

groups compared with uninfected mice (Fig. 2.B and C). The bacterial load in the lung at 20 h did not differ between the groups (Fig. 2.D). However, bacterial load (CFU) detected in BALF of d/d mice was two logs higher in d/d mice than in WT mice (Fig. 2.D). Increased CFU in BALF of d/d mice was associated with heightened neutrophil recruitment as compared with WT mice (Fig. 2.C). This correlated with increased epithelial injury in d/d mice, compared to WT mice (Fig. 2.E) indicating that pulmonary epithelial damage and protein release were enhanced in d/d mice compared to WT mice (Fig. 2.G and H). KC chemokine (CXCL1), known to attract neutrophils is increased in all groups after *P. aeruginosa* infection, as compared with uninfected mice (Fig. 2.F). In d/d mice, IL-1β and TNF-α levels were substantially higher in the lung than in WT mice. Histological analysis showed augmented cell infiltration in d/d mice as compared with WT mice after *P. aeruginosa* infection (Fig. 2.I and J).

3. IL-1β antibody neutralization attenuates *P. aeruginosa*-induced inflammation

First, to study IL-1β function at steady state in the lung, we investigated the effect of a long-lasting anti-IL-1β antibody treatment in wild type mice or in mice with F508del CFTR mutation (d/d). Following 8-week antibody treatment, the young adult mice (14-week-old females and males) were investigated. The treatment was well tolerated; no mortality or morbidity was observed in any of the experimental groups. Histological analysis did not reveal any significant differences of cell recruitment, mucus production and collagen deposition in the lung (Fig. 3.A-C) between WT and d/d mice treated with control PBS or anti-IL-1 antibody. Lung mRNA expression of *Il1b*, *Il6* and *Ccl2* was assessed by qPCR and no significant difference was found among the control and the anti-IL-1β antibody administered groups (Fig. 3.D and E). Therefore, this investigation suggests that under basal conditions, in the absence of stimulation, no significant difference is detected between d/d and WT control mice. The F508del CFTR murine model (on C57BL/6 background), displayed no significant spontaneous lung inflammation under these conditions. Furthermore, long-term IL-1β antibody treatment had neither a significant effect on pulmonary morphology, nor on basal expression of *Il1b*, *Il6* and *Ccl2*, suggesting that IL-1β plays no significant role in steady state conditions.

Subsequently, the role of IL-1β upon *P. aeruginosa* infection was studied using neutralizing antibodies in C57BL/6 mice. Mice were infected by intranasal instillation with 10^6 CFU of *P. aeruginosa* (strain PA011) in order to induce an acute inflammation analyzed at 20 h. To test the role of IL-1β, mice received two

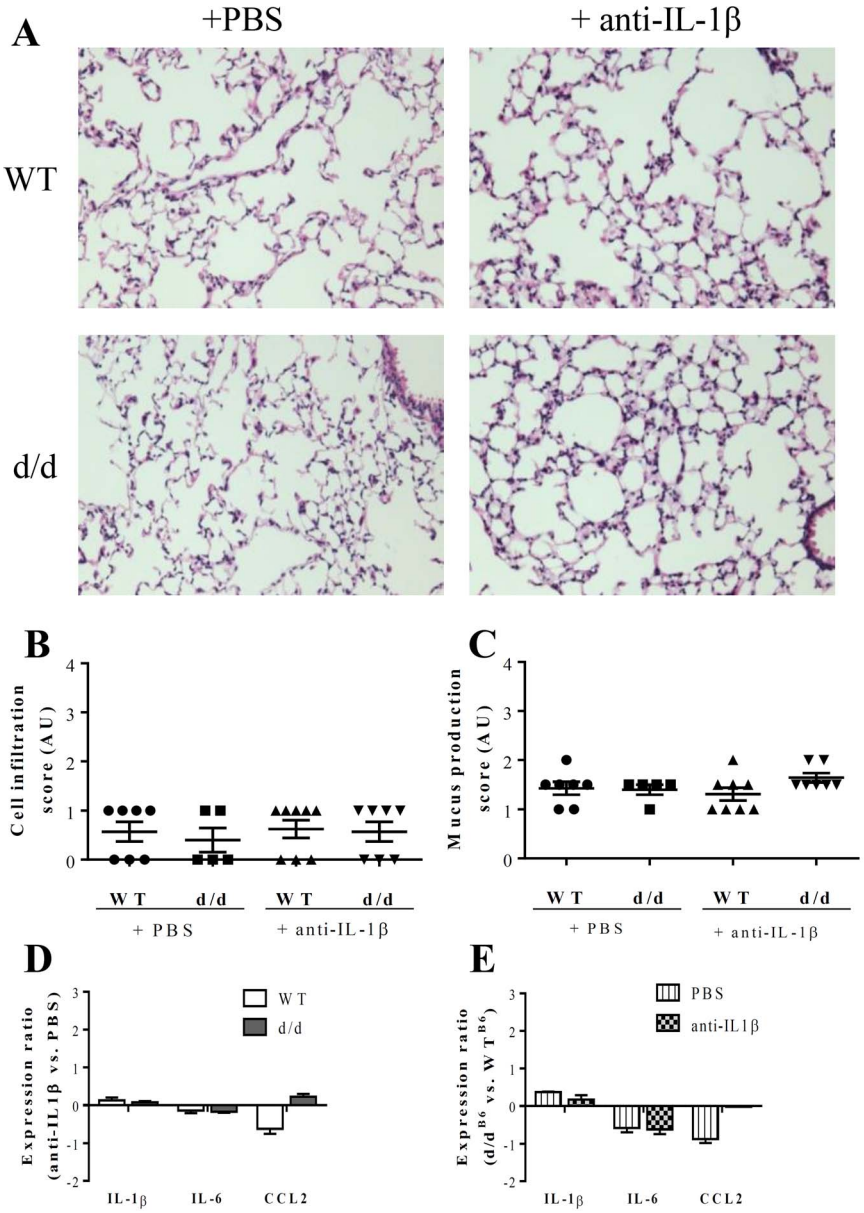

Fig. 3. Anti-IL-1β antibody treatment has no significant effect on lung ultrastructure, nor on IL-1β, IL-6 and CCL2 mRNA production in lungs. Wild type (WT) and F508del CFTR mutation homozygote (d/d) mice (14-week-old females and males) were treated intra-peritoneally, once a week for 8 weeks, an anti-IL-1β antibody (10 mg/kg), or PBS. Mice were euthanized 1 week after the last treatment and lung structure was observed on H&E stained slides observed at ×20 magnification (A). Cellular infiltration (B) was quantified on these H&E slides and mucus production (C) was analyzed on CAB stained lung slides. Lung injury score was recorded in anti-IL-1β antibody treated mice compared with PBS treated mice. (D and E) mRNA production of IL-1β, IL-6 and CCL2 was measured, and normalized with 2 housekeeping gene expression (*Hprt1* and *Gapdh*). The effect of anti-IL-1β antibody treatment compared to PBS control in WT and d/d animals is shown in (D), and the effect of F508del CFTR mutation compared to WT is shown in (E). (n=6–7)

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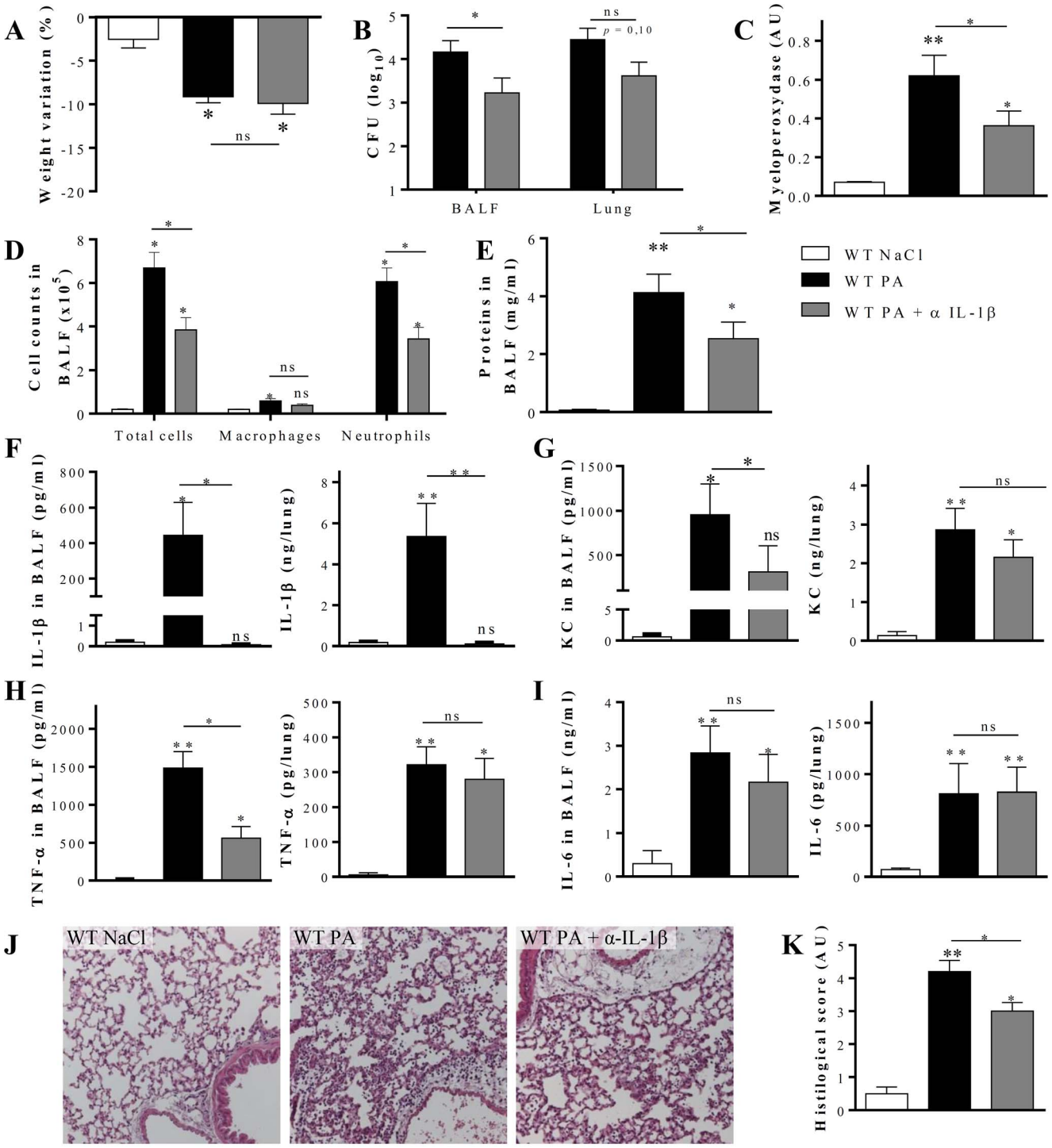

Fig. 4. IL-1β participates in *P. aeruginosa*-induced inflammation at 20 h in WT mice. WT mice received 10^6 CFU of *P. aeruginosa* PA011 intranasally, and WT mice treated with NaCl 0.9% were used as control. A group of WT mice was also treated intraperitoneally with anti-IL-1β antibody, 200 μg/mice, 15 h and 1 h before infection. Body weight variation 20 h after infection is shown in (A). Bacterial load was determined in BALF and in lung homogenate (B), myeloperoxidase activity was quantified (C) and absolute numbers of cells, (macrophages and neutrophils) were measured in BALF (D). Protein concentration was evaluated in BALF (E) and IL-1β (F), KC (G), TNF-α (H) and IL-6 (I), were measured in BALF and in lung homogenate. Cell infiltration was observed on H&E stained slide (J) and scored (K). (n=5–6) Values are in mean +/− SEM; * for $p<0.05$; ** for $p<0.01$ and *** for $p<0.001$. ns for non-significant.

doi:10.1371/journal.pone.0114884.g004

peritoneal injections of 200 μg neutralizing antibodies 15 h and 1 h before infection. As reported before (Figure 2 and in [30]), infection of C57BL/6 mice with *P. aeruginosa* caused a rapid weight loss (**Fig. 4.A**), with a bacterial load of 10^4 CFU in BALF and total lung (**Fig. 4.B**). *P. aeruginosa* infection provoked neutrophil recruitment (revealed by myeloperoxidase activity measurement and cell counts in BALF), and epithelial damage suggested by an increase of proteins in BALF in all groups post infection (**Fig. 4.C**-**E**). However, IL-1β neutralization had a distinct effect on infection and lung inflammation. Indeed, IL-1β blockade significantly reduced bacterial load in BALF, and to a lesser extent in lung (**Fig. 4.B**), suggesting a substantial difference in bacterial clearance between the two groups. This was correlated with a decrease in neutrophil recruitment (**Fig. 4.C** and **D**) and an inhibition of protein in BALF as measure of epithelial damage (**Fig. 4.E**). Moreover KC and TNF-α production in BALF were diminished (**Fig. 4.G** and **H**), whereas IL-6 was not modified (**Fig. 4.I**). In agreement with this, histological analysis showed reduced cell infiltration in anti-IL-1β treated mice as compared with WT untreated mice after *P. aeruginosa* infection (**Fig. 4.J** and **K**). These data suggest that IL-1β antibody neutralization may attenuate *P. aeruginosa*-induced acute lung inflammation.

Discussion

CF patients display increased susceptibility to chronic infections with opportunistic bacteria, excessive lung inflammation and fibrosis leading to fatal loss of function eventually [6]. *P. aeruginosa* has been shown to induce IL-1β through NLRC4 inflammasome activation [9, 10]. CFTR deficient mice were found to be more susceptible to *P. aeruginosa* infection (acute [14, 15] and chronic [16]) and have an exacerbated inflammatory response to LPS and *P. aeruginosa*. Furthermore, F508del CFTR mutant alveolar macrophages display an enhanced IL-1β production in response to LPS stimulation [18]. Thus, we were interested in clarifying the role of IL-1β in the resolution of *P. aeruginosa* infection, in a murine model with the most common CF mutation F508del CFTR [21–23]. Here, we show that, after *P. aeruginosa* infection, excessive activation of IL-1β in F508del CFTR (d/d) mice compared to WT was accompanied by increased CFU in BALF, inflammation and lung damage. Further we show that a therapeutic antibody administration attenuates inflammatory response in an acute model of infection using WT mice.

In CF patients bacterial infections persist and their chronicity facilitates lung inflammation, and subsequent tissue remodeling with severe loss of function [1]. CF lungs display excessive immune response, with increased neutrophil recruitment [8]. We first investigated the role of IL-1β under pathologic conditions, induced by repeated *P. aeruginosa*-LPS challenges. We observed LPS-induced mortality in d/d mice, that was absent in d/d x IL-1R1$^{-/-}$ double mutants, and a higher inflammation in d/d mice compared to the double mutant, suggesting a detrimental role of the IL-1R1 pathway in LPS-challenged d/d mice

(Fig. 1). The deficiency in IL-1R1 apparently protected the mice against LPS-induced mortality in d/d mice. *P.aeruginosa*-LPS challenges were responsible for leucocyte recruitment, including neutrophils. Despite increased cell infiltration in the lung of d/d mice, collagen deposition and mucus production were only slightly increased after multiple LPS challenges. Repeated LPS challenges did not cause epithelial damage and chronic inflammation, which we explain by tolerance induction [31], that is the reason to switch to *P. aeruginosa* infection.

No spontaneous inflammation was found in young adult F508del CFTR mutant on C57BL/6 genetic background under our conditions. Whereas signs of inflammation and tissue remodeling develop early in most CF patients [32], results on basal inflammation in murine models are variable [23]. These data are consistent with published data on the absence of spontaneous pathology such as mucus plugging, neutrophil accumulation or bronchiectasis, in young mice deficient or mutated for the *Cftr* gene [15, 33–35], but neutrophilic inflammation has been reported in absence of infection [36, 37]. Spontaneous lung pathology development seems to be dependent on the age of mice [37], genetic background and animal facility health status [23]. However, in CF mutant mice challenged with pro-inflammatory agents (*P. aeruginosa*, LPS) enhanced injury and inflammation compared to wild type was reported in several independent studies [14, 15, 26, 38].

Therefore, to get closer to a model of lung infections that occur in CF patients, we used an acute *P. aeruginosa* infection model, which is the most current pathogen found in CF patient [7], in F508del CFTR mice. Twenty hours after *P. aeruginosa* infection, d/d mice displayed increased neutrophil recruitment in BALF as compared with WT mice (Fig. 2). They also showed an increase in bacterial load and cytokine production, such as IL-1β and TNF-α, in BALF. These results suggest a higher sensitivity to infection in d/d mice, consistent with previous studies in CFTR mutant mice, showing an augmentation of bacterial load and production of pro-inflammatory cytokines after *P. aeruginosa* infection [14, 15, 38]. The enhanced production of IL-1β compared to WT after infection was associated with increased epithelial damage, cell infiltration and CFU (Fig. 2). Then, we analyzed the effect of IL-1β neutralization at steady state in the lung. We found that in d/d as well as in WT mice, IL-1β antibody had no significant effect on survival. Furthermore d/d and WT mice displayed no difference in basal *Il1b* mRNA expression, and repeated anti-IL-1β antibody administration had no significant effect on basal inflammatory parameters and cytokine production. However, our data suggest that IL-1β may cause an excessive and pathologic inflammation in challenged CF mutant lungs. IL-1β over-expression in d/d mice infected with *P. aeruginosa* was accompanied by a higher bacterial load. Indeed, excessive production of inflammatory cytokines has been associated with bacterial persistence in other studies as well. High concentrations of cytokine, such as IL-1β, IL-6 or TNF-α, enhance intracellular and extracellular bacterial growth of *P. aeruginosa*, or *S. aureus* in the presence of monocytes [39, 40]. Furthermore, IL-1β release was linked to mortality in a murine model of *P. aeruginosa* infection after thermal injury [41].

Therefore, we tested the effect of a therapeutic antibody against murine IL-1β in our acute *P. aeruginosa* infection model in WT mice. IL-1β neutralization has been successfully tested previously with this antibody in a model of intestinal inflammation [25]. Twenty hours after *P. aeruginosa* infection, IL-1β antibody administration caused a decrease in the inflammatory parameters and in bacterial load, as compared with NaCl-treated mice. This is consistent with studies by Schultz *et al.* who showed that inhibition of the IL-1R1 pathway by either the IL-1R1 mutation or application of the IL-1β antagonist IL-1RA improved antibacterial host defense and reduced pro-inflammatory cytokine production [42]. Reduced neutrophil recruitment by IL-1β neutralization could be due to reduced endothelial activation, as proinflammatory cytokines activate endothelial cells. For instance, TNF-α and IL-17 synergize to induce in cultured endothelial cells the expression of P- and E-selectin, as well as neutrophil chemokines, increasing neutrophil transmigration [43]. IL-1β produced by activated monocytes was shown to augment the expression of adhesion molecules (ICAM-1, E-selectin) *in vitro* [44]. Thus, the diminished leucocyte recruitment we observe with IL-1β neutralization could be related to a reduced endothelial activation. Abnormal recruitment and metabolic adaptation of neutrophils in human CF airways has been demonstrated [45], the molecular mechanism remains to be established but likely involves IL-1β signaling based on the arguments mentioned above. We cannot exclude a role of lung fibroblasts and myoblasts. Indeed, IL-1β stimulated human cardiac fibroblasts overexpress adhesion molecules and neutrophil chemoattractant [46].

In conclusion we propose that the IL-1β pathway is critical to drive excessive and detrimental inflammation in F508del mouse model of CF. We show here that antibody neutralization of IL-1β is well tolerated in mice, has no effect on the unchallenged lung in WT or F508del CFTR mice, whereas it can reduce pathology induced by acute bacterial lung infection.

Author Contributions

Conceived and designed the experiments: J. Palomo BJS BR. Performed the experiments: J. Palomo TM J. Piotet LF MR FR. Analyzed the data: J. Palomo MR. Contributed reagents/materials/analysis tools: J. Palomo MLB DT RBO MS VFJQ. Wrote the paper: J. Palomo BJS BR.

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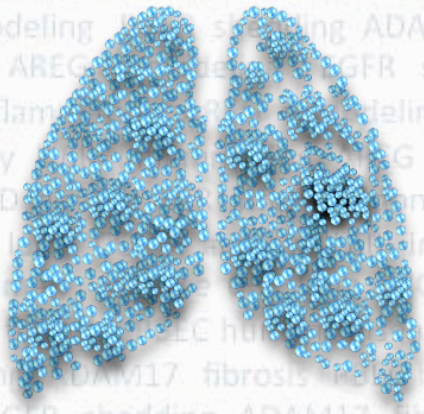
SUMMARY



SUMMARY

Cystic Fibrosis (CF) lung disease and Chronic Obstructive Pulmonory Disease (COPD) are progressive and eventually fatal lung diseases characterised by airway mucus hypersecretion, airway surface liquid dehydratation, chronic inflammation and lung tissue remodelling. Though the etiology of the two diseases is obviously different (CF results form CFTR deficiency, whereas COPD from chronic CS exposure), however, the molecular mechanisms involved in the progression of lung malfunction are likely overlapped, but not completely elucidated. By studying pathological processes in the CF and COPD airway model systems, we aim to establish novel therapeutic targets.

In our studies we focused on ADAM17 and EGFR which are both linked to lung pathology, by regulating inflammation, tissue regeneration, and mucus production. ADAM17 is a membrane bound protease that releases receptor-activating proteins from the cell surface by cleaving extracellular domains from a large number of target proteins. EGFR is a receptor for extracellular growth factors, which activates intracellular protein kinase cascades. They are both activated by external signals, including pollutants like cigarette smoke, viral and bacterial infections. Importantly, EGFR and ADAM17 function and activity are closely linked, so that they establish an EGFR/ADAM17 axis. ADAM17 sheds most of the EGFR ligands that activate the receptor in an autocrine and paracrine manner. When triggered by external signals, ADAM17 releases IL-6R and AREG from airway epithelial cells that transactivate IL-6st/gp130 and EGFR receptors respectively, on the underlying fibroblasts, myofibroblasts and smooth muscle cells. The IL-6/IL-6R/gp-130 and AREG/EGFR pathways converge in STAT3 activation, a transcription factor and genetic modulator of CF lung disease, involved in lung tissue repair, inflammation and fibrosis.

We hypothesized that hyperactivity of airway epithelial ADAM17/EGFR axis may contribute to the excessive inflammation and lung tissue remodelling characteristic of CF and COPD lung disease. Thus the focal point of this thesis was to establish the role of ADAM17 and EGFR in pathology of CFTR-related lung diseases: CF and COPD.

The aim of this thesis was to determine whether and how the COPD status and CF conditions affects basal and induced ADAM17/EGFR activity in airway epithelium. We addressed our hypothesis in advanced airway epithelial cell models: COPD and non-COPD primary bronchial airway epithelial cells exposed to cigarette smoke, and immortalized CF mutant CFBE41o- cells with induced CFTR expression, both cultured on permeable filters in air-liquid interface (ALI-HBEC, ALI-CFBE41o-). In ALI-HBEC we compared the effect of cigarette smoke exposure on ADAM17/EGFR in COPD and non-COPD ALI-HBEC cells, and studied the mechanism of this activation. In ALI-CFBE41o- cells we investigated whether CFTR deficiency hyperactivates ADAM17/EGFR as a cell autonomous defect, and we further defined the druggable targets upstream and downstream of this regulator to potentially apply them as an early intervention CF lung therapy.

Chapter 1 provides a general introduction to CF and COPD pathology and describes the importance of anti-inflammatory therapy in CF. In the second part, ADAM17 and EGFR are introduced and their role in lung inflammation and remodeling is underlined.

In **Chapter 2** we compared CS-induced ADAM17 mediated protein shedding and mRNA expression of sIL-6R and AREG in COPD and non-COPD (ex)smokers. We showed that CS significantly induces IL6R and AREG shedding into the basolateral medium from COPD cells, but not in non-COPD cells suggesting that COPD disease makes cells more sensitive to external triggers. Furthermore, by using selective inhibitors of ADAM17 and EGFR (TMI-2 and AG1478), we showed that they not only regulate shedding of IL-6R and AREG, but also their mRNA levels. Additionally, we showed that upon CS activation ADAM17 meets its substrate in an intracellular vesicular compartment. Also EGFR is internalized after CS exposure. This suggests that trafficking of ADAM17 and EGFR is one of the regulatory mechanisms of ADAM17/EGFR axis activation.

Chapter 3 shows that functional expression of CFTR in ALI-CFBE41o- cells reduces AREG release, suggesting that CFTR deficient cells have intrinsically altered ADAM17 dependent responses. In line with this, we also observed that CFTR deficiency elevates levels of EGFR phosphorylation. As in ALI-HBEC, AREG release depends on EGFR and ADAM17 to the same extent. In CFBE410-cells we observed that CFTR deficiency induces increased apical localization of EGFR, which may lead to abnormal activation of ADAM17/EGFR axis, however, these data need to be confirmed in differentiated primary airway epithelial cells. Finally, we observed that ADAM17 dependent shedding is sensitive to ROS scavengers, in particular extracellular glutathione (GSH). Using the fluorescent redox probes Grx1-roGFP expressed in cytoplasm and Grx1-roGFP-GPI expressed at the apical membrane, we showed that extracellular, but not intracellular redox potential is changed to a more oxidised state by deficient CFTR. Thus, based on these data we propose that deficient GSH secretion in CFTR deficient cells, shown by other authors in analogous systems, causes oxidation of extracellular and possible intravesicular membranes, which in turn leads to enhanced activation of ADAM17 by a known redox dependent conformation change, and concomitant abnormal EGFR signalling and localization.

Chapter 4 Illustrates the role of abnormal sphingosine metabolism in CF lung disease, providing an alternative intervention pathway. We show that unchallenged F508del CFTR mutant mice have enhanced infiltration with pro-inflammatory cells and abnormal dendritic cell activity which correlates with reduced sphingosine-1-phosphate S1P in lungs. Oral administration of an inhibitor of S1P-lyase (LX2931, Lexicon Pharmaceutics), increases tissue S1P levels and reduces inflammation and dendritic cell imbalance. Upon challenge with bacterial toxins (LPS-fMLP) LX2931 reduces induced MUC5AC production and pro-inflammatory cytokines in mutant mice (TNF-α, KC, IFN-γ, IL-12). The S1P receptor network is known to interact with the EGFR/ADAM axis through intracellular kinases, providing a mechanistic link between these observations.

Chapter 5 Summarizes and critically discusses the studies presented in this thesis and provides possible future directions in investigation of EGFR-ADAM17 axis in the context of CF and COPD lung disease.

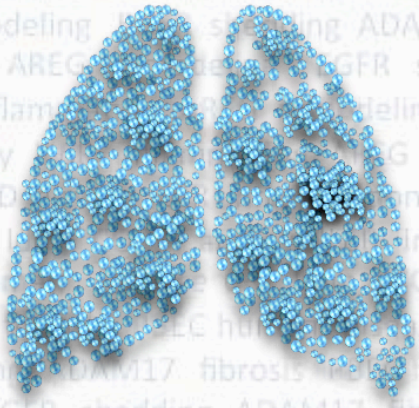
Appendix presents evidence that CFTR mutant mice show excessive inflammation, increased IL-1β production and reduced bacterial clearance. CFTR deficient mice with the IL-1R1 knock-out (double mutant F508del x IL-1R1) have attenuated inflammation, providing a

rationale for targeting the IL-1β/IL-1R1 pathway in CF patients. A therapeutic antibody reduces infection related pathology in this model. Importantly in the context of this thesis, activation of IL-1R also activates ADAM17/EGFR/IL8 signalling, in airway epithelial cell lines. Thus intervention in the IL-1β/IL-R1β pathway may be also beneficial in attenuation of EGFR/ADAM17 signalling in the CF or COPD disease state, which can be further evaluated in differentiated human airway epithelial cells.

Taken together, our data provide evidence for the involvement of the ADAM17/EGFR axis in CF and COPD lung disease. Although CF and COPD differ in primary cause, we showed that they share a molecular mechanism possibly leading to the development and progression of the disease. CS exposure in COPD and CFTR deficiency in differentiated airway epithelial cells induce ADAM17 and EGFR dependent protein shedding of IL-6R and AREG. ADAM17 dependent release of EGFR ligands may affect epithelial (autocrine) and subepithelial (trans-signaling) receptors. This implicates the ADAM17/EGFR axis in inflammation, lung tissue repair and fibrosis. Importantly we identified ROS, which inactivates phosphatases, as a highly plausible link between CFTR deficiency and EGFR/ADAM17 axis. Since we identified several potential druggable targets for intervention in this signalling pathway (GSH, PDI, SRC), future studies evaluating their exact mechanism and relevance in CF and COPD models are of importance.

Future studies are directed towards defining the role of CFTR deficiency in the abnormal regulation of redox potentials, bioactive lipid synthesis, and membrane protein trafficking, which results in inappropriate inflammatory and repair responses in CF lung tissue, and applying these in novel therapeutic approaches.

SAMENVATTING



SAMENVATTING

Longziekte bij cystische fibrose (CF) en COPD (*chronic obstructive pulmonary disease,* rokerslong) neemt toe met de leeftijd, is niet goed te behandelen, en leidt tot ernstig verlies van longfunctie met vaak de dood tot gevolg. Beide ziektes worden gekenmerkt door de ophoping van slijm in de luchtwegen, uitdroging van de luchtwegen, chronische ontsteking, moeilijk te bestrijden bacteriële infecties, en onomkeerbare verandering van de structuur van de longen. De primaire oorzaak van de ziektes is duidelijk verschillend. CF wordt veroorzaakt door een erfelijke afwijking in een chloride kanaal betrokken bij vloeistof transport door de slijmcellaag (CFTR). COPD wordt vooral veroorzaakt door blootstelling aan sigarettenrook. Toch vertonen de moleculaire mechanismes die een rol spelen bij het ontwikkelen van deze chronische longziektes overeenkomsten. Het doel van ons onderzoek was deze mechanismes in het laboratorium nader te onderzoeken, zodat we nieuwe geneesmiddelen kunnen testen voor beide ziektes.

We hebben ons geconcentreerd op de rol van twee eiwitten, waarvan we weten dat ze betrokken zijn bij ontstekingsreacties, slijmproductie, en weefselherstel in de long. ADAM17 is een enzym dat meerdere eiwitten van de celmembraan losmaakt, die op hun beurt via specifieke bindingseiwitten (receptoren) processen in de cel regelen. EGFR (epidermal growth factor receptor) is een van de ontvangers van die signalen, die via het celoppervlak worden doorgegeven aan een complex netwerk van eiwit fosforylerende enzymen (kinases) in de cel. Deze activiteit wordt in de long versterkt door diverse uitwendige prikkels zoals rook, beschadiging, en infecties. Zoals ook uit ons onderzoek blijkt beïnvloeden ADAM17 en EGFR elkaar wederzijds zodat ze samen een regelsysteem vormen (EGFR/ADAM17). ADAM17 knipt na stimulatie niet alleen EGFR bindende eiwitten van een cel los, waaronder Amphireguline (AREG), maar ook de receptor van een ontstekingsfactor (interleukine 6 receptor, IL6R). Hierdoor worden ook nabij-liggende cellen die een EGFR receptor of een interleukine receptor hebben geactiveerd. Op deze manier kunnen prikkels die het oppervlak van de long beïnvloeden worden doorgegeven aan het onderliggende bindweefsel en witte bloed cellen (trans-stimulatie). De ADAM17/AREG/EGFR en IL6/IL6R/gp130 receptor systemen activeren samen de productie van eiwitten nodig voor normale ontstekingsreacties en weefselherstel, onder andere via STAT3, een eiwit dat in de celkern de productie van specifieke messenger RNA's regelt, en waarvan bekend is dat het mede de ernst van CF longziekte bepaalt.

De Hypothese. We veronderstelden bij het begin van ons onderzoek dat een verhoogde activiteit van het ADAM17/EGFR systeem bijdraagt aan de heftige ontstekingsreacties en structuurveranderingen van de long bij CF en COPD. Daarom waren onze experimenten er vooral op gericht om de rol van ADAM17 en EGFR bij het ontstaan van chronische longziekte te analyseren.

We onderzochten daarvoor het effect van COPD en CF op de activiteit van ADAM17/EGFR in long-slijmhuid cellen (epitheel). Hiervoor kweekten we cellen van COPD patiënten, en van personen zonder COPD. Deze cellen werden onder gecontroleerde omstandigheden blootgesteld aan sigarettenrook. Zo konden we de activering van ADAM17/EGFR en het effect van chronische COPD onderzoeken. Daarnaast gebruikten we epitheel cellen oorspronkelijk geïsoleerd uit de long van een CF patiënt, waarin we het ontbrekende eiwit CFTR konden activeren. Op die manier konden we vaststellen dat het ontbreken van CFTR de activiteit van EGFR/ADAM17 inderdaad verhoogt. Door onder andere remmers van ADAM17 en van EGFR te testen, hebben we laten zien dat dit proces door experimentele geneesmiddelen beïnvloed kan worden.

Hoofdstuk 1 voorziet in een algemene inleiding over CF en COPD longziekte, en beschrijft het belang van anti-ontstekingstherapie bij de bestrijding hiervan. In het tweede deel worden ADAM17 en EGFR in detail beschreven, en hun mogelijke relatie met longziekte.

Hoofdstuk 2 beschrijft een onderzoek met longcellen die zodanig op filters zijn gekweekt, dat ze zich gedragen als slijmhuidcellen in de luchtwegen, door ze bloot te stellen aan lucht (*air-liquid interface culture*). Deze experimenten laten zien dat cellen afkomstig van COPD patiënten na behandeling met sigarettenrook een sterkere ADAM17/EGFR activiteit vertonen dan cellen van niet-CF patiënten, waarbij de groeifactor AREG en de ontstekingsfactor IL6R aan de bloedzijde van de cellaag worden uitgescheiden. Dit suggereert dat de longen van COPD patiënten een sterkere reactie op rook vertonen dan normaal. Verder hebben we laten zien dat remmers van zowel EGFR als ADAM17 niet alleen de uitscheiding van AREG en IL6R verminderen, maar ook de productie van de bijbehorende messenger RNA's, wat duidt op de betrokkenheid van transcriptiefactoren in de kern. Een onverwacht resultaat is dat ADAM17 na activering door sigarettenrook binnen in de cel een interactie aangaat met AREG en IL6R, niet op het oppervlak waar de producten worden uitgescheiden. Ook EGFR wordt na activering vanaf het celoppervlak naar binnen getransporteerd. Het transport van deze membraaneiwitten in de cel na stimulatie is blijkbaar een onderdeel is van het regelmechanisme. Dit moet nader onderzocht worden, en zal van belang zijn bij de ontwikkeling van nieuwe geneesmiddelen.

Hoofdstuk 3 laat zien dat onze hypothese juist was. De productie van de groeifactor amphireguline (AREG) door CF luchtweg epitheelcellen neemt af wanneer we ze een actieve vorm van eiwit CFTR laten maken. Productie van AREG is ook in deze cellen volledig afhankelijk van ADAM17 en EGFR. De activering van EGFR, gemeten als fosforylering door kinases is hoger in cellen zonder CFTR. Verder zien we meer EGFR op het aan de lucht blootgestelde deel van de celmembraan. Het verband tussen deze verhoogde activiteit en de afwezigheid van CFTR hebben we nader onderzocht. Onze metingen laten zien dat het CFTR defect een verhoogde oxidatie van de celmembraan veroorzaakt. We constateerden ook dat stoffen die de oxidatie van de celmembraan verminderen, met name glutathion, de uitscheiding van AREG sterk verminderen. Oxidatie veroorzaakt een stressreactie in de cel, en die beschermt zich daartegen onder andere door de uitscheiding van een natuurlijk antioxidant: glutathion. Andere onderzoekers hadden al laten zien dat glutathion transport in CF cellen minder is dan normaal. We concluderen dan ook dat in CF luchtweg cellen de oxidatie-stress veroorzaakt door onvoldoende glutathion transport leidt tot hyperactiviteit van EGFR/ADAM17. Waarschijnlijk komt dit door een verandering in de structuur van ADAM17, gecombineerd met een verandering van het intracellulaire transport van EGFR/ADAM17. Samenvattend betekent dit waarschijnlijk dat ook CF epitheel cellen in de long van een patiënt spontaan een verhoogde activiteit van het EGFR/ADAM17 vertonen, ook als er geen bacteriële infectie is in die long, met versterkte inflammatie en fibrose tot gevolg.

Hoofdstuk 4 maakt gebruik van een CF muismodel dat we eerder gemaakt hebben, met dezelfde erfelijke afwijking als de meeste CF patiënten (F508del CFTR). In dit model stelden we eerst met een geavanceerde methode vast dat de muizen longontsteking vertonen, zonder dat ze worden blootgesteld aan infecties of andere stressfactoren. Blijkbaar is het CFTR defect op zichzelf voldoende om witte bloedcellen te activeren tot een ontstekingsreactie. Verder vonden we dat de productie van een bekende signaalstof betrokken bij longontsteking, sphingosine-fosfaat (S1P) sterk verlaagd is in de long van de CF muizen. Wanneer we de S1P verhogen met behulp van een experimenteel medicijn tegen reumatische ontstekingen (LX2931 Lexicon Pharmaceutics), zien we een afname van

de longontsteking in CF muizen. Ook wordt de ontstekingsreactie op bacteriële toxines in de long minder langdurig. S1P en zijn receptoren maken deel uit van een groter signaalnetwerk, waar ook EGFR/ADAM17 deel van uitmaakt, en dat beïnvloed wordt door CFTR.

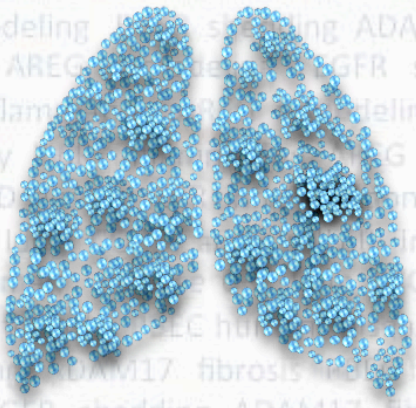
Hoofdstuk 5 geeft een samenvatting en een kritische analyse van de studies, en geeft aan wat de volgende stappen zijn in het onderzoek naar de relatie tussen EGFR/ADAM17 en chronische longziekte.

Appendix beschrijft in een gerelateerde studie dat F508del CFTR muizen meer moeite hebben om een bacteriële infectie te overleven dan gezonde muizen, en dat ze daarbij een verhoogde productie van het signaaleiwit IL1b vertonen. Een mutatie in een receptor van IL1b, en de toediening van een antilichaam tegen IL1b verminderen de gevoeligheid van muizen voor bacteriële infectie. Daarbij is van belang voor onze studie dat IL1b EGFR/ADAM17 activeert, en dus ook deel uitmaakt van dit CFTR afhankelijke regelnetwerk. Dit biedt een alternatieve mogelijkheid om in te grijpen in het proces van excessieve long inflammatie in CF en mogelijk bij COPD.

Samenvattend geven onze resultaten aan dat het EGFR/ADAM17 systeem betrokken is bij zowel CF als COPD longziekte. Hoewel deze ziektes verschillen wat betreft hun primaire oorzaak, laten we zien dat ze een gemeenschappelijk moleculair mechanisme hebben dat mede verantwoordelijk kan zijn voor het ontstaan en voortschrijden ervan. Sigarettenrook en CFTR defecten in luchtwegslijmhuidcellen activeren EGFR/ADAM17 afhankelijke uitscheiding van signaalmoleculen (IL6R en AREG). Dit kan de slijmproductie, het bindweefsel, en de witte bloedcellen in het weefsel activeren, wat kan bijdragen tot de typische ziekteverschijnselen van CF en COPD. Verder wijzen onze studies er op dat vooral oxidatiestress de verbindende schakel is tussen het CFTR defect en EGFR/ADAM17 activering. Dit biedt meerdere mogelijkheden tot nader onderzoek naar toepassing van nieuwe geneesmiddelen die dit regelsysteem beïnvloeden.

Toekomstige studies worden gericht op de rol van CFTR bij het regelen van oxidatiestress, de productie van signaalmoleculen, en het transport van membraan eiwitten, wat in de CF long leidt dit tot heftige ontstekingsreacties en abnormaal weefselherstel. De resultaten kunnen ons helpen om nieuwe geneesmiddelen te ontwikkelen.

CV & PhD PORTFOLIO



CURRICULUM VITAE

PERSONAL DETAILS

First and last name:	**Marta Stolarczyk**
Education level:	**MSc in Molecular Biology**
	(PhD candidate at ErasmusMC:
	University Medical Center Rotterdam)
Phone number:	**(+49) 162 153 184 6**
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Address:	Gustav-Kirchhoff Str 2b, 69120 Heidelberg, Germany
Date of Birth:	May 21th,1986, Iława, POLAND

EDUCATION

2011 – 2015 **PhD candidate, ERASMUS MC: UNIVERSITY MEDICAL CENTER ROTTERDAM, DEPARTMENT OF CELL BIOLOGY**
Thesis title: EGFR/ADAM17 axis in Lung Diseases: Cystic Fibrosis and COPD.

2008 – 2010 **MSc in Molecular Biology, UNIVERSITY OF WARSAW, BIOTECHNOLOGY, DEPARTMENT OF METABOLIC REGULATION**
Thesis title: Binding test optimization and examination of nuclear receptor PPARD activity changes under various candidate drugs stimulation

2005 – 2008 **BSc in Biotechnology, UNIVERSITY OF WARSAW, BIOTECHNOLOGY, DEPARTMENT OF METABOLIC REGULATION**
Thesis title: Agents stimulating insulin secretion

2003 – 2005 **High School No.2, Olsztyn**

PROFESSIONAL EXPERIENCE

2016 – present **PostDoc, UniversitatsKlinikum, Heidelberg, Hematology**
3D culture models for precision medicine of blood cancers

2011 – 2015 **PhD candidate, ERASMUS MC: UNIVERSITY MEDICAL CENTER ROTTERDAM, DEPARTMENT OF CELL BIOLOGY**
Investigation of the inflammatory responses mediated by ADAM17 in lung *ex vivo* models: primary bronchial human cells, CFBE cells, organoids.

2009 – 2010 **Pharmaceutical Company ,,ADAMED" - a contract for a specific task**
Investigation of the role of PPARbeta/delta agonists in diabetes type II in primary and stable cell lines (Hepa-1,6, HepG2, C2C12, 3T3L1)

2009 **Biotechnology Company "BIOTON" – a student training**
▪ transfection with bacterial DNA plasmid and plasmids isolation from bacterial culture, various electrophoresis techniques, PCR, electroporation, biochemical tests API (BioMerieux), SDS-PAGE electrophoresis, ELISA test for E.coli proteins, transfection with GFP

2009 **Fertility Clinic in Warsaw "NOVUM" – a student training**
▪ *in vitro* fertilization (IVF), cytogenetic and bacteriology analysis, blood tests, urine samples analysis

(INTER)NATIONAL CONFERENCES

2014	**North American Cystic Fibrosis Conference, NACFC, Atlanta** Poster presentation
2013	**Basic Science Meeting, Malta** Oral and Poster presentation
2013	**NRS Young Investigator Symposium, Animal models in pulmonary research, Utrecht** Poster presentation
2012	**The 7th European CF Young Investigator Meeting, Paris** Oral presentation: Adam17 a main player in CF lung disease?
2011	**FEBS, Sorrento** Poster Presentation: Cell Biology and Pharmacology of Mendelian Disorders
2011-2014	**Long Dagen, Utrecht** Poster presentations

FELLOWSHIPS

2015	**Short-term fellowship EMBO award** 4 month intership in Gergely L. Lukacs laboratory at McGill University, Montreal

WORKSHOPS AND MEETINGS

2011-2013	**PhD Workshops**, Maastricht (2011), Dusseldorf (2012), Luxemburg (2013)
2011-2015	**Regular meetings of the Netherlands CF groups,** Utrecht
2011-2015	**Regular meetings with CF experts in Sophia Hospital,** Rotterdam
2011-2014	**Journal Club Presentations**

SUPERVISING INTERNSHIPS

2012-2014	**Supervision of 3 internships** Duration: 9 months

COURSES AND TRAININGS

2016	**KNIME** Heidelberg Center for Human Bioinformatics (HD-HuB)
2014	**Photoshop and Illustrator C6 Workshop,** ErasmusMC, Rotterdam
2010	**Western Blotting Course** MERCK, Warsaw
2008	**Training in Animal Handling** University of Warsaw, Poland
2007	**Radiation Safety and Isotopic Techniques Training** University of Warsaw, Poland

PUBLICATIONS

In preparation	(1st author)	Stolarczyk et al, Lack of CFTR function in polarized bronchial epithelial cells results in increased extracellular redox potential leading to augmented ADAM17 and EGFR activity and enhanced AREG release.
2016	(1st author)	Stolarczyk et al., ADAM17 and EGFR regulate IL-6 receptor and amphiregulin in COPD bronchial epithelial cells, Physiol Rep
2016		Veltman et al., Correction of Lung Inflammation in a F508del CFTR murine Cystic Fibrosis model by the Sphingosine-1-Phosphate Lyase Inhibitor LX2931, Am J Physiol Lung Cell Mol Physiol
2014		Palomo, et al., Role of IL-1β in experimental cystic fibrosis upon P.aeruginosa infection, PLoS One
2011		Stolarczyk et al, Nuclear receptors PPAR as a drug target in metabolic disorders, Postepy Biochem.

PhD Portfolio

Summary of PhD training and teaching

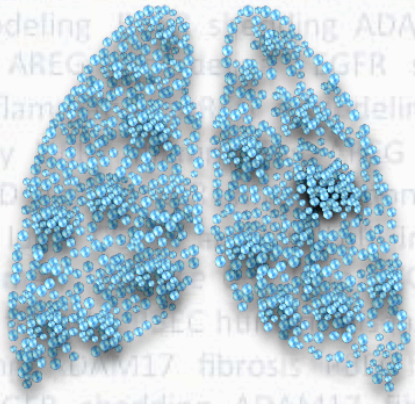
Name PhD student: Marta Stolarczyk	PhD period: 2011-2015
Erasmus MC Department: Cell Biology	Promotor(s): Prof. Dr. Frank Grosvelt
Research School: MGC Postgraduate School	Supervisor: Dr. Bob J Scholte

1. PhD training

	Year	Workload
General courses		
- Safety Laboratory Techniques	2011	0.3 ECTS
- Biophysics & Biochemistry	2011	3 ECTS
- Cell and Developmental Biology	2012	3 ECTS
- Genetic	2012	3 ECTS
- Laboratory Animal Science	2012	3 ECTS
- Literature discussion	2013	2 ECTS
- Statistic	2013	2 ECTS
- English for Academic Purpose	2014	3 ECTS
- Photoshop and Illustrator C6 Workshop	2014	0,3 ECTS
Seminars and workshops		
- PhD Workshop, Maastricht	2011	32 hours
- NRS Young Investigator Symposium, Animal models in pulmonary research, Utrecht	2011	0,3 ECTS
- PhD Workshop, Dusseldorf (poster presentation: The role of the sheddasses (Adam17) in Cystic Fibrosis – Chronic lung disease)	2012	32 hours
- NRS Young Investigator Symposium, Animal models in pulmonary research, Utrecht	2012	0,3 ECTS
- PhD Workshop, Luxemburg (oral presentation: COPD and Cystic Fibrosis a tale of two diseases)	2013	32 hours
Presentations		
- Oral presentation, Leiden (The role of ADAM17 and IL6 pathway in Cystic Fibrosis in HT29 adenocarinoma cells)	2011	1 ECTS
- Oral presentation, Utrecht (The role of ADAM17 and IL6 pathway in Cystic Fibrosis Lungs)	2011	1 ECTS
- One year evaluation presentation (The role of ADAM17 and IL6 pathway in Cystic Fibrosis and COPD)	2012	1 ECTS
- Oral presentation, Leiden (The role of ADAM17 and IL6 pathway in chronic lung disease: Cystic Fibrosis and COPD)	2012	1 ECTS
- Oral presentation Utrecht (Does CFTR regulate shedding of cytokines and growth factors in CF ling disease?)	2012	1 ECTS
- Monday morning meeting (CF)	2012	1 ECTS
- Monday morning meeting (COPD)	2013	1 ECTS
- Oral Presentation, Sophia Children Hospital (COPD and CF)	2013	1 ECTS
- Oral Presentation, Utrecht (The role of ADAM17 in chronic inflammatory lung diseases: CF and COPD)	2013	1 ECTS
- Oral Presentation, ErasmusMC, CF meeting (Metalloproteases in CF and COPD, primary bronchial epithelial cells in culture as a surrogate model for lung pathology)	2013	1 ECTS

- Monday morning meeting (The mode of action of ADAM17 in resolution of inflammation in CF. The pulmonary models to understand the mechanisms of lung fibrosis)	2013	1 ECTS
Long Dagen, Utrecht (poster presentation)	2013	0.5 ECTS
- NRS Young Investigator Symposium, Animal models in pulmonary research, Utrecht (poster presentation)	2014	1 ECTS
- Oral presentation, Utrecht CF meeting (ADAM17 and inflammatory responses in CFTR deficient cells and Lung organoids as a tool for drug screening in CF pathology)	2014	1 ECTS
- Monday morning meeting (The influence of CFTR potentiators and correctors on inflammatory responses in CF lung disease)	2014	1 ECTS
- Monday Morning meeting (The strategies in CF therapy)	2014	1 ECTS
- Long Dagen (poster presentation)	2014	1 ECTS
- Oral Presentation, Montreal (The mode of action of ADAM17 in resolution of inflammation in chronic lung diseases)	2015	1 ECTS
(Inter)national conferences		
- FEBS, Italy (Poster Presentation)	2011	1.4 ECTS
- The 7th European CF Young Investigator Meeting, Paris (Oral presentation)	2013	1.4 ECTS
- Basic Science Meeting Malta (Oral and Poster presentation)	2014	1.4 ECTS
- North American Cystic Fibrosis Conference, NACFC, Atlanta (Poster presentation)	2014	1.4 ECTS
Other		
- Short-term fellowship EMBO award *(4 month internship in Professor Lukacs GL Laboratory at McGill University (Montreal) under supervision of Veit G, Associate Professor)*	2015	4 months
2. Teaching		
Supervising practicals and excursions, Tutoring		
- Internship Technician (M. Veltman)	2011	9 months
- Internship Technician (S. Nieuwenhuize)	2012	9 months
- Internship Technician (A. Labasati)	2013	7 months
Other		
- Journal Club (presentation: “Cigarette smoke exposure induces CFTR internalization and insolubility, leading to airway surface liquid dehydration”)	2012	0.25 ECTS
- Journal Club (presentation: An HNF4a-miRNA Inflammatory Feedback Circuit Regulates Hepatocellular Oncogenesis)	2012	0.25 ECTS
- Journal Club (presentation: Cell surface annexin regulate ADAM-mediated ectodomain shedding of proAREG)	2013	0.25 ECTS
- Journal Club (Functional engrafment of colon epithelium expanded in vitro from a single adult Lgr5+ stem cell)	2014	0.25 ECTS
- Journal Club (A special population of regulatory T cells potentiates muscular repair)	2015	0.25 ECTS

ACKNOWLEDGEMENTS



ACKNOWLEDGEMENTS

After an intensive period of PhD programme, finally came the moment of the finishing touch on my thesis: writing the acknowledgements. As Murray Gell-Mann, an American physicist said: “No individual is alone responsible or a single stepping stone along the path of progress (…)”, also the progress of this thesis would not be possible without support, inspiration and motivation of many people who I met and encountered during my PhD path.

Firstly, I would like to express my sincere gratitude to my promoter **Prof. Frank Grosveld** for the opportunity to be enrolled in a PhD programme at the Cell Biology Department, for being the part of the committee and all advices given on a few occasional meetings. Dear **Prof. Dr. Huylebroeck,** you took a lead on my last years of PhD programme. I really appreciated your guidance, which helped me in all the time of the writing of this thesis.

My sincere thanks goes also to my co-promoter and daily advisor **Dr. Bob Scholte**. Bob thank you for providing me an opportunity to join your lab and the continuous support during my PhD research and also writing of this thesis (here goes also acknowledgements to the NCFS and Longfonds for sponsoring my project). I also appreciated your insightful comments and hard questions, which incentivized me to constantly deepen my knowledge. Finally, I thank you for allowing me to travel to various conferences and opening the opportunities for collaborations, which all had widened my perspective on the project. Finally, thank you for determination in allowing me to finish my PhD and all your help with the finalization of the manuscript, especially in the last year, when our lab encounter difficulties and I was not employed anymore. I took it for granted that in the morning I will get an e-mail from you with a question “Marta, how and where are you?”. You were always available for my questions. It was tough for both of us to finish this thesis without financial support, however, “I was taught that the way of progress was neither swift nor easy” (Maria Skłodowska-Curie, the first women to win the Nobel Prize, a Polish physicist and chemist).

Besides, I would like to thank the rest of the small thesis committee members: **Prof. Dr. Pieter S Hiemstra**, **Associate Prof. Dr Robbert Rottier** and **Prof. Dr. Ruud W Hendriks** for reviewing the thesis and providing comments, suggestions and critics to the manuscript.

Dear Prof. Dr. Pieter S Hiemstra and **Dr. Gimano D. Amatngalim** thank you for our nice collaboration. It was a pleasure to work with you. Your guidance and immense knowledge helped me in all the time of research. You always inspired me and gave a lot of motivation to widen my research.

Dear Associate Prof. Guido Veit and **Prof. Dr. Gergely L. Lukacs** thank you for pursuing the part of my project together and supplying us with your unique CF research model. **Guido** there is no words to express my sincere thanks to you. Thank you for inviting me to your laboratory (here goes also acknowledgements to the EMBO Founding Awards for supporting financially my visit). You were one of my best mentors during my PhD period. You not only gave me a good example in the laboratory practice, but also showed how to improve the organization skills. It was really a great pleasure to have a chance to work with you and have your expertise. We went through the cold winter up to the warm spring in Montreal ;). It is also a good moment to thank **Andrea** for your support and kindness during my stay in the Lukacs lab. You immediately made me feel as in my home lab. **Brian,** it was nice to share with you the office at McGill. Many thanks to **Pierre-Aurelien, Radu, Miklos, Pirjo, Hector, Haijin**, you were all very kind and helpful during this 4-month stay.

I would like to thank **Dr Gert Jansen** and **Dr Niels Galjart** for meetings, discussions and support.

Dear **Robbert**, it was great to share the lab with your group and always have a good support and feedback from you. I thank my fellow lab-mates for the stimulating discussions and for all the fun we had in the last four years. **Mieke**, it was great to have you as my first intern and later as a technician. Thank you for all your help and willingness for crazy parties! Thanks, smiley ☺! **Heelen**, you were not only a person to talk to, but also a person to dance and joke ☺. Take care of your family! **Marjon**, I loved your positive attitude everyday, simply do not change ;)! A lot thanks to **Joshua**, you was the first person showing me around. **Yadi**, it was nice to have evening's chats with you. **Kim** and **Anna** thanks for having you around. I will miss you all a lot! **Xiao**, I really enjoyed Food-Festival with you and our collaboration ☺! It was fun to do experiments together. Thanks to all the people on the 10th floor! **Alex, Nesrin, Luca, Jessica, Thomas, Andrea, Polynikis, Sreya, Liu, Dave**. It was great to have you around!

Marcel, thanks for enlightening me organoid cultures. **Hugo**, thank you for all your advices! **Paulina**, it was nice time in Atlanta! **Isabellus**, it was amazing trip to Antwerp. Good luck with finding nubs! I also would like to thank **Jeffrey, Johanna, Ludwik** for our regular CF meetings in UMC. I also thank all the people that I met during conferences, who I have not mentioned here by name, but they brought knowledge for fresh ideas ….

Marike, thank you for all your help. I will never forget your support with the final steps in order to submit my thesis. Thanks for all your actions, mails (sometimes late in the evening) and calming me down.

Dorota bralas udzial przy moim procesie rekrutacyjnym. Dziekuje za przetlumaczenie mojej polskiej pracy magisterskiej. Dzieki za pozytywny glos ☺! **Madzia** dziekuje za nasze mile spotkania i dzielenie sie ze mna Twoimi doswiadczeniami, ktore pomogly mi kierowac moja sciezka zawodowa. **Karolina,** dziekuje za wspolne rozmowy przy kawusi i nasze krotkie wycieczki. **Kasia,** dziekuje za wspolnie spedzone swieta, a Lukaszowi za pyszniusie jedzonko! Powodzenia w NL, trzymam kciuki ☺.

Finally I would like to thank to my current supervisor. Dear **Sascha**, thank you for trusting me and hiring me without PhD. I know that in Germany it is a challenge, so I am very thankful for your effort. It is a great pleasure to work with you. **Sophie, Caro, Lena**, girls, you are amazing! Thank you for unwind and relax moments, cinema evenings and eating-out "white pizza". No words to thank you for your support, especially in the last days before departure from Heidelberg to the Netherlands!

Least, but not last I would like to thank my close relatives for supporting me spiritually throughout writing this thesis and my life in general.

I will be never thankful enough to my dear parents. **Mama**, jestes niesamowita! Zawsze, mimo ciezkiej pracy, bylas kiedy Cie ptrzebowalam. Dziekuje za nasze cieple rozmowy i motywacje, ale tez konstruktywna krytyke. Nie ma slow ktore moga wyrazic moja wdziecznosc za to gdzie dzis jestem i kim jestem, a wszystko dzieki Tobie i Twoim staraniom! Dziekuje z calego serca ♥ ! **Tata**, dziekuje za wszelka pomoc i za to ze jestes zawsze, gdy tego potrzebuje.

My dear **Maciejasek**, we met at the end of my PhD contract. It was a period of my life that pushed me to the emotional and financial boarders (no job, no home, no money). There are no words to express my thanks for being with me in this hardest period of my PhD/life. We even did not know each other well and you decided to take care of me. Thank you for your kindness, warmness and love. I need to say here that without your financial support, this thesis would not be finished! Thanks for taking me for short trips and skis to have at least some fun in this hard situation. Dziekuje rowniez Twoim rodzicom za goscinnosc ☺. Thank you for all the tremendous, but also hard moments we shared. It's amazing to conquer the world together. Hope to see you soon in Heidelberg… ☺! I miss you a lot!

Piotrek, you encouraged me to apply for PhD in the Netherlands and shared with me all my ups and downs during this 4 year PhD period. Thank you for helping me out in our daily life and picking me up in the midnights from work when I had long-run experiments. Thanks that you were there for running, dancing, traveling, cooking, but also when I needed advises. Thank you for being my friend and partner. Our every year Asian journeys were amazing and inspiring. Thank you for all beautiful moments.

Mariusz, as typical older brother you were always demanding to your younger sister. But you also always believe in me and provide a lot of support and motivation. During your studies at the Michigan University, you helped me to snake into the genetic course. At that time I was in high school, but it was a moment when I decided to become a researcher. Thanks for your inspiration.

Dear **Raquel**, we met during one of The European CF Young Investigator Meeting in Paris. We approached each other and immediately realized that we found a soul mate. We were exploring Paris together and at that time we did not know that we would have a chance to meet each other again. When I got a short-term fellowship in Canada, you immediately made me feel like at home. Thank you for your amazing hospitality, our chats, delicious brunches, the Mexican B-day party, trips and tips. Thanks for encouraging your boyfriend to organize a trip to waterfalls …! You are amazing! I wish you all the best and "congratz" with your thesis ☺.

Martijn, I really enjoyed our tennis evenings and Saturday morning coffee. Thank you for helping me with the movement and keeping my Gazelle for half a year ☺!

Thanks to the **'Lindy Hop Gang'**, you were all like family to me. Without you my stay in the Netherlands would not be such a great fun. You always increased my positive energy level. Special thanks go to **Priscilla**, **Erik**, **Flo**, **Meine**, **Jamila**, **Spyros**, **Kayleigh**, **Mathijs**, **Alexandre**, **Jill** and all the others that I have not mentioned here. Thanks for our every Thursday fun, Brux exchange trip, our eating-out and all dance parties! It was amazing time with you, guys!

*This thesis was written in **the Netherlands** (Rotterdam), **Canada** (Montreal), **Poland** (Wroclaw, Olsztyn, Pawlowice) and **Germany** (Heidelberg).*

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...to be continued.