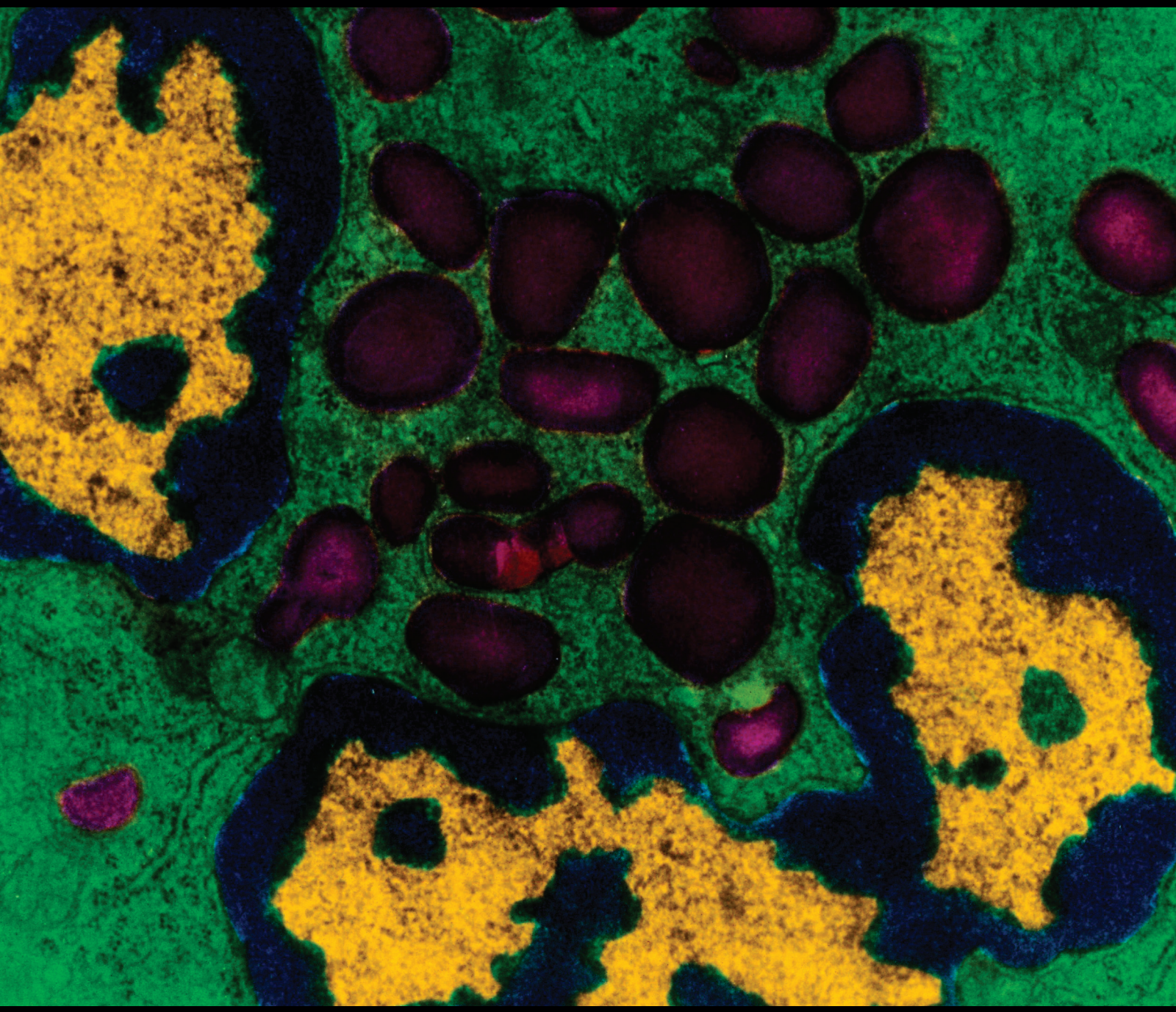
Mediators of Inflammation

New Developments in Cystic Fibrosis Airway Inflammation

Guest Editors: Nades Palaniyar, Hartmut Grasemann, Marcus A. Mall, Christian Taube, and Stefan Worgall



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Editorial Board

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Editorial

New Developments in Cystic Fibrosis Airway Inflammation

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Cystic fibrosis (CF) is an autosomal recessive disease that is caused by mutations in the cystic fibrosis conductance regulator (CFTR) gene and usually presents with multiorgan involvement. Although life expectancy of people with CF has significantly improved since the discovery of the CFTR gene in 1989, chronic progressive lung function decline remains the major contributor to CF morbidity and mortality. CFTR is a cAMP-dependent Cl^- channel that in epithelial cells also functions as a regulator of other ion channels such as the amiloride-sensitive epithelial Na^+ channel (ENaC). In CF airways, an imbalance in epithelial electrolyte secretion and absorption results in dehydration of the epithelial surface liquid layer, thickened secretions, defective mucociliary clearance, and a vicious cycle of mucous obstruction, infection, and inflammation. In recent years, there has been a tremendous advancement in the treatment options for patients with CF. With novel CFTR-targeting therapies, there are now opportunities to correct CFTR dysfunction and improve pulmonary function in some patients. However, airway inflammation remains a major factor in the disease and therefore this special issue is focused on the important role of airway inflammation in the development of chronic CF lung damage. The presented papers have been contributed by experts in the field and include both original research articles and state-of-the-art reviews.

One of the proposed pathophysiological mechanisms contributing to CF lung pathology is an imbalance between proteases and antiprotease in the airways. This concept has been recognized for decades but specific intervention studies so far have not been clinically successful. This important topic is thoroughly addressed in a review by M. S. Twigg et al. U. Müller et al., in their manuscript entitled "Changes of Proteases, Antiproteases, and Pathogens in Cystic Fibrosis Patients' Upper and Lower Airways after IV-Antibiotic Therapy," present an interesting clinical research study on microbiological patterns and disparity of changes in protease/antiprotease imbalance between upper and lower airways of CF patients treated with antimicrobial agents.

In recent years, it has become apparent not only that ribonucleic acid (RNA) functions as a messenger in transcription but also that small noncoding RNA molecules (microRNA) play important roles in posttranscriptional

regulation and modification. These microRNAs are now being studied in different diseases such as cancer, neurodegenerative conditions, and chronic lung diseases. The increasing body of literature on microRNAs in CF lung disease is summarized by P. J. McKiernan and C. M. Greene.

The regulatory mechanisms of CF airway inflammation are still not completely unraveled, but epithelial dysfunction seems to be pivotal for the development of CF-specific inflammatory responses. Current knowledge on CF epithelial function and host defense is summarized by F. Stanke. In addition, Z. Yu et al., in their original research study, provide novel data suggesting that ESE-1 may play a role in regulating CF airway inflammation via its effect on ICAM-1 expression.

Phosphoinositide 3-kinases are involved in cellular regulatory functions including cell growth, proliferation, differentiation, motility, survival, and intracellular trafficking. In this special issue, novel data are presented suggesting that phosphoinositide 3-kinase PI3Kγ may play a role in the regulation of neutrophilic inflammation in CF. Indeed, M. Galluzzo et al. report results from experimental studies in the mouse that PI3Kγ is implicated in chronic neutrophilic inflammation in CF-like lung disease. Their manuscript is entitled "Genetic Deletion and Pharmacological Inhibition of PI3Kγ Reduces Neutrophilic Airway Inflammation and Lung Damage in Mice with Cystic Fibrosis-Like Lung Disease."

Nitric oxide deficiency in CF airways is thought to be contributing to the increased risk of infections with certain pathogens including *Pseudomonas aeruginosa*. New experimental techniques for the assessment of the pulmonary L-arginine/nitric oxide metabolism in the mouse in vivo are described in the paper by H. Grasemann et al.: "Multitracer Stable Isotope Quantification of Arginase and Nitric Oxide Synthase Activity in a Mouse Model of Pseudomonas Lung Infection."

We hope that this special issue not only will be useful to the interested reader by providing insight into new and important aspects related to CF airway inflammation but also may stimulate new interest and the development of novel research ideas and therapeutic avenues in this specific area.

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Review Article

MicroRNA Dysregulation in Cystic Fibrosis

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The cystic fibrosis lung is a complex milieu comprising multiple factors that coordinate its physiology. MicroRNAs are regulatory factors involved in most biological processes and it is becoming increasingly clear that they play a key role in the development and manifestations of CF lung disease. These small noncoding RNAs act posttranscriptionally to inhibit protein production. Their involvement in the pathogenesis of CF lung disease stems from the fact that their expression is altered *in vivo* in the CF lung due to intrinsic and extrinsic factors; to date defective chloride ion conductance, endoplasmic reticulum stress, inflammation, and infection have been implicated in altering endogenous miRNA expression in this setting. Here, the current state-of-the-art and biological consequences of altered microRNA expression in cystic fibrosis are reviewed.

1. Introduction

Cystic fibrosis (CF) is a multifaceted autosomal recessive disease caused by mutations in the CF transmembrane conductance regulator (*CFTR*) gene. Although its pulmonary manifestations are responsible for the major morbidity and mortality associated with the disease, CF is also characterised by a multitude of clinical extrapulmonary manifestations. In addition, the great heterogeneity in disease severity among people with CF means that the design of therapeutic interventions is particularly challenging. Ultimately, a move toward personalised therapy will greatly enhance our treatment of CF. MicroRNAs (miRNA) are a class of regulatory biomolecules with important functions in numerous biological processes and are aberrantly expressed in many human diseases. Therefore, it is important to elucidate the roles of these molecules in CF pathophysiology.

2. MicroRNA

miRNAs are 20–25 nucleotide RNAs involved in the translational regulation of gene expression [1]. Although the term "microRNA" was first coined in 2001, the first miRNA, *lin-4*, was discovered eight years earlier by Lee and colleagues, in the nematode *Caenorhabditis elegans* [2]. Having been initially discovered to play important roles in developmental biology, interest in these small RNAs has dramatically increased since this time as they have been found to have significant roles in a range of other biological processes such as proliferation and apoptosis. The latest version of miRBase (http://www.mirbase.org/, v21 [3]), the most comprehensive microRNA bioinformatics repository, contains entries from 223 species corresponding to over 35,000 microRNAs. The database now contains over 2,000 human microRNA entries. Expression levels of miRNAs vary greatly between cells and tissues, and aberrant levels of miRNA are associated with many diseases in humans.

As a rule, mammalian miRNAs are initially transcribed in the nucleus into longer primary miRNA ("pri-mir") of up to 1000 nucleotides in length. These stem-loop structured pri-mirs are generally transcribed by RNA Polymerase II and subsequently undergo cleavage in two sequential steps. The initial processing occurs in the nucleus by the RNA endonuclease (RNase) type III enzyme Drosha with the involvement of other proteins, as part of the "microprocessor complex." Drosha cleaves the pri-mir liberating shorter hairpin pre-miRNA structures ("pre-mir"), which are approximately 70–100 nucleotides in length [4], and these are actively transported into the cytoplasm *via* a process involving the protein Exportin 5 [5]. Once in the cytoplasm, the pre-mir is further processed by the RNase III enzyme Dicer, resulting in a mature miRNA duplex with 5′ phosphate and two-nucleotide 3′ overhangs [6]. Duplexes consist of a mature miRNA

"guide" strand and a "passenger" miRNA* strand which, in general, is degraded.

Generally, microRNAs regulate gene expression post-transcriptionally by binding in a sequence-specific manner to miRNA responsive elements (MREs), particularly in the 3′ untranslated region (UTR), of a target mRNA. They are recruited by Argonaute (Ago) proteins, particularly Ago2 [7] to form the multiprotein RNA induced silencing complex (RISC) [8, 9]. miRNAs can guide the RISC to a target mRNA which then induces cleavage degradation or translational repression of that mRNA [10, 11]. Although most miRNA studies have largely focused on miRNA-mRNA interactions in the 3′UTR of target mRNA, these interactions can also occur in the 5′UTR and coding sequence (CDS) [12, 13].

As a single miRNA can regulate many target mRNAs and each mRNA may harbour several MREs, validation of targets can be difficult and time consuming. Since miRNA target interactions are complex, predictions are difficult. However, many computational tools are currently available for predictions and these are continuously improving. Peterson et al. [14] summarises the four approaches common to the target prediction tools currently used. These are the quality of seed match, evolutionary conservation of a particular microRNA, thermodynamics (specifically free energy) of miRNA::mRNA target binding, and site accessibility or mRNA secondary structure. Various online tools aid in these predictions and some well-known examples include TargetScan, PicTar, DIANA-microT, microrna.org, rna22, and RNAhybrid, which all utilise different algorithms and different sources of mRNA sequences. Yet, bioinformatic target prediction databases have high false positive and false negative rates, and experimental validation is ultimately required to truly determine miRNA::target mRNA binding and biological function.

It has been proposed that the expression and function of microRNAs themselves are regulated at three levels: transcription, processing, and subcellular localization [15]. At the level of transcription, miRNA expression can be controlled by many factors such as chromatin modifications, DNA methylation, and activity of transcription factors to name a few. miRNA processing can be affected by intrinsic or acquired alterations in the miRNA microprocessor machinery, thereby controlling miRNA function. A role for long noncoding RNA transcripts in the sequestration of miRNAs is emerging. These are termed "miRNA sponges," given their ability to soak up miRNAs and reduce their interactions with target mRNAs. Additionally, single nucleotide polymorphisms (miRSNPs) that affect miRNA binding and function are being increasingly reported.

2.1. miRNAs in Lung Inflammation and Cystic Fibrosis. Analysis of multiple organs and tissues suggests that miRNAs have dual roles as both regulators of development and in maintenance of homeostasis [9, 16, 17]. Their importance in lung development is undisputed. Widespread changes in miRNA expression have been observed during lung development, and Dicer knockout mice, who have disrupted miRNA processing, display a lethal phenotype as a result of impaired lung growth [18]. Various studies demonstrate that miRNA expression remains relatively constant over time in the adult lung [19], supporting the notion that miRNAs play a central role in maintenance of lung homeostasis in the developed lung [16]. However, expression of miRNA is altered in pathological states, such as lung inflammation and disease. miRNAs have been shown to play important roles in the regulation of innate immunity and inflammation. At the most basic level, miRNAs are important in haematopoiesis and differentiation of immune cells [20, 21]. Numerous miRNAs are induced in innate immune cells, with miRs-155, -146, and -21 being expressed at particularly high levels [22, 23]. With known roles in regulation of inflammation, miRNAs are increasingly being examined within the context of inflammatory lung diseases such as CF.

The CF airway lumen is a unique milieu (Figure 1). Lining the airway epithelium in the CF lung is a depleted airway surface liquid layer (ASL) and more mucus than normal. Impaired mucociliary clearance promotes bacterial colonisation and generates a highly proinflammatory environment wherein innate immune responses are frequently activated. Another characteristic of the CF airway lumen is the high numbers of infiltrating neutrophils which are inherently dysfunctional and contribute to the preexisting protease-antiprotease imbalance. Accumulation of misfolded CFTR may contribute to endoplasmic reticulum (ER) stress responses in the airway epithelium, and collectively these features are central to the pathology and physiology of CF lung disease. Our group was the first to examine miRNA expression in CF [24]. Numerous microRNAs had altered expression between CF and non-CF bronchial epithelium; the altered miRNAs were predicted to regulate expression of proteins involved innate immunity, inflammation, ion conductance, and ER stress, amongst others.

2.1.1. Innate Immunity. The airway epithelium acts as an anatomical barrier to or primary defense against infection. These cells contribute to the barrier function *via* three essential components: intercellular tight and adherens junctions (regulating epithelial permeability), secreted antimicrobial factors, and the mucociliary escalator [25]. Furthermore, it acts as a key mediator of both innate and adaptive immune responses toward invading pathogens. Toll-like receptors (TLRs) are a key group of pattern recognition receptors which mediate the recognition of and response to microbial infections and are highly expressed on myeloid cells. The expression of TLRs is, however, not confined to immune cells, and these receptors are also expressed at high levels on other cell types, including airway epithelial cells (AECs) such as tracheal, bronchial, and alveolar type II cells. In the CF lung, TLRs expressed by AECs contribute to the airway immune response by regulating the expression and secretion of cytokines, chemokines, and antimicrobial peptides and through enhancing the expression of cell surface adhesion molecules [26].

Target of Myb1 (TOM1) is a Tollip-binding protein recently shown to act as a negative regulator of TLR2, TLR4, and IL-1β induced signalling pathways in CF bronchial epithelial cells [24]. TOM1 was predicted to be regulated by miR-126, a miRNA that is significantly downregulated in CF bronchial brushings compared to controls. To validate this observation the coexpression of miR-126 and TOM1 was

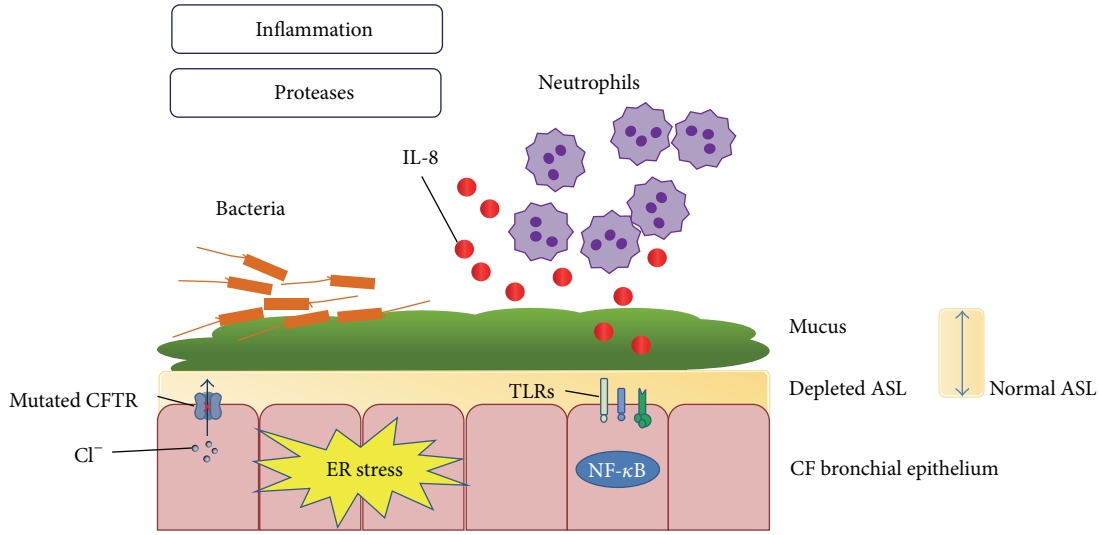

FIGURE 1: The CF airway lumen. Altered ion homeostasis in the CF airway due to mutated CFTR leads to impaired mucociliary clearance and a depleted ASL volume. This, coupled with intrinsic inflammation, leads to chronic bacterial infection and inflammation, with large numbers of neutrophils along with their secreted protease products being recruited to the lung. The high protease burden in the CF airway is damaging to lung tissue and leads to bronchiectasis and ultimately lung failure and death. IL-8: interleukin 8; ASL: airway surface liquid; TLRs: Toll-like receptors; NF-κB: nuclear factor-κB.

evaluated in CF and non-CF bronchial epithelial samples and cell lines, and a reciprocal expression pattern was evident; the effect of overexpression of miR-126 on *TOM1* gene and protein levels was examined in a CF bronchial epithelial cell line, and a miR-126::TOM1 mRNA interaction was functionally validated using a reporter system. This was the first report of altered miRNA expression affecting innate immune responses in the CF lung and suggests that decreased miR-126 may engender a TLR hyporesponsive state which could be important at times of infective exacerbations where a rapid and robust response is required.

In addition to the epithelium, bone marrow derived cells such as monocytes, macrophages, neutrophils, and dendritic cells are important in the CF lung. These are constantly recruited to the infected CF lung to clear pulmonary pathogens, but numerous studies have suggested an impairment of these cells in the context of the CF. It has been well established that the CF lung is dominated by a neutrophilic inflammation. Although neutrophils are required for antimicrobial defense, their accumulation over periods of time and poorly controlled release of their toxic granular content can lead to parenchymal lung tissue damage [27, 28]. Neutrophils from people with CF have been found to release more elastase [29] and have defective phagocytic capacity and oxidative burst compared to controls [30]. Impaired bacterial killing by CF neutrophils has been shown to be a result of excessive protease cleavage of important molecules such as the IL-8 chemokine receptor CXCR1 on neutrophils [31] and also impaired CFTR-dependent phagosomal chlorination [32]. Recent work has shown that neutrophils from people with CF have altered cytosolic ion concentrations resulting in impaired degranulation [33].

Monocytes originate from precursors in the bone marrow and circulate in the bloodstream, until they are attracted to infection or inflammatory signals in particular tissues, such as the lung, where they differentiate into macrophage or dendritic cell populations [34]. The monocyte/macrophage lineage of myeloid cells has three primary roles in the immune response: phagocytosis, antigen presentation, and immunomodulation [35]. In the lungs, monocytes primarily differentiate into alveolar macrophages. These are excellent phagocytes, effective at rapidly clearing bacteria from the airways. Their numbers are increased in BALF of young non-infected CF patients [36] and similarly in CF mouse models [37, 38]. Emerging evidences suggests that these cells are hyperresponsive in people with CF, when exposed to bacterial agonists [39–41]. CF macrophages also appear to be defective in intracellular bacterial killing [42–44] and efferocytosis (i.e., scavenging of apoptotic neutrophils) [45–47]. Therefore myeloid cells play important roles in driving pathogenesis of the CF airways.

Hector and colleagues have examined miRNA expression in CF myeloid cells (neutrophils and mononuclear cells) and found changes in specific miRNAs including decreased miR-9 in CF neutrophils and increased miR-126 in CF mononuclear cells versus the same cells from healthy control cells (Andreas Hector, University of Tuebingen, personal communication). Functional studies will define if these changes in miRNA expression impact on dysfunctional processes such as those described above.

2.1.2. Inflammation. The CF lung is a high protease milieu and bacterial-derived proteases can contribute to this protease burden. For example, *Pseudomonas aeruginosa* secretes the metalloproteases *Pseudomonas* elastase (PsE) and alkaline protease (APR), capable of cleaving a wide range of host proteins and of altering the physiology of the CF airways [48–50]. High numbers of neutrophils contribute significantly to

the abnormally high concentrations of neutrophil-derived proteases, for example, neutrophil elastase [51–53], proteinase 3 [54], and cathepsin G [55]; however a range of other endogenously expressed cysteine, metallo-, and aspartyl proteases generated by other cell types are also important. These include the cysteinyl protease cathepsin S [56] which can be expressed by bronchial epithelial cells and antigen presenting cells such as macrophages and dendritic cells. Weldon and colleagues [56] have recently found that the expression and activity of cathepsin S is increased in the BALF of children with CF, including a cohort of *Ps. aeruginosa*-negative preschool children, compared to non-CF children with recurrent infection, indicating that upregulation of cathepsin S may be CF-specific. Interestingly, they illustrated that this is due, in part, to decreased miR-31 which they have shown regulates the transcription factor interferon regulatory factor 1 (IRF-1), which controls cathepsin S expression. Levels of miR-31 were lower in CF versus non-CF cell lines, primary bronchial epithelial cells, and bronchial brushings [57].

Other studies have looked at alternative roles of miRNA in other aspects of inflammation in CF. Infection with *Ps. aeruginosa* induces the production of proinflammatory cytokines such as IL-8 in the CF airway epithelium. Fabbri et al. [58] found that miR-93 is decreased in CF bronchial epithelial IB3-1 cells during infection with this CF pathogen. They also demonstrated that the decrease in miR-93 expression is correlated with an increase in IL-8 levels and that miR-93 directly targeted IL-8 mRNA.

2.1.3. Ion Conductance. CFTR is the most important ion channel in CF. The *CFTR* gene encodes a membrane bound ion transport protein that belongs to the ATP-binding cassette (ABC) superfamily of transporter proteins [59]. The gene, containing 27 exons, was mapped by positional cloning in 1985 to the long arm of chromosome 7 (7q31) [60]. Its protein product, which is 1480 amino acids in length, primarily functions as an ion channel that, in concert with the Ca^{2+}-activated Cl^- channel (CaCC) [61], works to secrete Cl^- and fluid required to hydrate the airway mucus but has the additional ability to transport bicarbonate [62] and glutathione [63]. Although the mechanism is still not fully understood, evidence is emerging for the role of CFTR in regulating the epithelial Na^+ channel (ENaC) and the failure of mutated forms of CFTR in restricting salt absorption through ENaC [64]. Therefore, in the CF airway, epithelial CFTR dysfunction leads to airway surface liquid volume depletion due to an imbalance between CFTR-mediated Cl^- secretion and ENaC-mediated Na^+ absorption [27]. Indeed transgenic mice overexpressing the β-subunit of ENaC develop a CF-like lung pathology [65] and have been used as a model of CF lung disease [66].

CFTR expression is a carefully controlled process that is spatially and temporally regulated. Transcription can begin at different start sites depending on the tissue or developmental stage in question. For example, *CFTR* is positively regulated by a selection of transcription factors including C/EBP proteins and FOXA factors, amongst others; *CFTR* is also posttranscriptionally regulated by miRNAs. A number of studies have examined the role of microRNAs in the control of CFTR expression and various microRNAs were demonstrated to regulate CFTR [67–75]. Although different experimental situations were examined, such as different cell lines and response to cigarette smoke, miR-101, miR-145, miR-494, and miR-509-3p have been repeatedly implicated in many of these studies, strongly highlighting their roles in regulating CFTR expression. For example, Oglesby and colleagues [71] demonstrated that miR-145, miR-223 and miR-494 were upregulated in CF bronchial brushings and cell lines, inversely correlated with CFTR levels, and were shown to directly target *CFTR* mRNA. The expression of these miRNAs also correlated with p.Phe508del mutation and *Ps. aeruginosa* colonisation. Ramachandran et al. [73] showed that miR-494 and miR-509-3p are increased in CF primary airway epithelial cells, regulate CFTR, and are regulated by NF-κB. In the most recent study, Viart et al. identified miRNAs that participate in *CFTR* downregulation in the lung after birth [75]. Having compared the miRNA expression profiles of adult and foetal lungs, three miRNAs in particular (miR-145, miR-150, and miR-451) were found to have a temporal effect, being significantly upregulated in the adult lung and therefore contributing to downregulation of CFTR. They also demonstrated how inhibitors based on these miRNAs can affect *CFTR* gene expression and function in air-liquid interface culture and suggest that these may be developed as tools for CFTR correction in people with CF [76].

2.1.4. ER Stress. The ER is the site of protein translation, folding, and processing for transport to secretory vesicles. Misfolded variants of CFTR, for example, the class II p.Phe508del-CFTR protein, accumulate in the ER and fail to reach the apical surface of epithelial cells to function as anion channels. ER perturbation can lead to ER stress and the initiation of signalling networks aimed at restoring ER equilibrium. One such network is the unfolded protein response (UPR). Recent evidence has implicated miRNAs in regulation of the UPR, in contexts other than CF [77–80]. However one recent study has examined whether altered miRNA expression regulates expression of UPR genes in CF airway epithelium [81]. Activating transcription factor 6 (ATF6) is an ER resident transcription factor and a key component of the UPR [82]. Its activation leads to transcriptional induction of ATF6-regulated genes which function primarily to restore correct protein folding in the ER.

The role of miRNA in basal regulation of ATF6 was investigated in CF and non-CF bronchial epithelial cells *in vitro* and *in vivo*. miRNAs predicted to target the 3′UTR of the ATF6 mRNA were identified. Three of these, miR-145, miR-221, and miR-494, were upregulated in a p.Phe508del-CFTR versus non-CF bronchial epithelial cell line and also in p.Phe508del-CFTR versus non-CF bronchial brushings. Expression of ATF6 was reciprocally decreased in CF both *in vivo* and *in vitro*. After experimentally validating ATF6 as a molecular target of these miRNAs through the use of a luciferase reporter vector containing the full length 3′UTR of ATF6, the human studies were complemented by analysing the expression of key miRNAs in a mouse model of CF lung disease. Expression of miR-221, which is also predicted to regulate murine ATF6, was significantly increased in

native airway tissues of βENaC-overexpressing transgenic mice with CF-like lung disease versus wild type littermates, demonstrating structural and functional conservation between humans and mice. These findings implicate βENaC-overexpressing transgenic mice as a useful animal model for studies manipulating miR-221 levels *in vivo* using miRNA overexpression strategies to limit ER stress-mediated inflammation.

3. Concluding Remarks and Perspective

In this review, we have discussed current data regarding miRNA studies in cystic fibrosis. What is clear is that miRNA dysregulation exists in CF, with many studies highlighting an altered miRNA expression profile in the CF lung, be it in cell lines, primary cell cultures, or bronchial brush samples. Some of these aberrantly expressed miRNAs have been demonstrated to be involved in the regulation of key components of inflammatory signalling and, more recently, the UPR. Others have been shown to regulate the expression of CFTR itself. Such dysregulated miRNA may represent potential therapeutic targets. Although this is an emerging field, some work is beginning to be carried out with respect to the development of strategies to ultimately modulate miRNA levels *in vivo* in the CF lung, through the use of miRNA mimics and inhibitors [83]. Finally, the potential of miRNA as biomarkers of CF disease progression remains underexplored in comparison to other diseases such as cancers. The expression of these may become particularly useful for predicting and determining CF lung disease in infants and children, where currently used surrogate markers and biomarkers are of little use.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

The Contribution of the Airway Epithelial Cell to Host Defense

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In the context of cystic fibrosis, the epithelial cell has been characterized in terms of its ion transport capabilities. The ability of an epithelial cell to initiate CFTR-mediated chloride and bicarbonate transport has been recognized early as a means to regulate the thickness of the epithelial lining fluid and recently as a means to regulate the pH, thereby determining critically whether or not host defense proteins such as mucins are able to fold appropriately. This review describes how the epithelial cell senses the presence of pathogens and inflammatory conditions, which, in turn, facilitates the activation of CFTR and thus directly promotes pathogens clearance and innate immune defense on the surface of the epithelial cell. This paper summarizes functional data that describes the effect of cytokines, chemokines, infectious agents, and inflammatory conditions on the ion transport properties of the epithelial cell and relates these key properties to the molecular pathology of cystic fibrosis. Recent findings on the role of cystic fibrosis modifier genes that underscore the role of the epithelial ion transport in host defense and inflammation are discussed.

1. Introduction into the Defense Repertoire of the Epithelial Cell

The chloride and bicarbonate transporter CFTR, in healthy individuals as well as in F508del homozygous CF patients, is expressed on the apical surface of epithelial cells [1]. CFTR colocalizes with other ion channels such as the amiloride-sensitive epithelial sodium channel [2, 3]. The amount of liquid that covers the apical side of the epithelium is tightly regulated as the net ion transport through the apical membrane, driven by epithelial sodium and chloride channels, paralleled by water transport [3]. In turn, the amount and composition of the epithelial lining fluid determine the efficacy of mucociliary clearance [4], a mechanism by which the epithelium can detoxify pathogens and pollutants [4]. Host defense is mediated by resident macrophages that are localized on the epithelial surface [4] as well as by antimicrobial proteins that are secreted into the epithelial lining fluid [4]. The efficacy of mucociliary clearance depends on the beating of the epithelial cell's cilia [4] and on the viscoelastic properties of the fluid that covers the epithelium [4, 5]. Thereby, CFTR directly influences the extent to which the fluid on the airway's surface can be moved: firstly, chloride, and, in consequence, water, secreted via CFTR at the apical side of epithelial cells. Secondly, CFTR secretes bicarbonate, whereby the regulation of the pH determines whether secreted components such as mucins can unfold properly [6].

Apart from ion channels, the apical membrane of epithelial cells is equipped with a variety of receptors that sense the presence of pathogens or the inflammatory state: toll-like receptors that directly interact with bacterial or viral components [7, 8] as well as receptors for macrophage-derived cytokines such as TNFα [9] and IFNγ [10] are expressed by epithelial cells [7, 8, 11, 12]. Hence, the epithelial cell is well equipped to detect the presence of pathogens as well as the activity of macrophages that reside in the epithelial surface fluid. In other words, the airway epithelial cell is in a unique position to recognize the need for host defense as well as providing it via an activation of the chloride and bicarbonate channel CFTR.

Table 1: Effect of cytokines on ion transport in airway epithelial cells.

Model system	Cytokine	Ion channel	Techniques	Principle findings	Reference	EPub
Rabbit	TGFβ	ENaC	$^{22}Na^+$ efflux, electrophysiology, flow cytometry, surface biotinylation, and RT-PCR	TGFβ decreases ENaC-mediated sodium and fluid uptake	[25]	2014
Air liquid epithelial cell culture with human CFBE41o$^-$ and transfected cells	IL6, IL8, and CXCL1/2	CFTR, TMEM16A	Electrophysiology, immunocytochemistry, human cytokine antibody array, and cell-surface ELISA	CFTR and TMEM16A decrease secretion of IL6, IL8, and CXCL1/2	[24]	2012
Slc26a9-deficient mice	IL13	Slc26a9	Electrophysiology	IL13 increases Slc26a9-mediated Cl^- secretion	[23]	2012
BALB/c mice	IL13	ENaC	Electrophysiology, RT-PCR	IL13 decreases ENaC expression	[22]	2010
Human bronchial epithelial cells	IL17A	Bicarbonate transport	Electrophysiology, measurement of surface and intracellular pH	IL17A increases HCO_3^- secretion	[21]	2009
Human nasal epithelial cells	IL13	CFTR	Immunocytochemistry, western blot	IL13 increases CFTR expression	[20]	2007
Primary culture of rat alveolar epithelial type II cells, primary culture of human alveolar epithelial type II cells	Il1β	ENaC	$^{22}Na^+$ efflux, electrophysiology, western blot, and RT-PCR	IL1β decreases ENaC expression	[18]	2005
Rat alveolar epithelial cells	TNFα	ENaC	Electrophysiology, northern blot, and western blot	TNFα decreases ENaC expression	[19]	2004
Primary culture of rat alveolar epithelial type II cells, primary culture of human alveolar epithelial type II cells	TGFβ	ENaC	$^{22}Na^+$ efflux, electrophysiology, western blot, and RT-PCR	TGFβ decreases ENaC expression	[15]	2003
Primary culture of human bronchial epithelial cells	IL4, IL13	CFTR, ENaC	Electrophysiology, western blot	IL4 and IL13 increase CFTR expression IL4 and IL13 decrease ENaC expression	[16]	2002
Primary culture of human bronchial epithelial cells	IFNγ, TNFα	ENaC, CFTR	Electrophysiology, western blot, and transepithelial fluid transport	IFNγ decreases CFTR expression IFNγ decreases ENaC-mediated sodium transport TNFα increases CFTR expression	[17]	2000

2. Cytokines Alter the Ion Conductance Capabilities of Airway Epithelial Cells

In order to recognize that cytokines can alter the ion secretion properties of epithelial cells, two fields that are traditionally not well linked, that is, experimental immunology and electrophysiology, need to interact. Fortunately, several experiments wherein airway epithelial cells have been exposed to cytokines and the expression or function of ion channels such as CFTR, the amiloride-sensitive epithelial sodium channel ENaC, and calcium-activated chloride channels is monitored by comparative RT-PCR, western blot, or electrophysiology have been provided since the turn of the century ([15–25]; Table 1). Roughly summarized, the uptake of sodium by airway epithelial cells through ENaC is inhibited by TGFβ [15, 25], IL13 [16, 22], IL1β [18], TNFα [19], IL4 [16], and IFNγ [17]. In contrast, the secretion of chloride and/or bicarbonate via CFTR is increased by IL17 [21], IL13 [16, 20], IL4 [16], and TNFα [17] and via the chloride transporter SLC26A9 by IL13 [23]. In conclusion, the data generated by independent researchers paints a highly coherent picture of the cross-talk between immunologically relevant cells and

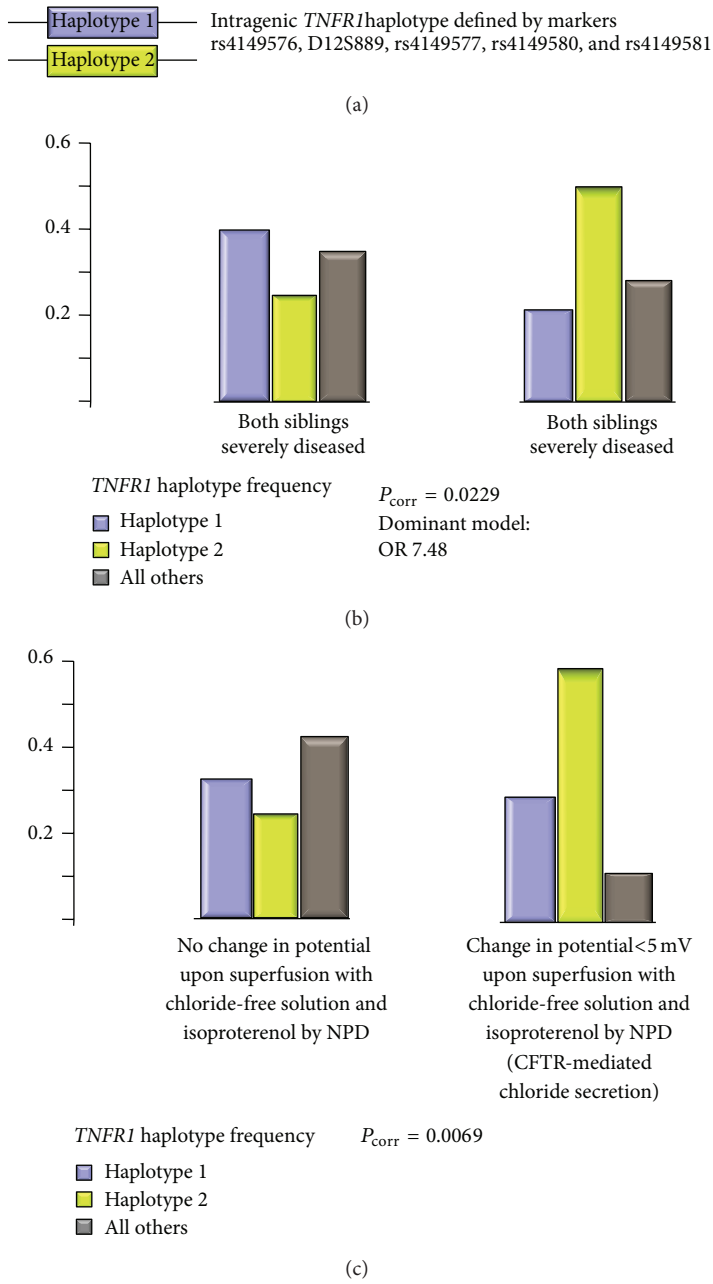

Figure 1: Association of *TNFR1* variants with CF disease severity and manifestation of CFTR-mediated residual chloride secretion in respiratory tissue among F508del-*CFTR* homozygous CF patients. (a) Definition of *TNFR1* variants. Two contrasting haplotypes, designated haplotype 1 and haplotype 2 within this figure, were described by typing the five markers rs4149576, D12S889, rs4149577, rs4149580, and rs4149581 among 101 families with a total of 171 F508del-*CFTR* homozygous CF patients [13]. Haplotypes were reconstructed using the software FAMHAP [14]. Association was judged by case-reference association whereby cases and references were defined based on the disease severity of the siblings (b) or the manifestation of a change in potential upon superfusion of the nasal epithelium with chloride-free solution and isoproterenol (c). All *P* values reported within this figure were calculated by FAMHAP and are corrected for sibling dependence and testing of multiple markers [14]. Please note that the case and control subpopulations compared within (b) and (c) were defined independently and were nonoverlapping (for details, please see [13]). Please also note that haplotype 2, depicted in green within this figure, is overrepresented among sib pairs with mild CF disease and among patients who display CFTR-mediated residual chloride secretion by NPD. As a causal interpretation, this might reflect the crosstalk between the cytokine pathway and the ion secretory properties of the epithelium [15–25], indicating that the mild *TNFR1* haplotype 2 is more susceptible to translating the action of the host defense modifier gene *TNFR1* into CFTR-mediated residual function. Alternatively, as CFTR-mediated residual chloride secretion causes a mild disease phenotype, the observed *TNFR1* association with the manifestation of the basic defect in NPD might reflect an overrepresentation of mild modifier alleles among patients with residual CFTR-mediated chloride secretion, which is equivalent to a replication study with confirmatory outcome.

Table 2: Immunologically relevant genes and transcription factors that were identified as modifiers of the CFTR-mediated basic defect among F508del-*CFTR* homozygous CF patients.

Functional category	Gene	Association observed with	Reference
Ligands	*CD14*	CFTR activity/intestinal tissue [C] Non-CFTR mediated residual chloride secretion/intestinal tissue [D]	[13]
	TNFA	CFTR activity/nasal epithelium [B]	[13]
	IL1B	CFTR activity/intestinal tissue [C]	[13, 39]
	TGFB1	Non-CFTR mediated residual chloride secretion/intestinal tissue [D]	[13]
	IL8	CFTR activity/nasal epithelium [B]	[13]
Membrane-bound receptors	*TLR4*	ENaC activity/nasal epithelium [A] CFTR activity/nasal epithelium [B] CFTR activity/intestinal tissue [C]	[13]
	TNFR1	ENaC activity/nasal epithelium [A] CFTR activity/nasal epithelium [B] CFTR activity/intestinal tissue [C] Non-CFTR mediated residual chloride secretion/intestinal tissue [D]	[13]
	CD95	ENaC activity/nasal epithelium [A] CFTR activity/nasal epithelium [B] CFTR activity/intestinal tissue [C] Non-CFTR mediated residual chloride secretion/intestinal tissue [D]	[13]
	IFNGR1	CFTR activity/nasal epithelium [B] CFTR activity/intestinal tissue [C]	[13, 39]
	TLR5	CFTR activity/nasal epithelium [B] Non-CFTR mediated residual chloride secretion/intestinal tissue [D]	[13]
	TLR9	CFTR activity/intestinal tissue [C] Non-CFTR mediated residual chloride secretion/intestinal tissue [D]	[13]
Transcription factors	*STAT3*	CFTR activity/intestinal tissue [C]	[13, 39]
	EHF	ENaC activity/nasal epithelium [A] CFTR activity/intestinal tissue [C]	[45]

[A]: the association was observed in a case-reference study comparing F508del-*CFTR* homozygotes with high versus low change of the potential upon superfusion of the nasal epithelium with amiloride as assessed by nasal potential difference measurement. Please see [13] for details.
[B]: the association was observed in a case-reference study comparing F508del-*CFTR* homozygotes with presence versus absence of a change of the potential upon superfusion of the nasal epithelium with chloride-free solution and isoproterenol as assessed by nasal potential difference measurement. Please see [13] for details.
[C]: the association was observed in a case-reference study comparing F508del-*CFTR* homozygotes with presence versus absence of DIDS-insensitive residual chloride secretion as assessed by intestinal current measurement of rectal suction biopsies. Please see [13] for details.
[D]: the association was observed in a case-reference study comparing F508del-*CFTR* homozygotes with presence versus absence of DIDS-sensitive residual chloride secretion of the intestinal tissue as assessed by intestinal current measurement of rectal suction biopsies. Please see [13] for details.

airway epithelial cells; cytokines, being released by immunologically active cells, will be interpreted by the epithelial cell as a signal to increase the epithelial surface fluid, thereby promoting mucociliary clearance and decreasing the amount of pathogens and inflammatory substances within the lung.

The clinical importance of these findings is underlined (a) by the susceptibility of CFTR-deficient individuals to nosocomial pathogens, as observed in cystic fibrosis, (b) by the susceptibility of ENaC-deficient patients who suffer from pseudohypoaldosteronism type I [26] to *P. aeruginosa* [27, 28], and (c) by the elevated susceptibility of patients with CF-like disease carrying partially dysfunctional CFTR and/or ENaC gene variants to respiratory disease [29]. Furthermore, the impaired regulation of lung fluid balance by the cytokine TGFβ has now been recognized as a direct cause for acute respiratory distress syndrome [30].

3. Cystic Fibrosis Modifying Genes That Determine Immunology and Inflammation Alter the CFTR-Mediated Basic Defect

Modifier genes of cystic fibrosis disease severity have now been studied for a decade [31, 32]. Many studies are candidate genes based; that is, the investigators rely on a hypothesis of which of the 22.000 protein-coding human genes [33] is likely to influence CF disease. Several researchers have selected genes encoding for cytokines such as *TNFA*, *IL1B,* and *TGFB1* as candidate genes because of their known role in infection, immunology, and inflammation [13, 34–38]. Among these immunologically relevant candidate genes, *IL1B* has been replicated in two truly independent studies [35, 39], albeit the molecular variant has not been mapped by the base yet. The cytokine receptors *TNFR1* and *IFNGR1* have been studied

as modifier genes in *European CF Twin and Sibling Study* [13, 39, 40]. Until now, the CF basic defect that can be assessed by nasal potential difference measurement in vivo has only been used by the *European CF Twin and Sibling Study* for an association study. Strikingly, the TNFα receptor 1 gene *TNFR1* was observed as a modifier of CF disease severity as well as of CFTR-mediated residual chloride secretion in the nasal epithelium, whereby the risk and the benign allele were identified consistently for both traits ([13, 40], Figure 1). Furthermore, several immunologically relevant genes were identified as modifiers of the CFTR-mediated basic defect ([13], Table 2). This observation parallels the aforementioned observed capabilities of cytokines to activate fluid secretion by airway epithelial cells in order to promote clearance: as functional experiments using human airway epithelial cell lines, primary airway epithelial cells, and animal models have demonstrated that cytokines can alter ion and fluid transport in the respiratory epithelium effectively [15–25], it must be expected that genetic variants in cytokines and their receptors show genetic association with the manifestation of the basic defect among cystic fibrosis patients [13].

4. Outlook

Functional data and genetic evidence indicate that the epithelial cell and the immune system interact to regulate ion and fluid secretion. It remains to be clarified by which molecular mechanism this is accomplished within the epithelial cell. Likely, part of the effect of cytokines on ion transport will be mediated by the signal transduction cascade that is set into motion by the contact between the soluble ligand and the membrane-bound receptor. While a target of such regulatory networks might be the CFTR gene itself, it is equally plausible that the configuration of the epithelial cell is altered to promote more efficient trafficking of the CFTR ion channel, known for its short half-life [41] and its prolonged residence in a subapical compartment [42–44], to the apical membrane. So far, an association with the CF basic defect has been described for genes encoding the two transcription factors *STAT3* [39] and the epithelial-specific transcription factor *EHF* [45], the latter being a positional candidate that has been selected for replication based on a genome-wide study undertaken to identify modifiers of CF lung disease severity [46]. However, in order to effectively select therapeutic targets in the future, the central molecular pathways that are used by host defense modifier genes which have an impact on CFTR-mediated residual function or ENaC activity in epithelial cells need to be identified. Ultimately, a drug that interferes with the activity of key regulatory elements—such as regulatory microRNAs and transcription factors—which translate the action of host defense modifier genes into CFTR-mediated residual function or ENaC activity will be an attractive instrument to counterbalance the susceptibility of CF patients to infection and inflammation.

Conflict of Interests

The author declares that there is no conflict of interests regarding the publication of this paper.

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Review Article

The Role of Serine Proteases and Antiproteases in the Cystic Fibrosis Lung

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Cystic fibrosis (CF) lung disease is an inherited condition with an incidence rate of approximately 1 in 2500 new born babies. CF is characterized as chronic infection of the lung which leads to inflammation of the airway. Sputum from CF patients contains elevated levels of neutrophils and subsequently elevated levels of neutrophil serine proteases. In a healthy individual these proteases aid in the phagocytic process by degrading microbial peptides and are kept in homeostatic balance by cognate antiproteases. Due to the heavy neutrophil burden associated with CF the high concentration of neutrophil derived proteases overwhelms cognate antiproteases. The general effects of this protease/antiprotease imbalance are impaired mucus clearance, increased and self-perpetuating inflammation, and impaired immune responses and tissue. To restore this balance antiproteases have been suggested as potential therapeutics or therapeutic targets. As such a number of both endogenous and synthetic antiproteases have been trialed with mixed success as therapeutics for CF lung disease.

1. Introduction to Cystic Fibrosis

Cystic fibrosis (CF) is an autosomal recessive genetic disorder caused by loss of expression or functional mutations to the cystic fibrosis transmembrane conductance regulator (CFTR) [1, 2]. CF affects multiple organs; however the majority of the pathology related to CF is due to its effect on the respiratory system. Nonfunctional CFTR channels in CF patients prevent the regulation of chloride and sodium ions across epithelial membranes leading to increased and dehydrated mucus secretions in the lungs [1]. CF patients have an impaired ability to clear this mucus due to damage caused to the cilia structures in the lungs and as such are therefore highly susceptible to chronic bacterial infections within the lung which are effectually impossible to eradicate [3]. The ramification of chronic bacterial infection is a sustained and detrimental inflammatory response from the body's innate immune system [4, 5]. There are many factors which mediate the inflammatory response to chronic bacterial infection in CF; these include proinflammatory cytokines such as IL-1β, IL-6, IL-8, GM-CSF, and TNF-α [6, 7].

One of the key immune cell mediators of this detrimental inflammatory response seen in CF patients is polymorphonuclear neutrophils [8]. In CF lungs, neutrophils represent ~ 70% of the inflammatory cell population in contrast to ~1% in epithelial lining fluid from healthy lungs [9]. Neutrophils are recruited to these sites of infection by increased expression of chemoattractants such as IL-8 by lung epithelial tissue [10]. Once recruited, neutrophils are activated and release a wide variety of molecules, such as proteases, DNA, and reactive oxygen species in an attempt to combat bacterial infection, further driving the inflammatory response and causing progressive tissue damage [8]. Evidence to date supports the hypothesis that CF neutrophils may be inherently defective [8, 11–13]. In addition to the release of proinflammatory mediators, neutrophils in the CF lung are not successfully

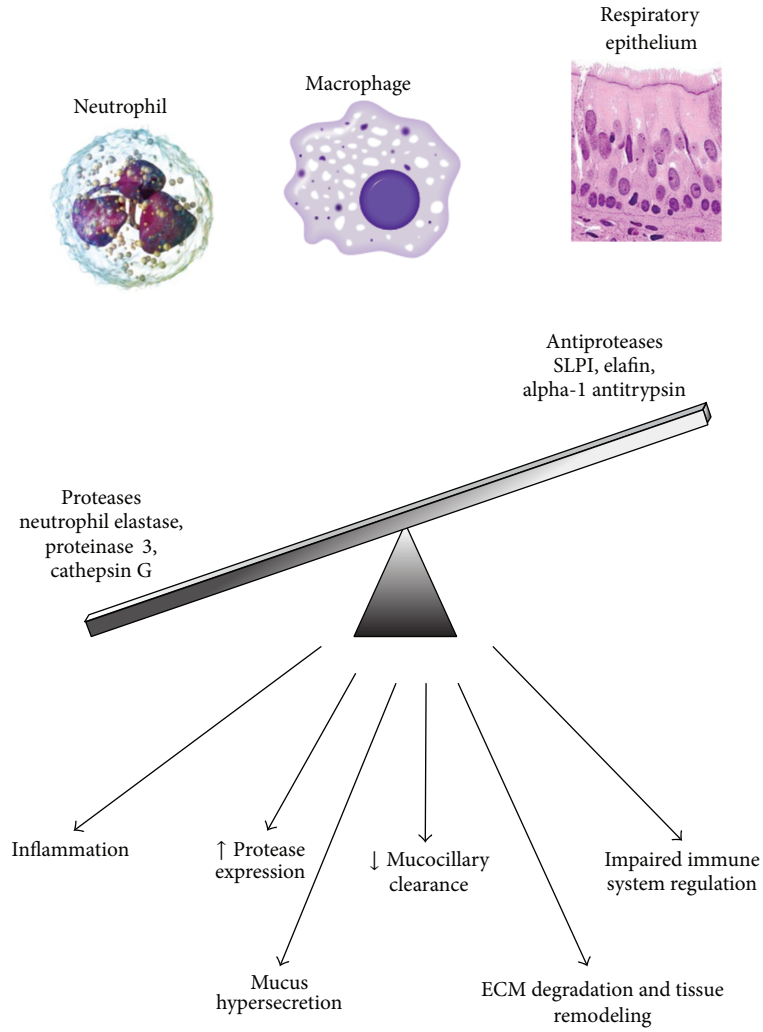

Figure 1: In the cystic fibrosis lung, antiprotease production by both innate immune cells and respiratory epithelial cells is overwhelmed by protease production resulting mainly from neutrophils. This leads to a disruption of the homeostatic protease/antiprotease balance resulting in a number of detrimental effects causing increase lung pathology.

cleared via macrophage phagocytosis [5, 14]. Neutrophil necrosis further increases the levels of proinflammatory mediators, increasing tissue damage and also increasing the viscosity of the CF patients sputum [14]. In healthy individuals tissue damage as a result of inflammation is in part controlled by homeostatic regulation of proteases via antiprotease activity. Inflammation observed in CF patients mainly as a result of neutrophil activity is highly disruptive to this protease/antiprotease balance as illustrated in Figure 1. The role that these serine proteases and their inhibitors play in the CF lung in either protecting the lung tissue or contributing to pathology will be the subject of this review.

2. Neutrophil Serine Protease Activity in CF

Proteases degrade proteins into either polypeptides or amino acids and are grouped on the basis of their catalytic residues. The 4 groups of proteases are serine proteases, cysteine proteases, metalloproteases, and the less common aspartic acid proteases [15, 16]. Neutrophil serine proteases are the main proteases implicated in the damage observed in the lungs of CF patients; these are neutrophil elastase (NE), proteinase 3 (PR3), and cathepsin G (Cat G) [17]. All three are members of the chymotrypsin family, and are expressed by neutrophils [17]. Upon translation these proteases appear as inactive precursor peptides referred to as zymogens. All three serine proteases undergo a two-stage posttranslational modification process in order to produce their active mature forms. The initial stage is the cleavage of an N-terminal signal peptide by a signal peptidase. The second stage is the cleavage of a prodipeptide from the N-terminal by the cysteine protease cathepsin C, which is required for enzymatic activity, and the cleavage of a C-terminal propeptide which may be required for packaging of the mature protein [18–22].

The mature forms of NE, PR3, and Cat G are stored in azurophilic granules within the cytoplasm of neutrophils. The activities of all three of these proteases are reliant on an amino acid triad composed of aspartate, histidine, and serine

residues [18]. These residues are interspersed at different positions in the primary structure of each of the three serine proteases; however these residues are brought together in an active site region in the tertiary structure [15]. Serine proteases act either intracellularly, degrading microbial proteins in the phagosome, or extracellularly, regulating the immune system and aiding the degradation of extracellular matrix (ECM) components [18]. Owing to their broad range activity, the lack of regulation of these proteases in the CF lung is highly detrimental. The majority of research into the role of neutrophil serine proteases in the lung has focused on NE; however PR3 and Cat G are found at high concentrations in the sputum and bronchial alveolar lavage fluid (BALF) of CF patients so they therefore should not be discounted [23, 24].

2.1. Neutrophil Elastase. NE is a 29 kDa serine protease expressed by neutrophils from the gene *ELANE*, located on chromosome 19 [25]. NE is secreted upon neutrophil activation, into the phagosome during phagocytosis or released during neutrophil necrosis. Due to the heavy neutrophil burden associated with CF discussed previously, the levels of NE in the CF airway have been shown to reach micromolar concentrations [26]. Increased levels of NE in the CF lung have been attributed to elevated neutrophil numbers; however, defective neutrophil degranulation may also play a role [27, 28]. CF neutrophils were shown to release greater levels of elastase than non-CF controls despite the fact that the total complement of NE in the CF neutrophil was similar between groups [28]. This increased release may be attributable in part to the inflammatory milieu in the CF lung or as a result of CFTR mutation and/or dysfunction in the cell [27–29]. Further work is needed to fully understand the mechanisms of elevated NE activity in CF. In a healthy individual NE functions to cleave microbial peptides liberated during phagocytosis [30]. However in CF, the elevated level of NE overwhelms the host's cognate regulation of this protease and as such has profound detrimental effects. The general effect of increased NE levels in the CF lung can be grouped into the following categories: impaired mucociliary clearance, airway remodeling, proinflammatory activity, and the impairing of both the innate and adaptive immune system. The impairment of mucociliary clearance mainly revolves around the interactions between NE and mucins. Mucins are a family of highly glycosylated proteins produced by epithelial cells and are the main components of the mucus found clogging the airways of CF patients [31, 32]. NE has been shown to regulate the mucins MUC5AC and MUC2 via activation of TNFα-converting enzyme which upregulates the expression of these mucins via the epidermal growth factor receptor (EGFR) pathway [33–35]. The mucins MUC4 and MUC1 are also upregulated by NE; however their function in the lung is less understood [36–38]. NE has also been shown to cause the hypersecretion of both MUC5AC and MUC5B via the activation of protein kinase pathways further increasing mucus production and its secretion into the CF airway [39, 40]. Finally NE has the ability to reduce ciliary beat frequency and degrade cilia structures. The combined effect of reducing ciliary beat frequency and degradation of cilia prevents mucus from being removed from the airways therefore increasing mucus plugging in CF patients, providing potential colonization sites for bacteria [41–43].

When at high concentrations as found in the CF lung, NE causes airway remodeling owing to the degradation of ECM proteins in the airway such as elastin and fibronectin [44]. The disruption of cell surface structures by NE aggravates neutrophil mediated inflammation increasing expression of the proinflammatory cytokine IL-8 by airway epithelial tissue [28, 45–47]. This increase in IL-8 may be mediated via NE activation of the TLR-4 or EGFR cell signaling pathways, or through the TLR-2 pathway due to the cleavage of CXCR1 receptors from neutrophil cell surfaces [46, 48, 49]. NE release from neutrophils in the lung induces IL-8 expression leading to further neutrophil recruitment resulting in a self-perpetuating and detrimental cycle of neutrophil mediated inflammation. In addition to having proinflammatory activity via acting upon IL-8 levels, NE has also been shown to directly upregulate the proinflammatory matrix metalloproteases (MMPs): MMP-9 and MMP-4 [50]. Indirect activation of MMP-9 can also be mediated by NE due to its ability to inactivate the cognate inhibitor of MMP-9: TIMP-1 [51].

Excess NE levels in the CF lung may negatively affect both the innate and adaptive immune systems. NE both cleaves and downregulates flagella, an important bacterial pathogen-associated molecular pattern (PAMP) [52, 53]. Flagella cleavage has the effect of reducing the innate immune system's ability to detect pathogens such as *Pseudomonas aeruginosa* via TLR signaling pathways [54]. Detection and clearance of pathogens is also inhibited by excess NE due to cleavage of opsonizing peptides C3bi, CR1, and C5 receptor site, rendering reduced phagocytic ability [55, 56]. NE has also been shown to degrade antimicrobial peptides such as lactoferrin and β-defensins directly inhibiting bacterial killing [57, 58]. Finally NE has been shown to reduce the ability of macrophages to clear apoptotic cells due to its ability to cleave macrophage apoptotic cell receptors such as CD36 [59]. In addition to inhibition of the innate immune system, NE inhibits the adaptive immune system as research has shown that NE cleaves T cell receptors CD2, CD4, CD8, and CD14, impairing monocyte activation and also blocking dendritic cell maturation and antigen presentation [60, 61]. The combined detrimental effects of excess NE in the CF lung may result in increased bacterial survival rates heavily contributing to the state of chronic infection associated with CF.

NE can be found associated with the DNA structures secreted from activated neutrophils called neutrophil extracellular traps (NETs). NETs are known to be produced as a result of reactive oxygen species; however downstream of this, NE has been shown to regulate the formation of NETs, with studies showing that NE knockout mice have an inability to form NETs in a *Klebsiella pneumonia* infection model [62, 63]. The translocation of NE to the nucleus upon neutrophil activation and its subsequent degradation of specific histones promotes chromatin decondensation; this process has been shown to be further driven by another enzyme associated with neutrophil granulocytes: myeloperoxidase

[63, 64]. The role of NE directly associated with NETs in the CF lung is however poorly understood and currently under investigation. Due to the high neutrophil burden present in the CF lung these NET structures account for a significant proportion of the DNA content found in mucus [65]. The presence of this extracellular DNA in mucus increases its plasticity increasing mucus plugging [66]. NE associated with the NET structures has been shown to be less active when it is associated with DNA; however it is also significantly less susceptible to the actions of cognate and therapeutic protease inhibitors [67]. It is therefore speculated that NET associated NE could act as a reservoir for NE in the CF lung [67]. DNase treatment of sputum has been shown to significantly increase the activity of NET associated NE but also render it susceptible to inhibition to cognate protease inhibitors. A combination of DNase treatment and protease inhibitor may be a potential therapeutic treatment to alleviate the detrimental inflammatory burden of NE in CF patients [67, 68]. Due to the wide variety of detrimental effects associated with high levels of NE found in CF patients, NE is regarded as the main protease responsible for inflammatory tissue damage in the CF lung.

2.2. Proteinase 3. PR3 is a 29 kDa, 222-amino acid serine protease expressed from the *PRTN3* gene by activated neutrophils [69, 70]. The biological role of PR3 in degrading microbial peptides is similar to that of NE; however, PR3 has been shown to degrade IL-8 resulting in a truncated form of the chemokine with more potent neutrophil chemoattraction activity [71]. This increase in chemoattraction has the potential to further potentiate the detrimental cycle of inflammation previously described for NE. PR3 has also been shown to increase the interaction between neutrophils and the IL-8 receptor CXCR1 [71]. The increases in both neutrophil recruitment and receptor interaction with IL-8 caused by PR3 have clear detrimental implications for the cycle of neutrophil derived inflammation which results in tissue damage within the CF lung.

2.3. Cathepsin G. The remaining serine protease produced by neutrophils implicated in CF lung disease is Cat G. Although Cat G is a serine protease, it belongs to a larger family of proteases which encompass the cysteine cathepsins B, C, H, L, S, K, O, F, X, V, and W and aspartic acid proteases (cathepsins D and E) [72]. Mature Cat G is 28.5 kDa in size and composed of 235 amino acid residues [73]. Cat G is released upon neutrophil activation and possesses the ability to degrade structural components of the extracellular matrix when at high concentrations [17]. Cat G will inhibit the actions of macrophages in clearing apoptotic cells from CF airways; this leads to a rise in neutrophil necrosis and therefore the uncontrolled release of proteases into the lung [17]. Finally Cat G in CF BALF has been shown to have the highest potency of all three neutrophil serine proteases to degrade surfactant protein A, a peptide that facilities microbial clearance by macrophages, the result of which is a reduction in macrophage phagocytic activity, and therefore increased bacterial survival [74].

3. Antiprotease Activity in CF

In a healthy lung, antiproteases maintain a homeostatic balance with proteases, preventing the associated inflammatory damage which results from excess protease activity. In the CF lung it is the inability of these antiproteases to regulate their cognate proteases that is in part responsible for the pathology associated with infection and inflammation. A number of antiproteases associated with CF are discussed below.

3.1. Introduction to the WFDC Protein Family. The whey acidic protein (WAP) four disulphide core (WFDC) proteins are a family of putative multifunctional host defense proteins. These proteins possess a WAP domain composed of approximately 50 amino acid residues with eight conserved cysteine residues which form four disulphide linkages [75]. To date there have been 18 WFDC proteins identified and, in humans, the majority of these proteins are transcribed from genes located on chromosome 20. However two WFDC proteins have been found to be transcribed from genes of chromosome 17 [76, 77]. The best characterized members of this family are secretory leukocyte protease inhibitor (SLPI) and elafin, both heavily implicated in the protease/antiprotease balance in the lung.

3.2. SLPI. SLPI is an 11.7 kDa cationic serine protease inhibitor which forms a constituent part of the body's antiprotease screen [78]. Following posttranslational processing, the mature SLPI peptide consists of 107 amino acid residues and possesses two WFDC domains, each of which has four disulphide linkages [75]. SLPI has been detected in a variety of patient samples and is produced by a number of cell types including neutrophils, macrophages, serous cells of bronchial submucosal glands and nonciliated bronchial epithelial cells [79–81]. SLPI is expressed in response to various stimuli such as bacterial lipopolysaccharides, NE, and a number of cytokines [80–84]. SLPI exerts antiprotease activity against NE, cat G, trypsin and chymotrypsin, mediated via its C-terminal WFDC domain [85]. The key active site amino acid residue in SLPI is Leu^{72} since point mutation of this residue abolishes the antiprotease activity of SLPI [85].

In addition to protease inhibition SLPI has also been shown to inhibit inflammatory responses via a number of differing mechanisms, both extracellularly and intracellularly. SLPI acts extracellularly directly binding bacterial lipopolysaccharide (LPS) and lipoteichoic acid (LTA), preventing TLR activation [86]. SLPI acts intracellularly by preventing the LPS/LTA induced activation of NF-κB, competing with p65 for binding to NF-κB sites in the promoter regions of pro-inflammatory genes such as IL-8 and TNFα therefore preventing the expression of these proinflammatory cytokines and inhibiting the degradation of IκBα and IRAK which in turn prevents NF-κB activation [86–88]. The antiprotease and anti-inflammatory functions of SLPI can

however be disrupted due to cleavage by excess concentration of its cognate substrates. SLPI cleavage by the cysteine proteases cathepsins B, L, and S between residues Thr^{67} and Tyr^{68} disrupts the active site of SLPI abolishing its ability to inhibit NE [89]. Further work demonstrated that SLPI levels are reduced in CF patients infected by the opportunistic pathogen *P. aeruginosa* [90]. Western blot analysis of BALF from these patients showed SLPI to be cleaved, an observation not seen in CF patients who were negative for *P. aeruginosa* [90]. Further investigation identified this cleavage of SLPI to be caused by excessive levels of NE present as a result of *P. aeruginosa* infection [90]. NE-cleaved SLPI loses the ability to bind LPS and NF-κB consensus oligonucleotides [90]. NE-cleaved SLPI however maintains some antiprotease activity as this activity is mediated by the C-terminal domain which remains intact after cleavage [90]. The proteolytic cleavage of SLPI by NE could have implications for its use as an anti-inflammatory therapeutic in CF patients.

3.3. Elafin. Elafin, like SLPI, has been shown to be expressed in macrophages and neutrophils [77]. Mature elafin is formed from the cleavage of a 12 kDa, 117-amino acid long precursor peptide called preelafin or trappin-2 by tryptase [91, 92]. The mature elafin peptide has a relative mass of 6 kDa and possesses 57 amino acid residues [91, 93]. Elafin has two conserved domains, a cementoin domain which acts as a substrate for the enzyme transglutaminase, mediating the incorporation of elafin into extracellular matrix proteins, and the characteristic WFDC domain [76, 94]. The high level of homology seen in the WFDC domain between SLPI and elafin would suggest that like SLPI, elafin functions as a protease inhibitor. This was shown to be the case as elafin, like SLPI, possesses antiprotease activity against NE, trypsin, and chymotrypsin [93, 95]. However, unlike SLPI elafin possesses antiprotease activity against PR3 but not against Cat G [93].

In addition to antiprotease activity, elafin also possesses anti-inflammatory activity, and similar to SLPI, functions in both an intracellular and extracellular manner. Elafin has been shown to inhibit NF-κB activation in monocytes stimulated by LPS and LTA therefore causing a reduction in inflammatory cytokine expression [96]. Elafin was also shown to inhibit proteosome pathways evidenced by the buildup of ubiquitinated IRAK-1 and IκBα in LPS-stimulated monocytic cells [96]. Elafin, like SLPI, will also neutralize LPS. *In vivo* recombinant trappin-2 has been shown to reduce proinflammatory cytokines MIP-2, KC (murine IL-8 homologue), and TNF-α in LPS-treated mice [97]. Neutrophil influx into the lung and protease activity was also seen to be reduced in mice treated with recombinant trappin-2 and stimulated with LPS [97, 98]. Elafin has been proposed as a therapeutic in the treatment of pulmonary arterial hypertension with Proteo Biotec Inc. carrying out phase I clinical trials of elafin with measured success. Elafin treatment for pulmonary arterial hypertension is currently in phase II trials. If the trialling of elafin to treat pulmonary arterial hypertension is successful, it could be used as a potential treatment in CF. Experimental evidence has however shown that like SLPI, elafin is proteolytically cleaved by excess NE in the BAL fluid of CF patient infected with *P. aeruginosa* [99, 100]. The effects of this cleavage were the inactivation of elafin's anti-neutrophil elastase activity due to cleavage of the protease-binding loop. Interestingly the antibacterial properties of elafin were not affected by cleavage [100]. Proteolytic cleavage of elafin may have implications for its efficacy when being used as a therapeutic *in vivo*. Recent work has shown that mutating key residues at the NE cleavage site in elafin results in a peptide with similar antiprotease activity as the wild-type form but with significantly increased stability and anti-inflammatory activity [101]. When compared to wild-type, the mutant forms of elafin were shown to have improved LPS neutralizing activity *in vitro* and increased anti-inflammatory activity when employed in an acute model of pulmonary inflammation induced by *P. aeruginosa* LPS [101].

In addition to their antiprotease and anti-inflammatory function both SLPI and elafin have been shown to possess antimicrobial activity against both Gram negative and Gram positive organisms [102]. Both SLPI and elafin are cationic peptides and this property may mediate their antibacterial activity. Bacterial species reported to be susceptible to SLPI and elafin include *P. aeruginosa* and *Staphylococcus aureus* both of which are heavily implicated in the colonization of adult and juvenile CF patients, respectively [102, 103]. Although SLPI's antibacterial activity is mainly mediated by the N-terminus of the peptide, antibacterial activity was observed to be maximal when the peptide was complete with mutation of the C-terminal WFDC domain shown to result in a slight reduction in antibacterial function [102]. The antibacterial activity of elafin is mediated by both the WFDC domain and the cementoin domain [104]. Interestingly the precursor peptide to elafin, trappin-2, has been shown to possess greater antibacterial activity than the mature peptide [103].

3.4. Alpha-1 Antitrypsin. Alpha-1 antitrypsin (AAT) is a serine protease inhibitor shown to be active against trypsin, plasmin, Cat G, MMP-12 in addition to NE [105–108]. AAT is expressed as a 418-amino acid protein which undergoes posttranslational cleavage of a 24-amino acid signal peptide and glycosylation to form a 52 kDa mature peptide [109]. Expression of AAT mainly takes place in hepatocytes; however, AAT expression has been observed in respiratory epithelial cells, macrophages, and neutrophils [109, 110]. Aerosolized AAT therapy has been proposed as a treatment for the inflammatory damage caused by neutrophil serine proteases in CF patients for a number of years. However a trial conducted with aerosolized AAT showed only a tending relationship toward reduced NE in patients treated with AAT after 4 weeks and did not identify any anti-inflammatory effects of AAT treatment [111]; this trial did however show significant reductions in NE/AAT complexes and myeloperoxidase in the AAT treated cohort [111]. Furthermore, CF patients receiving aerosolized AAT did not exhibit significant reductions in *Pseudomonas* counts in comparison to the placebo group [111]. The results of the Martin et al. [111] study were however contradicted by a

second clinical trial published one year later which showed AAT to have significant anti-inflammatory effects [112]. This trial involved the aerosolized delivery of AAT to CF patients and found a significant reduction in NE, IL-8, IL-1β, and TNF-α levels after 4 weeks of treatment in comparison to baseline; however no increase in FEV1 was observed [112]. In addition, AAT treatment reduced neutrophil counts and there was a reduction in *P. aeruginosa* CFUs [112]. The ability of AAT to regulate its cognate proteases in the CF lung may however be curtailed by high reactive oxygen species levels as a result of increased neutrophil burden [112, 113]. Oxidation of residue Met358 in the active site of AAT renders the protease inactive [47]. AAT can also be cleaved and inactivated by MMPs [114, 115]. If MMP cleavage of AAT takes place in the CF lung the effects could potentially be decreased inhibition of neutrophil serine proteases and therefore continuing the detrimental effects of these peptides. Martin et al. [111] and Griese et al. [112] are the two most recent clinical trials using ATT in CF patients; however there are a number of NIH funded trials currently in progress in the US investigating the use of ATT in CF treatment [111, 112]. The results of these trials may provide further clarification on the efficacy of using this antiprotease as a CF therapeutic.

3.5. Synthetic Serine Protease Inhibitors. In addition to the endogenous protease inhibitors such as SLPI and elafin being trialled for use as anti-inflammatory therapeutics in CF, a number of synthetic inhibitors of NE have been developed and trialed. NE was chosen as a target for inhibition by synthetic compounds due to its being widely recognized as the key serine protease connected to lung pathology in CF. DX-890 is a small protein inhibitor of NE, which has been shown to be tolerable in rat and in humans after a phase I clinical trial [116, 117]. This compound was shown to inhibit NE released from both healthy and CF neutrophils when treated at concentrations above 100 nM [118]. DX-890 also reduced IL-8 release from both healthy and CF neutrophils and reduced neutrophil transmigration through the epithelial barrier [118]. A second trial using DX-890 as a NE inhibitor in CF showed it to be only partially effective at inhibiting NE in CF sputum; however this study only tested DX-890 against low molecular ratios of NE, which do not adequately represent the levels observed in the CF lung [119]. Another compound trialled is AZD9668, an orally administered reversible inhibitor of NE [120]. A phase II clinical trial looking at its efficacy in bronchiectasis showed reductions in proinflammatory cytokines IL-6 and IL-8 and improvements in FEV_1; however sputum neutrophils were not shown to be decreased [121, 122]. A trial looking at AZD9668 use as a CF therapeutic showed similar results, with reports of a reduction in pro-inflammatory cytokines which was postulated to be due to inhibition of elastin cleavage by NE [123].

4. Conclusion

An increasing volume of experimental evidence points to the importance of proteases and their cognate protease inhibitors in CF lung disease. The imbalance of the protease/antiprotease balance in favor of the neutrophil serine proteases results in a self-perpetuating cycle of inflammation and respiratory tissue damage. This evidence also points to therapeutic options for the treatment of CF patients to reduce the inflammatory tissue damage in the form of antiprotease therapy using either synthetic antiproteases or mutated endogenous antiproteases. Clinical trials have shown that the application of antiproteases such as SLPI and AAT results in the reduction of inflammation due to the restoration of the protease/antiprotease balance. The therapeutic and diagnostic applications of research into proteases and antiproteases in the CF lung continue to attract significant interest.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Epithelium-Specific Ets-Like Transcription Factor 1, ESE-1, Regulates ICAM-1 Expression in Cultured Lung Epithelial Cell Lines

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Cystic fibrosis (CF) patients suffer from chronic airway inflammation with excessive neutrophil infiltration. Migration of neutrophils to the lung requires chemokine and cytokine signaling as well as cell adhesion molecules, such as intercellular adhesion molecule-1 (*ICAM-1*), which plays an important role in mediating adhesive interactions between effector and target cells in the immune system. In this study, we investigated the relationship between *ICAM-1* and epithelium-specific ETS-like transcription factor 1 (*ESE-1*) and found that *ICAM-1* expression is upregulated in cell lines of CF (IB3-1) as well as non-CF (BEAS-2B and A549) epithelial origin in response to inflammatory cytokine stimulation. Since *ESE-1* is highly expressed in A549 cells without stimulation, we examined the effect of *ESE-1* knockdown on *ICAM-1* expression in these cells. We found that *ICAM-1* expression was downregulated when *ESE-1* was knocked down in A549 cells. We also tested the effect of *ESE-1* knockdown on cell-cell interactions and demonstrate that the knocking down *ESE-1* in A549 cells reduce their interactions with HL-60 cells (human promyelocytic leukemia cell line). These results suggest that *ESE-1* may play a role in regulating airway inflammation by regulating *ICAM-1* expression.

1. Introduction

Airway inflammation is a hallmark of the cystic fibrosis (CF) lung disease. The airways of CF patients are initially colonized by viruses, fungi, or bacteria, including *Staphylococcus aureus*, *Haemophilus influenzae,* and *Klebsiella pneumonia* [1]. Most patients later become infected with mucoid strains of *Pseudomonas aeruginosa* and some with *Burkholderia cepacia* [2].

In CF patients, the number of neutrophils and the levels of cytokines such as tumor necrosis factor-α (TNF-α), interleukin- (IL-) 6, and IL-8 in the airways are increased compared to non-CF individuals [3, 4]. Cultured CF lung epithelial cells (IB3-1) show downregulation of the anti-inflammatory cytokine IL-10 and an exaggerated upregulation of IL-8 in response to a variety of external stimuli, such as TNF-α and bacterial products [5, 6]. Overproduction of IL-8 is likely a major cause of excessive neutrophil infiltration, since IL-8 is a potent chemoattractant for neutrophils [7].

Neutrophil migration in response to inflammatory stimuli requires cell adhesion molecules, such as intercellular adhesion molecule-1 (*ICAM-1*, also known as CD54) [8, 9]. Migration of neutrophils out of the vascular system occurs in distinct phases: rolling, firm adhesion, and transmigration [10].

Four types of cell adhesion molecules are involved in this process, namely, E-selectin, *ICAM-1*, vascular cell adhesion molecule-1 (VCAM-1), and platelet-endothelial cell adhesion molecule-1 (PECAM-1). Neutrophil rolling is the first step of the migration process and E-selectin is the key molecule involved in slowing down the circulating neutrophils. This step is critical to ensure firm adhesion of neutrophils to the endothelial cell layer. Firm adhesion is mediated through *ICAM-1* expressed on endothelial cells, which interacts with CD11a/CD18 (LFA1) or CD11b/CD18 (Mac-1) as counter-receptors on neutrophils [11]. The final phase of transmigration of neutrophils through the endothelium is triggered by PECAM-1 and VCAM-1 [10]. Currently, the mechanism by which neutrophils migrate to the airway lumen is unclear, but they are thought to travel through the intercellular space [12, 13]. Other cell adhesion molecules such as ICAM-2 and ICAM-3 are also involved in the migration of monocytes [14] or dendritic cells [15].

ICAM-1 is a 114 kD inducible surface glycoprotein that belongs to the immunoglobulin superfamily [9] and it plays an important role in innate and adaptive immune responses [16]. Although the role of *ICAM-1* in endothelial cells as well as in adaptive immunity [17–20] is well established, the function of epithelial *ICAM-1* during inflammation is not fully understood. Since epithelial *ICAM-1* is expressed on the airway lumen [21–24], a role for leukocyte transmigration is not expected. On the other hand, cell adhesion studies [25, 26] indicate that epithelial *ICAM-1* is important for leukocyte homing. Because neutrophils and macrophages are enriched at the sites of injury or inflammation, it is possible that homing of these cells is part of the resolution of inflammation.

Among the adhesion molecules, *ICAM-1* may play a more important role in the infiltration of leukocytes during airway inflammation. For example, Hubeau et al. performed quantitative analysis of inflammatory cells infiltrating the CF airway mucosa in lung tissues collected at the time of transplantation and found that *ICAM-1*, but not VCAM-1 or E-selectin, was overexpressed on the epithelium surface [27]. In addition, a recent *in vitro* study also showed that *ICAM-1* is expressed in a higher percentage of cultured airway epithelial cell lines (IB3-1, C38 and BEAS-2B) than other cell adhesion molecules, such as VCAM-1 or E-selectin [28].

ICAM-1 is expressed at a very low level in airway epithelial cells. Interestingly, CF-deficient airway epithelial cells have a slightly higher basal level of *ICAM-1* expression [28]. Upon stimulation with proinflammatory cytokine (e.g., TNF-α or IL-1β) [29, 30] or other stimulatory substances (e.g., LPS) [31], *ICAM-1* expression is significantly induced in both human primary bronchial epithelial cultures and epithelial cell lines. This induction is mediated by activation of nuclear factor-kappa B (NF-κB) signaling transduction pathway. In addition, *ICAM-1* induction can also be mediated through the STAT signaling pathway since IFN-gamma can significantly elevate its expression in epithelial cells [16]. CF airways have chronic inflammation, which contributes to the overexpression of *ICAM-1* [27]. Since epithelial *ICAM-1* may be critical for neutrophil homing and epithelial killing, it is important to understand its regulation and function in airway epithelial cells in order to identify potential drug targets for the CF lung disease.

The E26 transformation-specific (ETS) family of transcription factors is characterized by a highly conserved 85 amino acid DNA binding domain, which is known as the ETS domain [32]. It is comprised of 27 and 26 members in humans and mice, respectively. The ETS domain is usually located in the carboxyl-terminal region of the protein as a winged helix-turn-helix structural motif and binds to purine-rich DNA that has a core consensus sequence of GGAA/T- within the promoter and enhancer regions of target genes [33]. ETS transcriptional factors act as both positive and negative regulators of gene expression in various biological processes, such as cellular proliferation, differentiation, apoptosis, metastasis, hematopoiesis, and angiogenesis [34]. Although many of ETS family members are expressed in nonepithelial cells, such as hematopoietic and endothelial cells, *ESE-1* is mainly expressed in epithelial-rich tissues, such as lung, kidney, stomach, small intestine, colon, pancreas, trachea, salivary gland, prostate gland, mammary gland, uterus, and skin [35], but it can be upregulated in nonepithelial cells by proinflammatory cytokines such as TNF-α and IL-1β. Our previous work has shown that *ESE-1* can be highly induced in epithelial cells by inflammatory cytokines [36].

In this study, we investigated the regulation of *ICAM-1* expression by *ESE-1*. Here we demonstrate that the expression of both *ICAM-1* and *ESE-1* is upregulated in human bronchial epithelial cells (BEAS2B), CF cells (IB3-1), and lung cancer cells (A549) by inflammatory cytokines. We also show that *ICAM-1* expression is downregulated upon *ESE-1* knockdown in A549 cells and that *ESE-1* regulates the *ICAM-1* expression at the transcriptional level. Finally, we demonstrate that the downregulation of *ICAM-1* by knocking down *ESE-1* in A549 cells results in a reduced capacity of A549 cells to interact with HL-60 cells.

2. Materials and Methods

2.1. Cell Culture and Reagents. Human bronchial epithelial cells (BEAS-2B), human lung carcinoma cells (A549), and CF bronchi epithelial cells (IB3-1) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS). Recombinant human tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) were obtained from R&D Systems (Minneapolis, MN) and reconstituted in phosphate buffered saline (PBS) containing 0.1% bovine serum albumin (BSA). For cytokines stimulation, TNF-α and IL-1β were used at 10 ng/mL each.

2.2. Viral Transduction and shRNA-Mediated Gene Knocking Down. ESE-1 gene was knocked down in A549 cells using a shRNA helper-dependent adenoviral vector expressing two shRNAs from murine U6 gene promoter as described previously [36]. A C4HSU empty vector was used as control. For viral transduction, A549 cells were seeded at 2×10^5 cells per well in 6-well plates overnight, and then cells were transduced at 40–60% confluency with viral vector at

2500 particles/cell (or 50 moi) under serum-free conditions for two hours, followed by addition of media to a final concentration of 10% FBS. We usually achieved near 100% transduction at this vector concentration. Cells were then collected for protein extraction or RNA isolation at desired time points.

2.3. Transient Transfection and Luciferase Reporter Assay. For cell transfection and cotransfection experiments, BEAS-2B cells were seeded in 6-well plates and transfected at 50–60% confluency by using PolyFect transfection reagent (Qiagen) according to the manufacturer's protocol. After 24 h, cells were harvested and luciferase activity was measured using a Dual Lucifcerase kit (Promega) and a luminometer (EG&G Berthold, BadWildbad, Germany) as described previously [37].

2.4. RNA Analysis. Cells were lysated and RNAs were extracted by using RNA spin Mini kit (GE Healthcare) according to manufacturer's instructions. Total RNA (1 μg) was reverse transcribed using random hexamers and Super-Script II reverse transcriptase (Invitrogen), and then the resulting template (20 ng cDNA) was used for each real-time PCR reaction (ABI Prism 7700, Applied Biosystems). Primers for human *ESE-1* and *ICAM-1* were purchased from ACGT, Toronto. For relative quantification, PCR signals were compared between groups after normalization with GAPDH as an internal reference. Fold changes were calculated according to Livak and Schmittgen [38]. Primers used were hICAM1-F: GGAAGGTGTATGAACTGAGCAA, hICAM1-R: GGAGTCCAGTACACGGTGA, hese1-F: GGCGTCTTCAAGTTCCTGCG, hese1-R: CTCCCGTTTGTAGTAGTACCT, hGAPDH-F: GAAGGTGAAGGTCGGAGTC, hGAPDH-R: GAAGATGGTGATGGGATTTC.

2.5. Western Blotting Analysis. Cell lysates were run on an 8% SDS-polyacrylamide gel at 10 μg each lane and transferred to nitrocellulose membrane (Bio-Rad) following electrophoresis. The membrane was then blocked with Tris-buffered saline with Tween-20 (TBST) containing 5% milk and probed with goat anti-human *ICAM-1* antibody (R&D System, Minneapolis, USA) at 1 : 1000 dilution and rabbit anti-human *ESE-1* antibody (R&D System, Minneapolis, USA) at 1 : 5000 dilution. Rabbit anti-human GAPDH antibodies (Trevigen, Gaithersburg, USA) were used at 1 : 3000 as a protein loading control. The horseradish peroxidase-conjugated secondary antibodies for rabbit anti-goat IgG were from Kirkegaard & Perry Laboratories and for goat anti-rabbit IgG were from Bio-Rad Laboratories. Detection of proteins was performed with enhanced chemiluminescence reagents (Amersham Pharmacia Biotech, Baie-d'Urfe, CA).

2.6. Cell Adhesion Assay. At day 0, 1×10^6 A549 cells were seeded on a 10 cm dish and virus vector particles were added at 100 moi (5000 particles per cell) for 1 hour in serum-free media. Fetal bovine serum was then added to final concentration of 10%. At day 5, the treated cells were harvested and seeded at 250,000 to each well of a-24-well plate. The reason for waiting for 5 days was to minimize the influence of inflammatory cytokines induced by the viral transduction. For visualization of cell-cell interactions, HL-60 cells were labeled with carboxyfluorescein succinimide ester (CFSE) at 5 μM in the cell culture for 5 min at 37°C and then washed 3 times with RPMI 1640. The labeled HL-60 cells were put back in the cell culture condition for 24 hours. The A549 cells were then stimulated with IL-1β and TNF-α each at 10 ng/mL for 6 hours and labeled HL-60 cells were activated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA) at 5 ng/mL for 4 hours. After washing both cells, 1 million HL-60 cells in 0.5 mL of RPMI 1640 were added to each well of the treated A549 cells under cell culture conditions. Following adhesion for 1 hour, nonattached HL-60 cells were washed away with 1x PBS for 3 times at room temperature. Cells were fixed with 1% Paraformaldehyde in 1x PBS for 10 min and washed 3 times with 1x PBS. Cells were photographed using a Leica Fluorescence Microscope (model, DMIRE2).

2.7. Statistical Analysis. Data were analyzed by using Mann-Whitney *U* test (Kruskal-Wallis test for more than two groups). $P < 0.05$ was considered statistically significant.

3. Results

3.1. ESE-1 and ICAM-1 Expression Are Upregulated in BEAS2B, IB3-1, and A549 Cells in Response to Inflammatory Cytokines. To examine whether both *ESE-1* and *ICAM-1* are upregulated under proinflammatory conditions, BEAS-2B, IB3-1, and A549 cells were stimulated with a combination of proinflammatory cytokines TNF-α and IL-1β (10 ng/mL each) for 2 hours, and their mRNA levels were assessed by qRT-PCR. As shown in Figures 1(a) and 1(b), levels of mRNA for both *ESE-1* and *ICAM-1* were increased significantly (except for *ESE-1* expression in A549 cells). We also observed a higher level of *ESE-1* mRNA expression in A549 cells than BEAS-2B and IB3-1 cells. To further investigate at which time point *ESE-1* and *ICAM-1* start responding to cytokine stimulation, we completed a time course study. As shown in Figures 1(c) and 1(d), the *ESE-1* mRNA expression peaked at 2 hours after stimulation in A549 cells while *ICAM-1* mRNA expression reached a plateau at 4 hours after stimulation.

3.2. ICAM-1 Expression Is Downregulated by the ESE-1 Knockdown in A549 Cells. Since both *ESE-1* and *ICAM-1* mRNA expression were upregulated when stimulated by proinflammatory cytokines and given that *ESE-1* is a transcription factor, we questioned whether *ICAM-1* expression is transcriptionally regulated by *ESE-1*. We used a helper-dependent adenovirus (HD-Ad) vector to knockdown *ESE-1* gene expression in A549 cells and examined the changes in *ICAM-1* expression. We selected A549 cells for the knockdown experiment, as these cells constitutively express relatively high levels of *ESE-1*, at both mRNA and protein levels. As expected, we found a significant reduction of *ESE-1* mRNA following HD-Ad vector transduction (Figure 2(b)). Interestingly, *ICAM-1* expression was also downregulated compared to the empty vector (C4HSU) control group

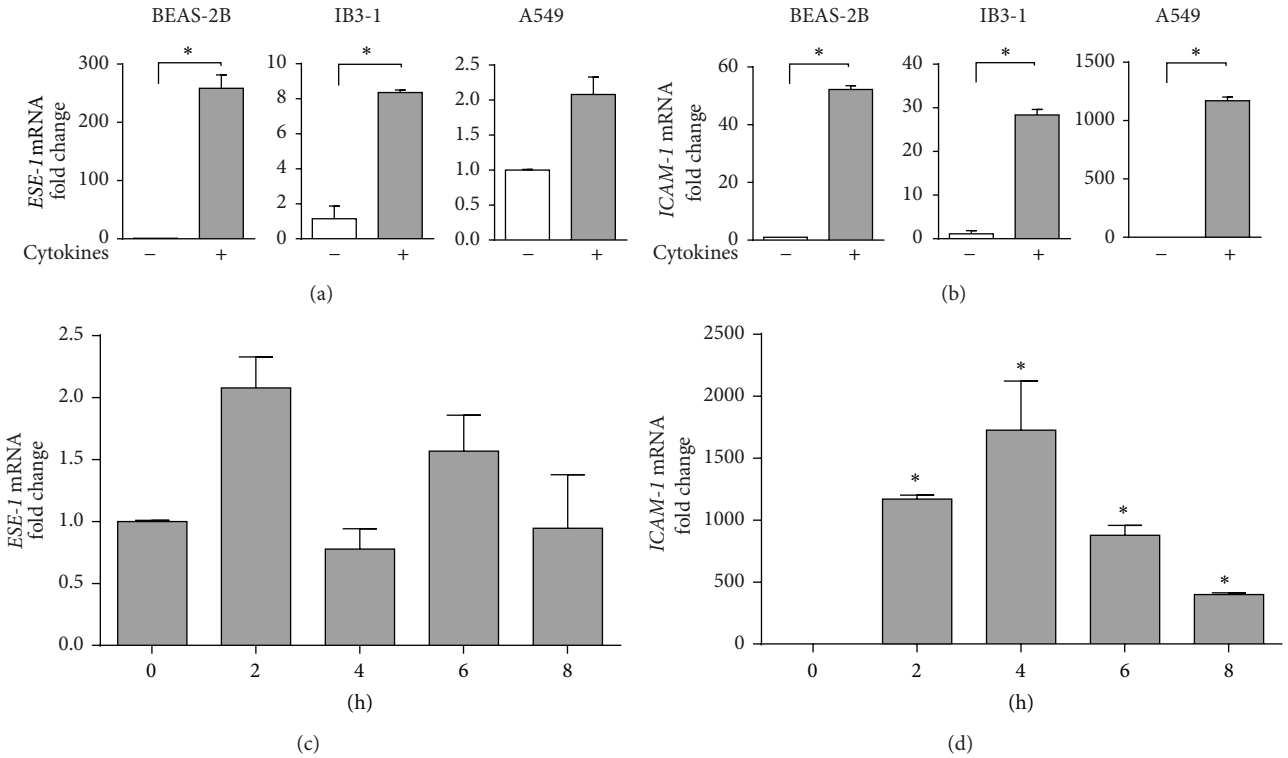

Figure 1: Induction of *ESE-1* and *ICAM-1* mRNA expression by TNF-α and IL-1β in different cell lines. (a, b) *ESE-1* and *ICAM-1* mRNA expression in BEAS2B, IB3-1, and A549 cells after stimulation with TNF-α and IL-1β (10 ng/mL each) for 2 hours. The mRNA expression levels were determined by real-time quantitative RT-PCR and the fold of change was based on the mRNA level of non-induced cells. Data were normalized to GAPDH and values were shown in $2^{-\Delta\Delta CT}$ as the mean ± SD, $n = 3$, $^*P < 0.05$. Statistics was performed as described in Materials and Methods. (c, d) *ESE-1* and *ICAM-1* mRNA expression in A549 cells at different time points following cytokine stimulation. Cells were lysed at time points as indicated after TNF-α and IL-1β (10 ng/mL each) stimulation. The fold of change was based on the expression level at time 0 hour. Data were collected and analyzed as described in (a) and (b).

(Figure 2(c)). We examined *ESE-1* and *ICAM-1* expression at days 4 and 5 after transduction because viral vectors alone can induce the production of proinflammatory cytokines that can last for more than two days after viral transduction. We also used longer time points for cytokine stimulation in protein analysis (16 h) than in mRNA analysis (2 h), as changes in protein expression lag that of the mRNA.

3.3. ESE-1 Gene Regulates ICAM-1 Expression at the Transcriptional Level. To further investigate the regulatory mechanism, we developed an *ICAM-1* promoter reporter assay to examine the role of *ESE-1* in the regulation of *ICAM-1* expression. As shown in Figure 3(a), we made the reporter construct by inserting a 1.4 kb human *ICAM-1* promoter in front of the luciferase reporter gene in pGL-3-basic vector (Promega). BEAS-2B cells were cotransfected with the *ICAM-1* promoter luciferase reporter plasmid and pcDNA3-ESE-1 or pcDNA3-empty as an empty vector control. BEAS-2B cells are noncancerous human bronchial epithelial cells which express minimal basal levels of *ESE-1*, allowing us to specifically overexpress the protein by transfection. Twenty-four hours after cotransfection, the luciferase activity was measured and as shown in Figure 3(b), the level of luciferase activity was increased in the *ESE-1* overexpression group compared to empty vector control group, suggesting that *ESE-1* regulates *ICAM-1* expression at the transcriptional level.

3.4. Knockdown of ESE-1 Expression in A549 Cells Reduces Binding of HL-60 Cells. Since *ICAM-1* is involved in cell-cell interactions, we performed a cell adhesion assay to investigate whether knockdown of *ESE-1* gene could affect cellular adhesion. We found that, following transduction with ESE-1-RNAi viral vector, A549 cells had less binding of HL-60 cells compared with C4HSU empty vector transduced group (Figure 4).

4. Discussion

In response to environmental perturbations, airway epithelia produce and release a variety of inflammatory cytokines, including TNF-α, IL-1, IL-6, and IL-8 [39]. A number of studies have shown that the epithelium of patients who have airway inflammatory diseases is structurally and functionally altered, and that bronchial epithelial cells that are isolated from patients with asthma or cystic fibrosis (CF) express increased levels of cytokines (IL-8, IL-25) [40, 41]. It is thus important to fully understand the gene regulation in

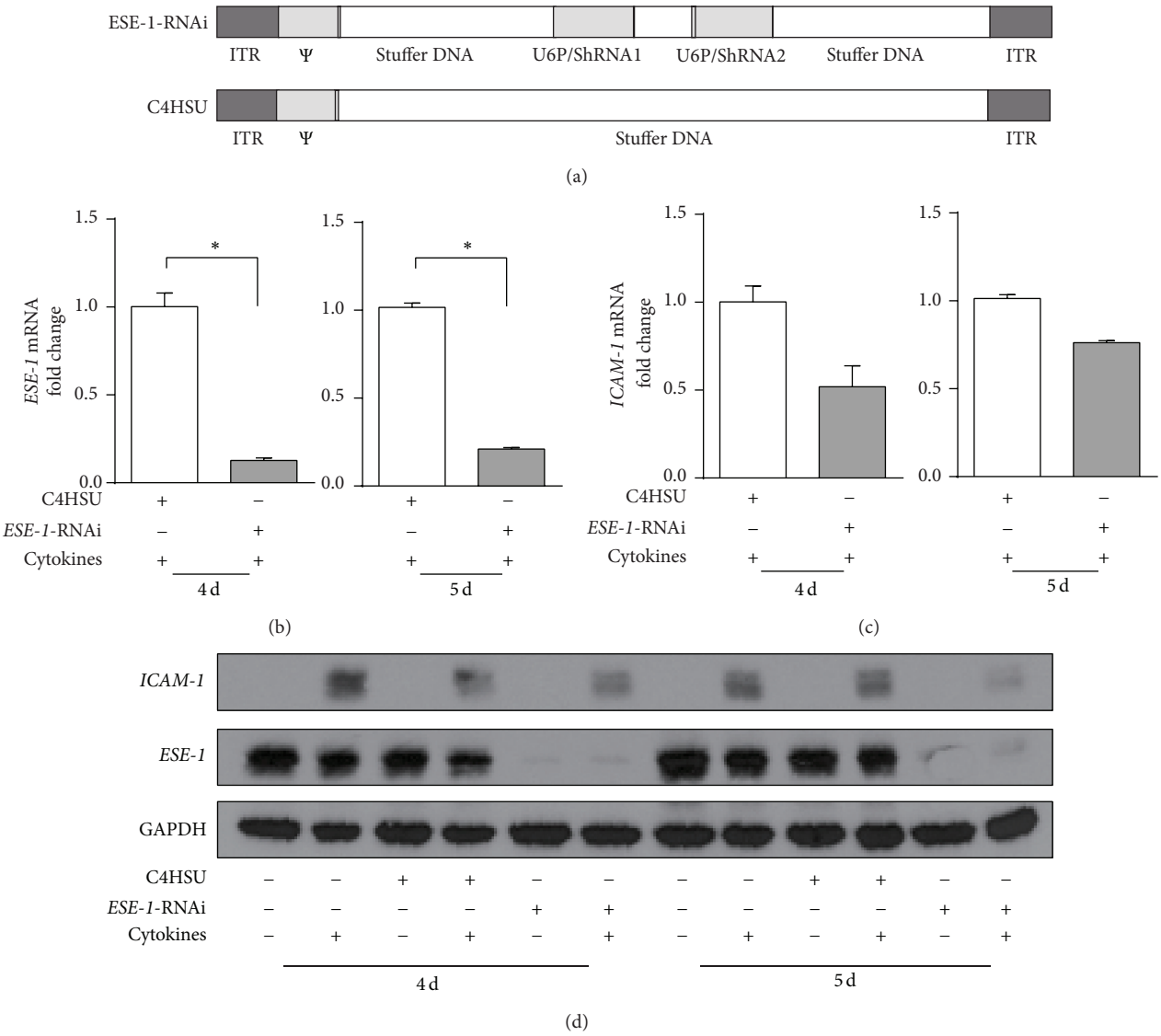

Figure 2: Effects of *ESE-1* knockdown in A549 cells on the expression of *ICAM-1*. (a) Schematic diagrams of the helper-dependent adenovirus vectors (HD-Ad) that were used for the *ESE-1* knockdown experiments. ESE-1-RNAi expresses two shRNAs from the murine U6 gene promoter, and C4HSU is used as an empty vector control which does not express any transgene. ITR: inverted terminal repeat; Ψ: packing signal. (b, c) *ESE-1* and *ICAM-1* mRNA expression in A549 cells 4 and 5 days after transduction with ESE-1-RNAi vector compared to that of C4HSU. Both groups were stimulated with TNF-α and IL-1β at 10 ng/mL each for 2 hours before cell lysis. The mRNA expression levels were normalized to GAPDH and the values were presented in $2^{-\Delta\Delta CT}$ as the mean ± SD, $n = 3$, $^{*}P < 0.05$. Statistics was performed as described in Materials and Methods. (d) A representative western blot analysis of *ICAM-1* expression with (+) and without (−) ESE-1-RNAi, compared to that of the C4HSU vector control group on day 4 and day 5 after transduction. Since *ICAM-1* levels were very low, subgroup of cells were stimulated with TNF-α and IL-1β (10 ng/mL each) for 16 hours to visualize ICMA-1 protein expression before cell lysis.

airway epithelium in order to alleviate airway inflammatory diseases.

In this study, we show that both *ESE-1* and *ICAM-1* mRNAs are upregulated in lung epithelial cell lines after treatment with TNF-α and IL-1β. However, while both genes can be activated by the NF-κB [42], we questioned whether there was any regulatory relationship between the two. The regulation of *ICAM-1* gene expression is primarily at the transcriptional level by several signaling pathways including protein kinase C (PKC), mitogen activated protein (MAP) kinase (JNK, ERK, and P38), and NF-κB. The human *ICAM-1* gene promoter contains binding sites for many transcription factors, including AP-1, C/EBP, Ets, NF-κB, STAT, and Sp1 [42]. Since *ESE-1* is a member of Ets family transcription factors which shares DNA binding sites, we decided to examine whether *ESE-1* regulated *ICAM-1* expression. To investigate the relationship between these two genes, we used helper-dependent adenovirus vector containing shRNA-ESE-1 to knockdown *ESE-1*. We observed that *ICAM-1* expression in A549 cells was indeed decreased after knocking down *ESE-1*.

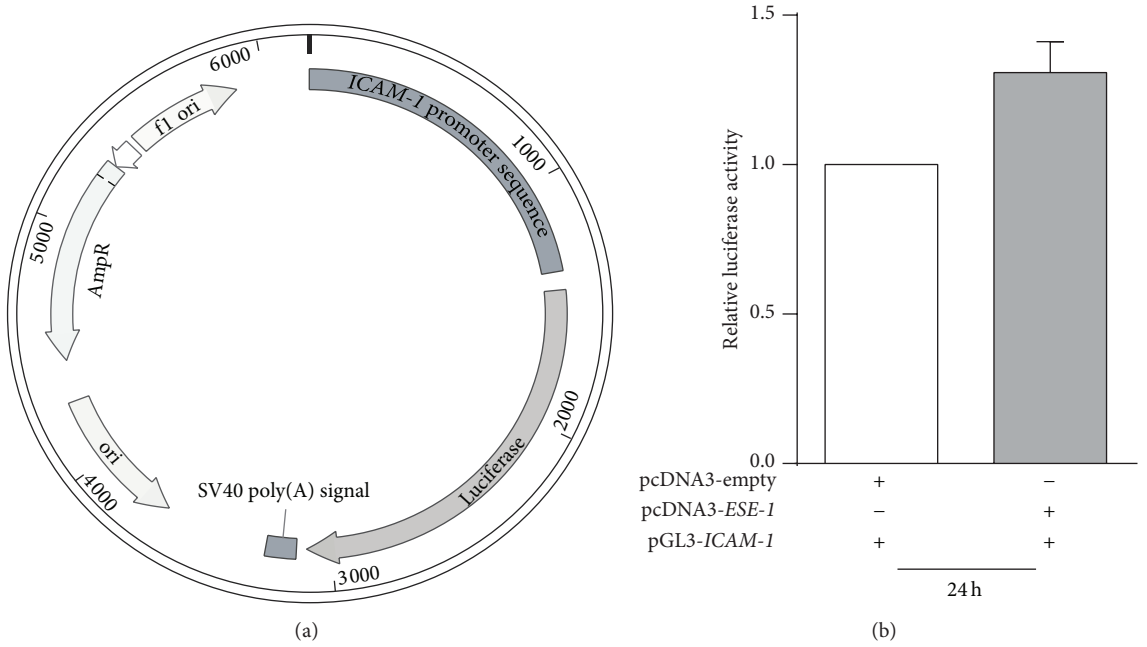

Figure 3: Luciferase assay on BEAS2B cells after cotransfection with pcDNA3-ESE-1 and PGL3-ICAM-1 vector. (a) Schematic diagram of pGL3-ICAM-1 promoter reporter plasmid that contains an *ICAM-1* promoter sequence expressing the luciferase gene in the pGL3-Basic vector (Promega). (b) Luciferase activity assay. BEAS-2B cells were cotransfected with pGL3-ICAM-1 luciferase reporter plasmid and pcDNA3-ESE-1 or pcDNA3-empty vector [37]. The luciferase activity was measured 24 h after transfection.

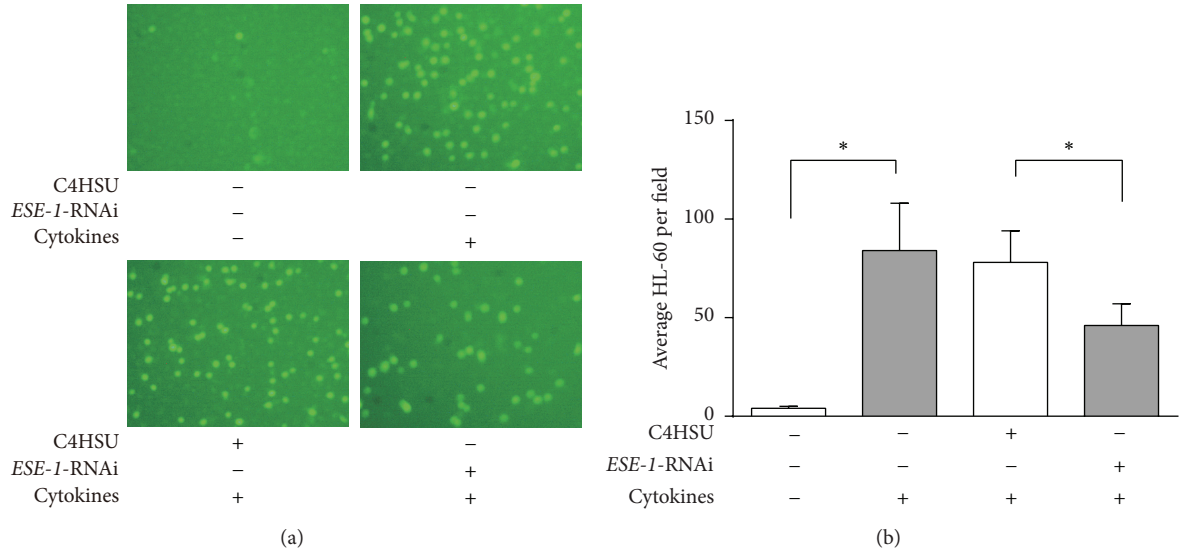

Figure 4: Cell adhesion assay of the effect of *ESE-1* knocking down on HL-60 binding to A549 cells. (a) Images showing HL-60 cells attached to A549 cells at 40x magnification. (b) Quantification of cell binding. The average number of HL-60 cells per field was compared among groups with *ESE-1* knocking down or C4HSU empty vector transfection or nontransfected groups with or without cytokine stimulation (TNF-α and IL-1β at 10 ng/mL each). Cell numbers were counted under a microscope and data from six wells were presented as mean ± SD, $^{*}P < 0.05$.

To further investigate on the mechanism of this relationship between *ESE-1* and *ICAM-1*, we used *ICAM-1* promoter luciferase reporter assays to determine whether this upregulation was at the transcription level. Upon cotransfecting the luciferase reporter plasmid with an *ESE-1* gene expressing plasmid into BEAS-2B cells, we observed an increase in luciferase activity when *ESE-1* gene was overexpressed compared to the cells cotransfected with an empty vector plasmid. This result suggests that *ESE-1* regulates *ICAM-1* expression at the transcriptional level. However, we noted that

the level of upregulation was not dramatic, but under chronic inflammatory conditions, a small change in *ICAM-1* levels may have a significant impact on disease progression.

We also performed a cell adhesion assay to investigate whether knocking down *ESE-1* could affect cell adhesion of epithelial cells. We show that, following transduction with ESE-1-RNAi vector, A549 cells exhibited less binding of HL-60 cells compared to groups of transduced with C4HSU empty vector or untransduced. This suggests that knocking down *ESE-1* in A549 cells causes downregulation of *ICAM-1* gene expression which in turn results in a reduced binding interaction between HL-60 and A549 cells.

ICAM-1 is a key molecule that contributes to the control of inflammatory process. Although our finding of *ESE-1* regulating *ICAM-1* expression in this work is interesting, there are limitations to its clinical applications. First, *ESE-1* knocking down was carried out only in A549 cells, not in primary CF lung epithelial cells. Second, similar experiments have not been performed in any CF animal model. Future studies on this regulation in other model systems will be important for the development of effective anti-inflammatory strategies suitable for therapeutic intervention of inflammatory conditions such as CF. Since *ESE-1* is an transcription factor, it could be a potential drug target for screening small molecules that inhibit its expression.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Genetic Deletion and Pharmacological Inhibition of PI3Kγ Reduces Neutrophilic Airway Inflammation and Lung Damage in Mice with Cystic Fibrosis-Like Lung Disease

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Purpose. Neutrophil-dominated airway inflammation is a key feature of progressive lung damage in cystic fibrosis (CF). Thus, reducing airway inflammation is a major goal to prevent lung damage in CF. However, current anti-inflammatory drugs have shown several limits. PI3Kγ plays a pivotal role in leukocyte recruitment and activation; in the present study we determined the effects of genetic deletion and pharmacologic inhibition of PI3Kγ on airway inflammation and structural lung damage in a mouse model of CF lung disease. *Methods*. βENaC overexpressing mice (βENaC-Tg) were backcrossed with PI3Kγ-deficient (PI3Kγ^{KO}) mice. Tissue damage was assessed by histology and morphometry and inflammatory cell number was evaluated in bronchoalveolar lavage fluid (BALF). Furthermore, we assessed the effect of a specific PI3Kγ inhibitor (AS-605240) on inflammatory cell number in BALF. *Results*. Genetic deletion of PI3Kγ decreased neutrophil numbers in BALF of PI3Kγ^{KO}/βENaC-Tg mice, and this was associated with reduced emphysematous changes. Treatment with the PI3Kγ inhibitor AS-605240 decreased the number of neutrophils in BALF of βENaC-Tg mice, reproducing the effect observed with genetic deletion of the enzyme. *Conclusions*. These results demonstrate the biological efficacy of both genetic deletion and pharmacological inhibition of PI3Kγ in reducing chronic neutrophilic inflammation in CF-like lung disease *in vivo*.

1. Introduction

Cystic fibrosis (CF), the most common genetic disease in Caucasian populations, results from mutations in a single gene encoding for 1480 residues transmembrane glycoprotein, the cystic fibrosis transmembrane conductance regulator (CFTR), that regulates cAMP-mediated chloride conductance at the apical surface of secretory epithelia [1, 2]. Impaired CFTR-mediated secretion of Cl- and bicarbonate results in dehydration and acidification of the airway surface liquid, which in turn causes impaired mucociliary clearance and bacterial killing. These defects trigger a progressive lung disease characterized by airway mucus obstruction, chronic neutrophilic inflammation, bacterial infection, and structural lung damage that remains the major cause of morbidity and mortality in patients with CF [3].

A growing number of *in vitro* and *in vivo* studies support the notion that chronic neutrophilic inflammation with the release of damaging neutrophil products, such as neutrophil elastase, constitutes a key risk factor in early structural lung damage and lung function decline in CF [4–6]. Neutrophilic airway inflammation is augmented after onset of chronic bacterial infection with *Pseudomonas aeruginosa* and other pathogens. In this context, the inflammatory response in the CF lung is nonresolving and self-perpetuating, and a vicious cycle of neutrophilic inflammation, noxious mediator release, and overwhelmed defenses amplifies inflammation, perpetuates infection and contributes to irreversible lung damage and disease progression [7–9]. Therefore, anti-inflammatory therapy, combined with antibiotic therapy, appears crucial to prevent chronic lung damage. However, traditional therapeutic strategies, as well as more recently studied anti-inflammatory drugs, have shown several limitations and limited clinical benefit [8–10]. Clearly, novel approaches have to be undertaken to provide effective anti-inflammatory therapy to CF patients. One possibility is to interfere with leukocyte trafficking into CF airways. Trafficking of leukocytes is controlled by chemotactic factors which bind to heterotrimeric G-protein-coupled receptors (GPCR) and trigger a complex set of signaling pathways inside the cell involving the generation of second messengers like phosphoinositides. Phosphoinositides are substrates of the phosphoinositide 3-kinases (PI3Ks), enzymes that catalyze the phosphorylation of the phosphatidylinositol at the 3rd position of the inositol ring. PI3Ks modulate a wide number of cellular functions such as proliferation and survival, cytoskeletal remodeling, and membrane trafficking and represent important mediators in the signaling cascade leading to the initiation of the inflammatory response [11–14]. PI3Ks can be divided in three classes (I, II, and III) based on their biochemical properties. Leukocytes express all four known isoforms of class I PI3Ks, namely, PI3K α, β, δ, and γ [14]; nonetheless PI3Kγ plays a fundamental role in leukocyte migration and function by acting as a chemokine sensor and regulating neutrophil oxidative burst, T cell proliferation, and mast degranulation. We therefore hypothesized that PI3Kγ plays a pivotal role in mediating leukocyte recruitment and activation and may thus represent a potential target for anti-inflammatory treatment to reduce neutrophilic airway inflammation and lung damage in CF. To test this hypothesis, we used transgenic mice with airway-specific overexpression of the epithelial Na^+ channel (ENaC) and determined the effects of genetic deletion and pharmacologic inhibition of PI3Kγ [15–17].

2. Materials and Methods

2.1. Mice. PI3Kγ^{WT}/βENaC-Tg (βENaC-Tg) [15–18] and PI3Kγ-deficient (PI3Kγ^{KO}, Harlan, Italy) mice on the C57BL/6 background were intercrossed to generate βENaC-Tg/PI3Kγ^{KO} mice. All experiments were performed in 7- to 8-week-old adult mice. βENaC-Tg, PI3Kγ^{KO}, PI3Kγ^{KO}/βENaC-Tg, and wild-type (PI3Kγ^{WT}) mice were housed in a pathogen-free animal facility at the Istituto per la Ricerca e la Cura del Cancro, University of Turin, in accordance with the Institutional Animal Welfare Guidelines and Italian legislation. The animal study protocols were reviewed and approved by the Institutional Animal Ethics Committee of the Istituto per la Ricerca e la Cura del Cancro, University of Turin, Turin, Italy, and performed according to the Institutional Animal Welfare Guidelines and Italian legislation.

2.2. Assessment of Inflammatory Cells in Bronchoalveolar Lavage. Inflammatory cell numbers were assessed in the broncoalveolar fluid (BALF) of PI3Kγ^{WT} mice and of PI3Kγ^{WT}/βENaC-Tg, PI3Kγ^{KO}, and PI3Kγ^{KO}/βENaC-Tg mice. Briefly, mice from each genotype were sacrificed and BALF was then collected by lavaging lungs in situ with 3 × 1-mL volumes of PBS. After centrifugation of the BALFs, cell pellets, in 500 μL of RPMI medium, were deposited onto glass slides using a Cytospin Cytocentrifuge. Slides were then stained using the Diff-Quick system (MICROPTIC S.L., Spain) and a differential cell count was performed as previously described [19]. In addition, BALF inflammatory cells were also analyzed in mice treated with the PI3Kγ inhibitor AS-605240 [5-(quinoxalin-6-ylmethylidene)-1,3-thiazolidine-2,4-dione] (Sigma, Germany). PI3Kγ^{WT} and PI3Kγ^{WT}/βENaC-Tg mice were treated once daily for 3 days with the AS-605240 by intraperitoneal injection of 10 mg/kg of the drug or vehicle (0.5% carboxymethyl cellulose, 0.25% Tween) alone.

2.3. Lung Histology and Morphometry. Animals of each group were sacrificed under anaesthesia with pentobarbital (60 mg/Kg) and the lungs fixed intratracheally with buffered formalin (5%) at a constant pressure of 20 cm H_2O. Lung volume (V) was measured by water displacement according to Scherle [20]. Sagittal sections of each pair of lungs were cut and stained with haematoxylin/eosin. The slides were coded to prevent bias. Morphometric evaluations included determination of the average interalveolar distance (mean linear intercept: Lm) [21] and internal surface area (ISA) estimated by the Lm method at postfixation lung volume by the formula $4V/\mathrm{Lm}$, where V is the postfixation lung volume [22]. For the determination of the Lm for each pair of lungs, 40 histological fields were evaluated both vertically and horizontally. The development of goblet cell metaplasia was evaluated by periodic acid-Schiff reaction (PAS) according to standard histological protocols [23]. The total number of cells, as well as the percentage of PAS-positive cells, was determined. The number of cells in airways that demonstrated PAS staining was determined by examining eight intrapulmonary airways per section and counting at least 3,000 cells/section. Data were reported as the percentage of positive cells per total cells.

2.4. Statistical Analysis. Statistical analyses were performed using one-way analysis of variance. Survival curves were compared using Kaplan-Meier log rank analysis. $P < 0.05$ was considered statistically significant and "n" represents the number of mice in each experimental group. Data are expressed as mean ± SD.

3. Results

3.1. Genetic Deletion of PI3Kγ Reduces Neutrophilic Airway Inflammation and Mortality in βENaC-Tg Mice. As observed in previous studies, βENaC-Tg (PI3Kγ^{WT}/βENaC-Tg; Figure 1(a)) mice on the C56BL/6J background exhibited a spontaneous mortality of ~23% [18, 24]. Deletion of PI3Kγ had no effect on survival in wild-type mice; however, in the presence of the βENaC transgene (PI3Kγ^{KO}/βENaC-Tg), PI3Kγ loss significantly reduced the mortality by ~50%, since at 60 days the survival rate is more than 85% ($P < 0.05$, Figure 1(a)).

To determine the effect of genetic deletion of PI3Kγ on airway inflammation, we compared inflammatory cell numbers in BAL fluid from surviving PI3Kγ^{WT}/βENaC-Tg and PI3Kγ^{KO}/βENaC-Tg mice. As expected, in homozygous PI3Kγ^{WT} and PI3Kγ^{KO} control mice, neutrophils were rarely detected in the BALF (Figure 1(b)) as well as in the airways lumen (Figure 1(c)). The number of neutrophils, in BALF and in the airways lumen, was markedly elevated in PI3Kγ^{WT}/βENaC-Tg mice (Figures 1(b) and 1(c)). On the contrary, the absence of PI3Kγ expression in PI3Kγ^{KO}/βENaC-Tg mice led to a large reduction of neutrophil recruitment into the lung if compared to PI3Kγ^{WT}/βENaC-Tg mice (Figure 1(b)). Nonetheless, deletion of PI3Kγ did not affect macrophage and lymphocyte recruitment as no differences were detected between PI3Kγ^{KO}/βENaC-Tg and PI3Kγ^{WT}/βENaC-Tg mice in BALF (Figures 1(d) and 1(e)).

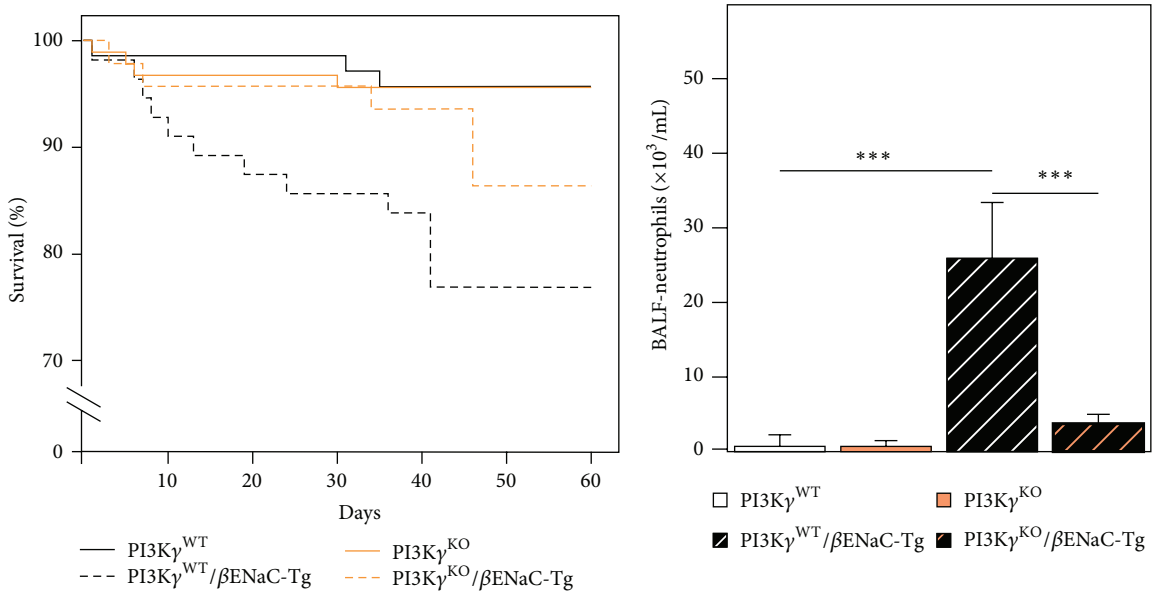
3.2. Genetic Deletion of PI3Kγ Reduces Structural Lung Damage in βENaC-Tg Mice. Chronic inflammation, in PI3Kγ^{WT}/βENaC-Tg mice, triggers emphysema with distal airspace enlargement and alveolar destruction resulting in reduced lung tissue density and increased lung compliance [6, 17, 19]. To assess the protective effects of the genetic deletion of PI3Kγ on emphysema-like changes in PI3Kγ^{KO}/βENaC-Tg mice, we determined the averaged interalveolar distance (mean linear intercept, Lm) and the internal surface area (ISA) estimated by the Lm method at postfixation lung volume. ISA and Lm were not altered in the lungs of controls PI3Kγ^{WT} and PI3Kγ^{KO} mice (Figures 2(a) and 2(b)), and morphological analysis showed a well-fixed normal parenchyma with normal airways (data not shown). As expected from previous studies [6, 17, 19], PI3Kγ^{WT}/βENaC-Tg mice lungs showed significant emphysematous changes (Figures 2(a)–2(c)) while the genetic deletion of PI3Kγ in PI3Kγ^{KO}/βENaC-Tg mice resulted in a significant reduction of the degree of emphysema, as assessed by both morphometric analyses (ISA: $P < 0.0002$ versus PI3Kγ^{WT}/βENaC-Tg mice; Lm: $P < 0.0003$ *versus* PI3Kγ^{WT}/βENaC-Tg mice; Figures 2(a) and 2(b)) and morphology (Figure 2(c)).

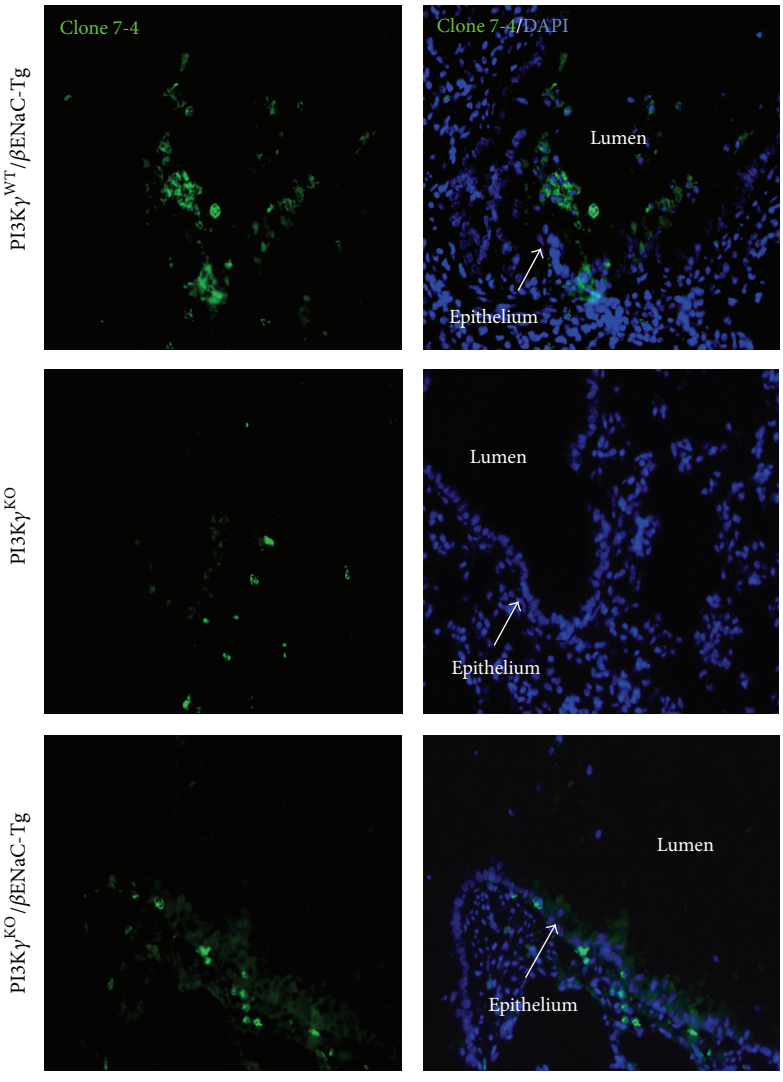
In addition to neutrophilic inflammation, goblet cell metaplasia and mucus obstruction were a common feature of the airways of adult PI3Kγ^{WT}/βENaC-Tg mice [19]. Since neutrophil products, such as neutrophil elastase, have been implicated in goblet cell metaplasia and mucin hypersecretion in CF [25, 26], we assessed the effects of genetic deletion of PI3Kγ on goblet cell metaplasia. Goblet cells were not observed in PI3Kγ^{WT} and PI3Kγ^{KO} mice; in PI3Kγ^{KO}/βENaC-Tg mice, the goblet cell metaplasia appeared reduced compared to PI3Kγ^{WT}/βENaC-Tg mice; however, this difference was not statistically significant, based on the variability and the number of mice included in our studies (data not shown).

3.3. Pharmacological Inhibition of PI3Kγ Reduces Neutrophilic Airway Inflammation in βENaC-Tg Mice. Next we tested effects of pharmacological inhibition of PI3Kγ by using the inhibitor AS-605240 on airway inflammation in βENaC-Tg mice. Treatment of βENaC-Tg mice with AS-605240 but not with vehicle alone reduced neutrophil infiltrates in BALF of βENaC-Tg mice (Figure 3(a)). In contrast, as observed in PI3Kγ^{KO}/βENaC-Tg mice, the PI3Kγ inhibitor had no effect on the recruitment of macrophages or lymphocytes into the lung (Figures 3(b) and 3(c)).

4. Discussion

Progressive lung disease is the major cause of morbidity and mortality in CF and is characterized by chronic airway infection and associated airway inflammation leading to irreversible lung destruction and early death [1–3]. Accumulating evidences suggest that CFTR dysfunction impairs mucociliary clearance and bacterial killing as crucial innate defense mechanisms of the lung leading to chronic bacterial infection and nonresolving inflammation in CF airways [3]. The main feature of airway inflammation in CF is a persistent influx of neutrophils that release a variety of oxidants and granule-associated enzymes, thus contributing to the development of lung injury and to the chronicity of pulmonary infection [7–9]. Repeated episodes of exacerbation of chronic infection and inflammation occur during the natural history of the disease, further increasing the structural damage in the CF lung [27, 28]. Therefore, anti-inflammatory therapy, combined with antibiotic therapy, offers a rational approach to prevent chronic lung damage. However, current anti-inflammatory drugs have shown several limits. The use of oral corticosteroids has been limited by severe adverse effects and studies using inhaled corticosteroids in CF have not been particularly successful [8, 9]. In addition, nonsteroidal anti-inflammatory drugs, such as ibuprofen, although revealing beneficial effects in younger CF patients [29], are difficult to dose and thus are not widely used [30]. Likewise, a phase 3 study of the LTB_4 receptor antagonist BIIL 284 had been stopped due to adverse effects in the treatment group [31]. An alternative approach to decrease chronic inflammation is to use a more targeted anti-inflammatory therapy directed at reducing neutrophil trafficking in the CF lung. In this context, class I PI3K member, PI3Kγ, has been demonstrated to play a pivotal role in mediating leukocyte recruitment and activation into sites of inflammation [11]. Therefore PI3Kγ may represent an innovative and appropriate target to interfere with the excessive neutrophil-mediated inflammation and damage in CF. Of note, recently developed small-molecule





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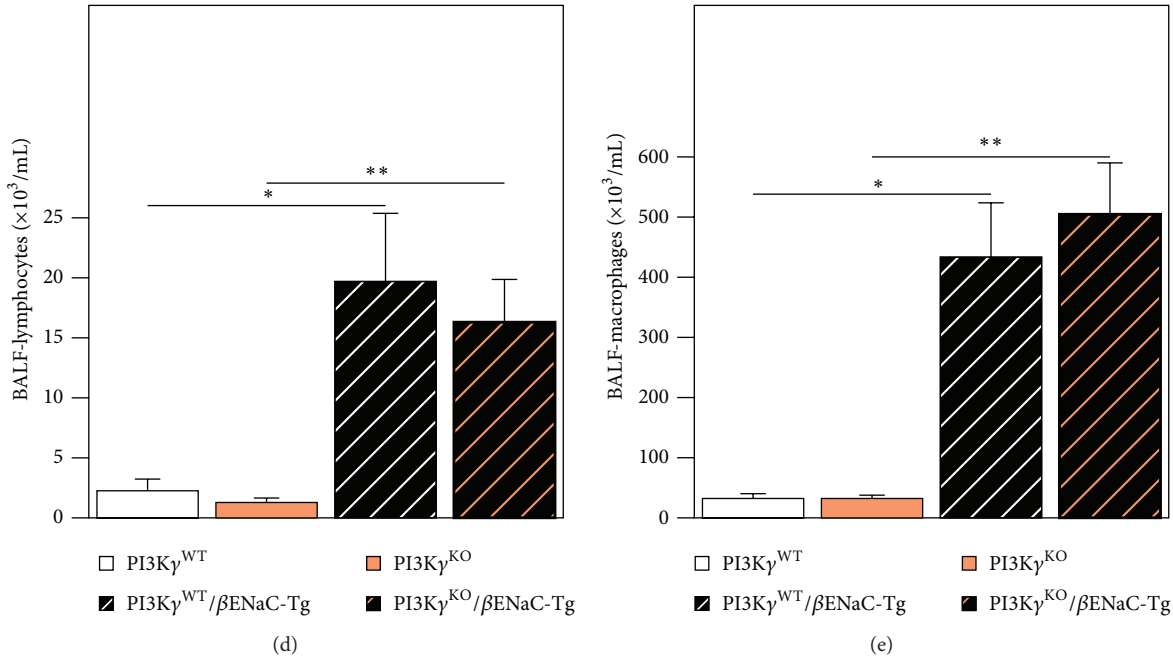

Figure 1: *Effect of genetic deletion of PI3Kγ on mortality and airway inflammation in βENaC-Tg mice*. (a) Survival curves for the different groups of mice studied ($P < 0.05$). (b) Neutrophil numbers were assessed in BALF of PI3Kγ^{WT}, PI3Kγ^{WT}/βENaC-Tg, PI3Kγ^{KO}, and PI3Kγ^{KO}/βENaC-Tg mice. Neutrophils are expressed as cell numbers per mL of BALF ($n = 10$ mice for each group). Comparison between the different groups was performed by one-way analysis of variance. $^{***}P < 0.001$ PI3Kγ^{WT} *versus* PI3Kγ^{WT}/βENaC-Tg and $^{***}P < 0.001$ PI3Kγ^{WT}/βENaC-Tg *versus* PI3Kγ^{KO}/βENaC-Tg. (c) Immunofluorescent detection of neutrophils in lung tissues of PI3Kγ^{WT}/βENaC-Tg, PI3Kγ^{KO}, and PI3Kγ^{KO}/βENaC-Tg mice. Neutrophils were stained by using monoclonal rat antibodies to neutrophils (clone 7/4, Acris) and nuclei with DAPI. (d) Lymphocyte numbers were assessed in BALF of PI3Kγ^{WT}, PI3Kγ^{WT}/βENaC-Tg, PI3Kγ^{KO}, and PI3Kγ^{KO}/βENaC-Tg mice. Lymphocytes are expressed as number of cells per mL of BALF ($n = 10$ mice for each group) $^{*}P < 0.05$ PI3Kγ^{WT} *versus* PI3Kγ^{WT}/βENaC-Tg and $^{**}P < 0.01$ PI3Kγ^{KO} *versus* PI3Kγ^{KO}/βENaC-Tg. (e) Macrophage numbers were assessed in BALF of PI3Kγ^{WT}, PI3Kγ^{WT}/βENaC-Tg, PI3Kγ^{KO}, and PI3Kγ^{KO}/βENaC-Tg mice. Macrophages are expressed as number of cells per mL of BALF ($n = 10$ mice for each group). $^{*}P < 0.05$ PI3Kγ^{WT} *versus* PI3Kγ^{WT}/βENaC-Tg and $^{**}P < 0.01$ PI3Kγ^{KO} *versus* PI3Kγ^{KO}/βENaC-Tg.

PI3Kγ inhibitors were shown to be effective in suppressing joint inflammation in mouse models of rheumatoid arthritis [32]. In the present study we evaluated the effects of genetic deletion and pharmacologic inhibition of PI3Kγ in the βENaC-Tg mouse as a model of CF lung disease [15, 16, 33]. Such model phenocopies the airway surface dehydration and mucociliary dysfunction characteristic of CF airways. βENaC-Tg mice develop spontaneous CF-like lung disease with early onset goblet cell metaplasia and airway mucus obstruction, reduced bacterial clearance, and chronic neutrophilic inflammation triggering emphysema-like structural lung damage [15, 17, 34, 35]. Genetic deletion of PI3Kγ resulted in decreased neutrophil numbers in BALF of PI3Kγ^{KO}/βENaC-Tg mice, and reduced neutrophilia was associated with reduced emphysematous changes in these mice. Taken together, these data support an important role of PI3Kγ for transmigration of neutrophils from the blood into the airway lumen and a crucial role of neutrophilic airway inflammation in the *in vivo* pathogenesis of lung damage. Several leukocyte-derived proteases including neutrophil elastase have been shown to cause emphysema in mice [36–38]. Furthermore, previous studies demonstrated that overexpression of several proinflammatory mediators in genetically modified mice induces an imbalance in the pulmonary protease/antiprotease system and emphysema in these mice [39, 40]. Thus, it is likely that neutrophil-dominated chronic pulmonary inflammation and the disruption of protease/antiprotease balance contribute to the development of emphysema in PI3Kγ^{WT}/βENaC-Tg mice. Neutrophil elastase (NE) is the major product of activated neutrophils and has been implicated in the pathogenesis of key features of CF lung disease, such as chronic airway inflammation, mucus hypersecretion, goblet cell metaplasia, and structural damage [41–47]. We hypothesize that deletion of PI3Kγ decreases lung damage through the reduction of neutrophilic inflammation and neutrophil-associated active elastase. Consistently, a recent study demonstrated that NE activity is increased at the surface of airway neutrophils in PI3Kγ^{WT}/βENaC-Tg mice and patients with CF [6] and that genetic deletion of NE results in a significant reduction of emphysema-like changes in PI3Kγ^{WT}/βENaC-Tg mice, suggesting that NE is implicated in emphysema associated with chronic neutrophilic airway inflammation *in vivo*.

Recently, selective PI3Kγ inhibitors have been developed and investigated in different mouse models of chronic inflammation [48–51]. Therefore, we evaluated the efficacy

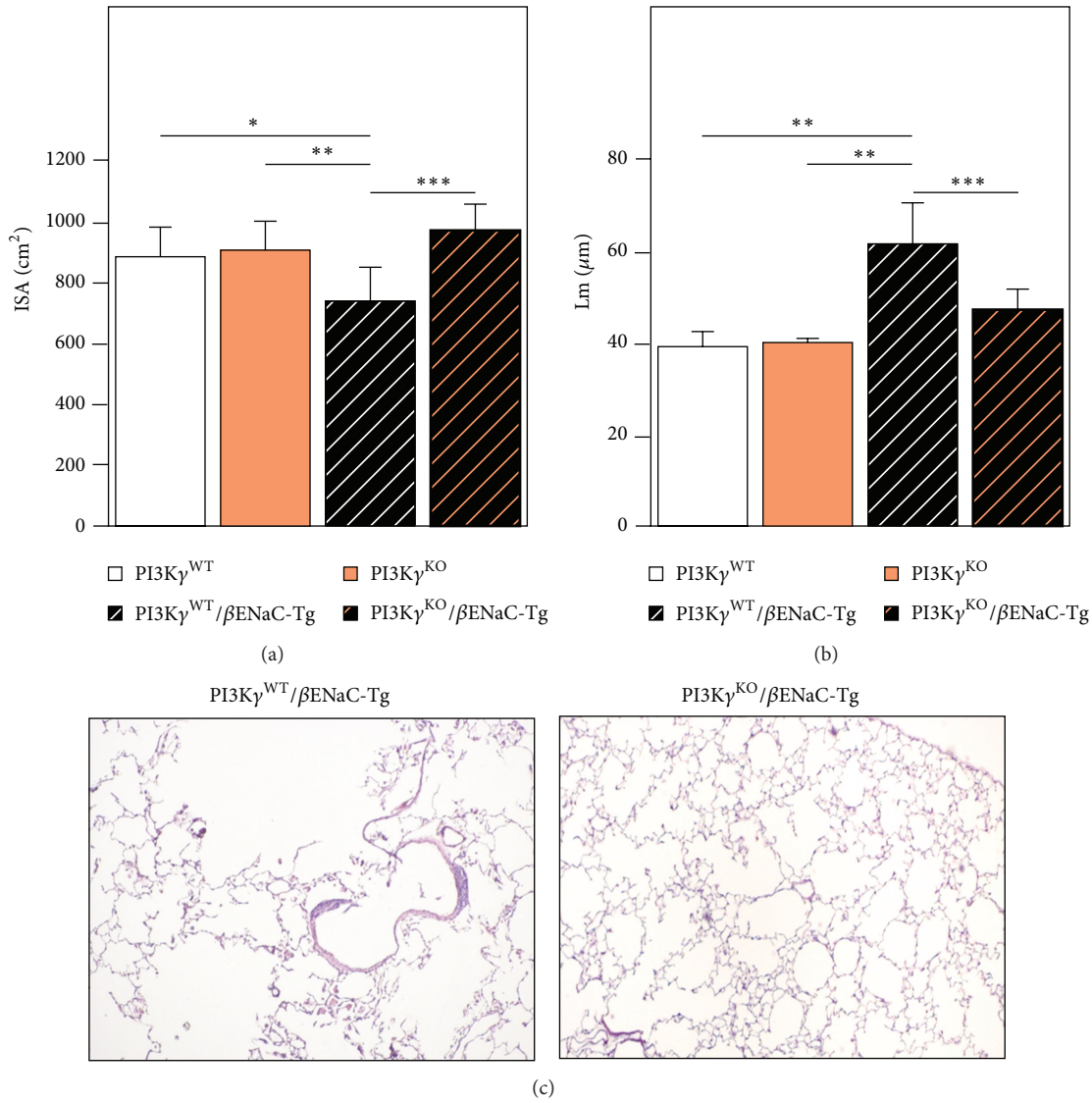

Figure 2: *Genetic deletion of PI3Kγ decreases emphysema in βENaC-Tg mice*. Mouse lungs were fixed in 4% formalin and embedded in paraffin and 5 μm sections were stained with hematoxylin/eosin; assessment of emphysema included the internal surface area (ISA) at postfixation lung volume and the morphometric assessment of the average inter-alveolar distance (mean linear intercept: Lm). (a) ISA and (b) Lm from 8-week-old wild type (PI3Kγ^{WT}), βENaC-Tg, PI3Kγ^{KO}, and PI3Kγ^{KO}/βENaC-Tg mice (n = 8–10 mice for each group). Comparison among groups was performed using one-way analysis of variance. (a) $^{*}P < 0.05$ PI3Kγ^{WT} *versus* PI3Kγ^{WT}/βENaC-Tg, $^{**}P < 0.01$ PI3Kγ^{KO} *versus* PI3Kγ^{WT}/βENaC-Tg, $^{***}P < 0.001$ PI3Kγ^{WT}/βENaC-Tg *versus* PI3Kγ^{KO}/βENaC-Tg; (b) $^{**}P < 0.01$ PI3Kγ^{WT} *versus* PI3Kγ^{WT}/βENaC-Tg, and $P < 0.01$ PI3Kγ^{KO} *versus* PI3Kγ^{WT}/βENaC-Tg, $^{***}P < 0.001$ PI3Kγ^{WT}/βENaC-Tg *versus* PI3Kγ^{KO}/βENaC-Tg mice. (c) Representative histological sections from the lung of 8-week-old βENaC-Tg mousee (left) showing evident areas of emphysema and PI3Kγ^{KO}/βENaC-Tg (right) mouse showing a focal areas of mild emphysema. Haematoxylin and eosin stain. Original magnification ×40.

of the PI3Kγ inhibitor AS-605240 on airway inflammation in βENaC-Tg mice; we decided to use AS-605240 for its well characterized *in vivo* profile of efficacy and selectivity, indicated by the so far largest number of reports of pharmacological PI3Kγ inhibition in mice [48–53]. We showed that treatment with the PI3Kγ inhibitor decreased the number of neutrophils in BALF of βENaC-Tg mice, thus reproducing the effect observed with the genetic deletion of PI3Kγ. Several technical problems limit the assessment of the increased PI3Kγ activity in βENaC mice; however, the findings that PI3Kγ^{WT}/βENaC-Tg inflamed lungs have more leukocytes than PI3Kγ^{KO}/βENaC-Tg controls are an indirect indication of increased PI3Kγ activity in these mice. Taken together, our data demonstrate the biological efficacy of both genetic deletion and pharmacological inhibition of PI3Kγ in reducing chronic neutrophilic inflammation in CF-like lung disease *in vivo*.

Whereas blockade of PI3Kγ activity by small-molecule inhibitors may represent a valid approach to modulate excessive leukocyte accumulation in inflamed tissues where

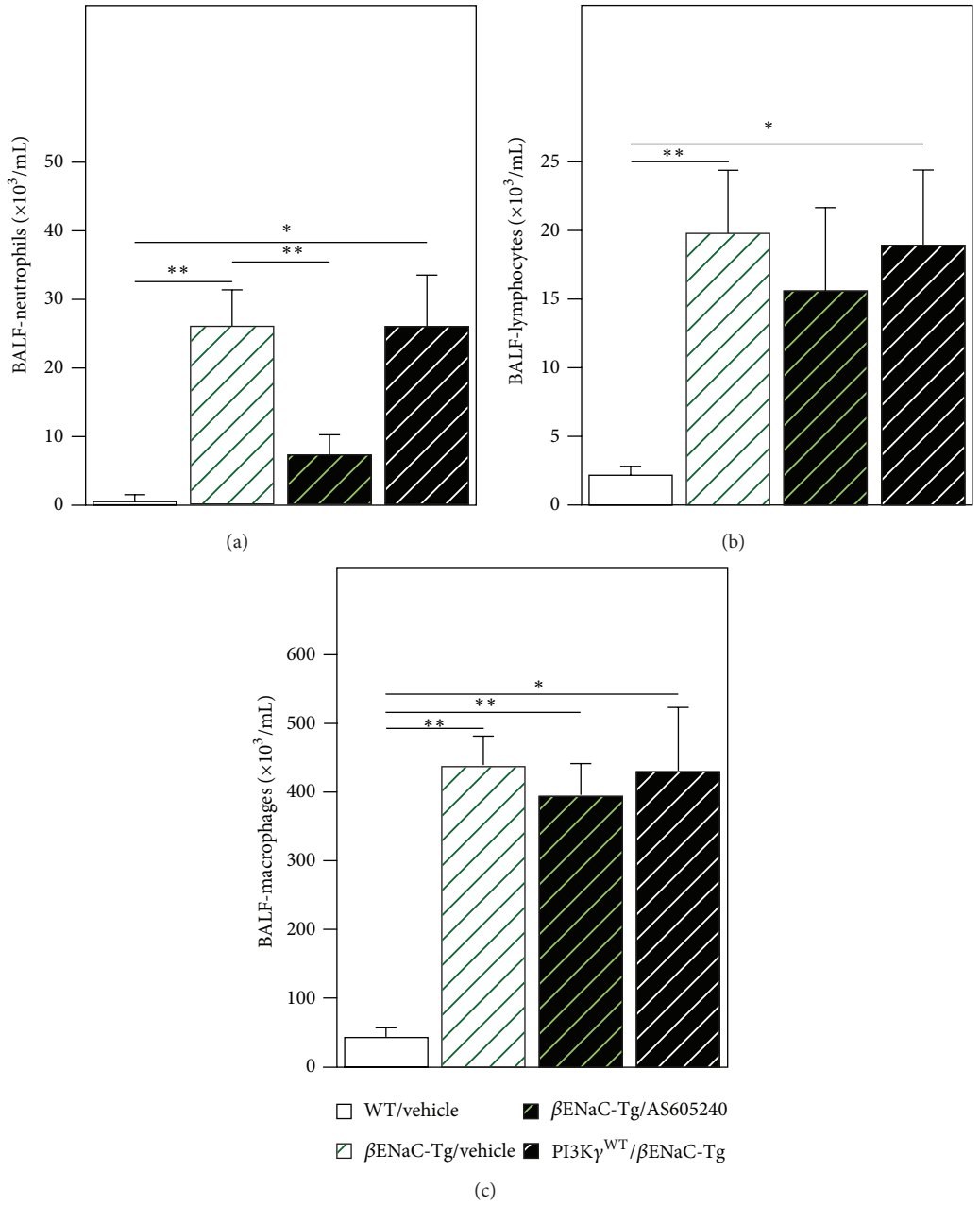

Figure 3: *Pharmacological inhibition of PI3Kγ decreases neutrophilic airway inflammation in βENaC-Tg mice*. Neutrophils (a), lymphocytes (b), and macrophages (c) numbers were determined in BAL fluid of control (WT/Vehicle) and βENaC-Tg mice untreated or treated with the PI3Kγ inhibitor AS-605240 (βENaC-Tg/AS-605240) or with vehicle (βENaC-Tg/Vehicle). Cells are expressed as number per mL of BAL fluid. (a) $^{**}P < 0.01$ WT/Vehicle *versus* βENaC-Tg/Vehicle, $^{**}P < 0.01$ βENaC-Tg/Vehicle *versus* βENaC-Tg/AS-605240 and $^{*}P < 0.05$ WT/Vehicle *versus* PI3Kγ^{WT}/βENaC-Tg. (b) $^{**}P < 0.01$ WT/Vehicle *versus* βENaC-Tg/Vehicle and $^{*}P < 0.05$ WT/Vehicle *versus* PI3Kγ^{WT}/βENaC-Tg. (c). $^{**}P < 0.01$ WT/Vehicle *versus* βENaC-Tg/Vehicle, $^{**}P < 0.01$ WT/Vehicle *versus* βENaC-Tg/AS-605240, and $^{*}P < 0.05$ WT/Vehicle *versus* PI3Kγ^{WT}/βENaC-Tg.

leukocyte recruitment is correlated with disease progression, on the other hand increased susceptibility to infection might be a potential side effect of the use of these molecules. In this context, a previous study [54] showed that either gene deletion or pharmacologic inhibition of PI3Kγ in mice infected with *S. pneumoniae* caused an impaired exudate macrophage recruitment associated with a reduced lung pneumococcal clearance and an impaired resolution/repair process, leading to progressive pneumococcal pneumonia. Thus, whereas pharmacological inhibition of PI3Kγ, eventually in association with antibacterial treatment, may be a viable strategy to inhibit chronic inflammation and limit lung damage in stable CF lung disease, it might have adverse effects on host defense in acute infections when high bacterial

burden occurs. In view of a clinical application of PI3Kγ inhibitors, target validation will be an important future aspect to discriminate between specific effects of the drug and potential side effects.

5. Conclusions

Neutrophil-dominated airway inflammation has been implicated as a key feature of progressive lung damage in CF. Thus, reducing airway inflammation is a major goal to prevent lung damage and maintain lung function in CF. Current therapeutic strategies that aim to reduce chronic neutrophilic inflammation in the airways of CF patients have been largely unsuccessful. This study shows that genetic deletion and pharmacological inhibition of PI3Kγ decrease neutrophilic airway inflammation and structural lung damage in a mouse model of CF lung disease. These results provide insight into the molecular mechanisms of chronic airway inflammation and suggest a novel treatment strategy to reduce inflammation and lung damage in patients with CF and potentially other neutrophilic airway diseases. Further studies with emerging PI3Kγ inhibitors [49–51] are required to confirm the efficacy of these molecules and exclude their potentially adverse effects on host defense.

Disclosure

Emilio Hirsch and Virginia De Rose are co-senior authors.

Conflict of Interests

Emilio Hirsch has equity ownership in Kither Biotech S.r.l. which is developing products related to the research being reported. Marcus Mall is inventor of a patent filled by the University of North Carolina and related to βENaC transgenic mice. All other authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Maria Galluzzo and Elisa Ciraolo contributed equally to this work. Gerd Doring is deceased.

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Research Article

Changes of Proteases, Antiproteases, and Pathogens in Cystic Fibrosis Patients' Upper and Lower Airways after IV-Antibiotic Therapy

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Background. In cystic fibrosis (CF) the upper (UAW) and lower airways (LAW) are reservoirs for pathogens like *Pseudomonas aeruginosa*. The consecutive hosts' release of proteolytic enzymes contributes to inflammation and progressive pulmonary destruction. Objectives were to assess dynamics of protease : antiprotease ratios and pathogens in CF-UAW and LAW sampled by nasal lavage (NL) and sputum before and after intravenous- (IV-) antibiotic therapy. *Methods*. From 19 IV-antibiotic courses of 17 CF patients NL (10 mL/nostril) and sputum were collected before and after treatment. Microbiological colonization and concentrations of NE/SLPI/CTSS (ELISA) and MMP-9/TIMP-1 (multiplex bead array) were determined. Additionally, changes of sinonasal symptoms were assessed (SNOT-20). *Results*. IV-antibiotic treatment had more pronounced effects on inflammatory markers in LAW, whereas trends to decrease were also found in UAW. Ratios of MMP-9/TIMP-1 were higher in sputum, and ratios of NE/SLPI were higher in NL. Remarkably, NE/SLPI ratio was 10-fold higher in NL compared to healthy controls. SNOT-20 scores decreased significantly during therapy ($P = 0.001$). *Conclusion*. For the first time, changes in microbiological patterns in UAW and LAW after IV-antibiotic treatments were assessed, together with changes of protease/antiprotease imbalances. Delayed responses of proteases and antiproteases to IV-antibiotic therapy were found in UAW compared to LAW.

1. Introduction

Cystic fibrosis (CF) is the most common lethal autosomal recessive inherited chronic disease in the Caucasian population and is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*CFTR*, 7q31). Defective ion channels lead to production of viscous secretions from exocrine glands. In CF, the innate immunity is ineffective because of impaired mucociliary clearance and immune cellular causes [1]. This allows chronic pathogen colonization and in airway, inflammation which results in progressive pulmonary destruction as main reason for increased morbidity and mortality in CF [2–5]. Pathogen colonization with *Staphylococcus (S.) aureus* and *Haemophilus influenzae* commonly begins in the first few months of life [6]. Later on, gram-negative organisms dominate, as *Pseudomonas (P.) aeruginosa* which chronically colonizes the lungs of 70–80% of adult CF patients [7]. *P. aeruginosa* enhances inflammation

in CF airways, for example, by causing the release of different proinflammatory and immunological active components, promoting secretion of mucus and impairing ciliary function [8].

Inflammation in CF airways is neutrophil-dominated; thus high levels of the proteolytic enzyme neutrophil elastase (NE) and oxidants can be found in the airway surface liquid [13]. At the same time enzymes with protective function in CF airways like α_1-antitrypsin and secretory leukocyte protease inhibitor (SLPI) can be inactivated by NE [39]. Furthermore, NE can enhance pulmonary inflammation and destruction by degrading extracellular matrix components. NE also serves as a biomarker for inflammation in CF [2, 19, 40–42]. The release of NE by neutrophils can be stimulated upon different cytokines and chemoattractants, for example, TNF and IL-8 [43]. High concentrations of NE and IL-8 in the airway surface liquid overwhelm and inactivate the antiprotease defense system, deranging the balance of proteases and antiproteases which is required for equilibration of defense mechanisms and prevention of tissue damage [8]. Recently, concentrations of NE in CF patients' lower were found to be elevated, compared to concentrations in the upper airways (LAW/UAW) [16]. In the UAW especially the serine protease SLPI is a major antagonist of NE. Contained in mucosal lining fluids SLPI is produced by macrophages, neutrophils, and epithelial cells of the respiratory and alimentary tract. Due to its high cationicity, SLPI can disrupt microbial membranes, affecting opportunistic pathogens in the lungs such as *S. aureus* and *P. aeruginosa* as well as skin pathogens, for example, *S. epidermidis* and *Candida albicans*, to become established [12, 44]. Increased concentrations of SLPI can be found in infection, for example, in pneumonia, whereas downregulation is triggered by interferon-gamma (IFN-γ) [45]. Elevated ratios of NE/SLPI in CF-UAW compared to LAW have been reported previously by Hentschel et al. assuming a greater benefit of NE inhibitors in the sinonasal than in the pulmonary compartment based on a more pronounced imbalance, than for the MMP-9/TIMP-1 ratio [16].

Furthermore, proteolytic active mediators, such as human cysteine cathepsins, are involved in lung injury and tissue remodelling in CF patients' pulmonary inflammation. So far, increased levels of cathepsins were found in sputum of CF patients, allowing their use as inflammation markers [9]. The cathepsins, including cathepsin S (CTSS), are produced by macrophages and are involved in matrix remodeling and antigen processing [10]. The acid pH-value of the airway surface liquid in CF provides an optimal condition for their activity [11]. Cathepsins cleave and inactivate antimicrobial peptides or proteins such as SLPI, which leads to an inactivation of SLPI anti-NE capacity [12].

Altogether, chronic inflammation in the CF airways is characterized by an imbalance of proteases and antiproteases, such as NE and SLPI or MMP-9 and tissue inhibitor of metalloproteinase-1 (TIMP-1). MMP-9 as a biological active enzyme is known to be released, especially in the airways, by neutrophils, macrophages, and epithelial cells in response to inflammation and takes part in the remodeling and degradation of extracellular matrix proteins [4, 13, 14]. Particularly in chronic lung disease, asthma, bronchopulmonary dysplasia, and α_1-antitrypsin deficiency, this imbalance and an overproduction of MMP-9 play an important role in the pulmonary pathogenesis [13, 15]. So far, MMP-9 and TIMP-1 as its major physiological inhibitor by forming specific complexes with pro-MMP-9 were determined in the bronchopulmonary compartment of CF patients. Elevated levels of MMP-9 and TIMP-1 as well as an increase in MMP-9/TIMP-1 ratio have been reported previously in NL fluid, sputum, and bronchoalveolar lavages (BAL) [13, 16–18].

MMP-9 is known as the predominating MMP in bronchopulmonary secretions from CF patients. This may be due to the ability of NE to cleave and activate MMP-9 as well as to inactivate TIMP-1 [13, 15]. Moreover, in healthy subjects' induced sputum, higher levels of TIMP-1 were detectable when compared to CF patients, which emphasizes the relative lack of antiproteases in CF lungs [19]. As previously described from the bronchopulmonary compartment of stable CF patients, increased NE in sputum is related to increased MMP-9/TIMP-1 ratio and the implication of this an imbalance on proteolytic dysregulation has been discussed [19]. Additionally, we have recently described a correlation of TIMP-1 and MMP-9 to *P. aeruginosa* colonization of CF patients' airways [20].

At the same time, the impaired mucociliary clearance also has a considerable effect on the patients' UAW and paranasal sinuses, frequently causing chronic rhinosinusitis (CRS) and nasal polyps [21]. As a consequence, symptoms like chronic nasal congestion, rhinorrhoea with anterior and postnasal drip, mouth breathing, anosmia, facial pain, and sleep disorders affect the quality of life (QoL) [22]. Approaches including medical therapy and extensive endoscopic sinus surgery are measures to improve sinonasal disease in CF [23]. Beyond that, the defective sinonasal mucociliary clearance makes the UAW a gateway for primary pathogen colonization and a reservoir for descending infection of the lower respiratory tract [24–26]. So far, a recent series of studies found concordant strains of *P. aeruginosa* in the UAW and LAW of CF patients. Consequently, the authors postulate to treat the UAW and LAW as one airway system [21, 27, 28]. In this regard, early detection of pathogen colonization and an effective eradication by antibiotic prophylaxis or therapy may prevent subsequent descent to the LAW or exacerbations [29].

In order to preserve a good pulmonary function and to improve the QoL, particularly the treatment against a chronic infection with *P. aeruginosa* is a main focus of attention in CF care. There is evidence that a systemic intravenous- (IV-) antibiotic therapy, either applied in a more preventive elective regimen or applied symptomatically at acute pulmonary exacerbations (APE), combined with long-term nebulized antibiotic therapy benefits CF patients chronically colonized with *P. aeruginosa* [29]. The elective IV-antibiotic treatment of colonized patients for eradication and/or reduction of the pathogen burden and the resulting pulmonary inflammation belong to standards of care in many European CF centers [30, 31]. However, regimes vary regarding duration and dosage of

therapy and there is no final evidence for superiority of one concept [30].

Previous studies of our group compared proteases/antiproteases relations in the UAW and LAW in a cross-sectional study [16].

The main purpose of the present longitudinal study was to analyze changes of proteases and antiproteases in sputum and NL together with changes in pathogens detected with conventional microbiological tools in both the upper and lower airway compartments. Levels of MMP-9, TIMP-1, SLPI, NE, and CTSS were quantified in NL and sputum from CF patients before and after a 14-day IV-antibiotic therapy and compared to results from healthy controls. Furthermore, we assessed the impact of the treatment on sinonasal symptoms (health-related QoL). We hypothesized that in CF patients' UAW and LAW non-invasively assessed by NL and sputum chronic imbalance of proteases and antiproteases can be adjusted after a 14-day IV-antibiotic therapy.

2. Patients, Materials, and Methods

2.1. Study Population. The prospective case control study conducted at the Jena University Hospital CF Center, Germany, included 17 CF patients who underwent 19 IV-antibiotic treatments between August 2012 and January 2013. Inclusion criteria were a diagnosis of CF confirmed by two positive sweat tests and/or a molecular genetic identification of two disease-causing *CFTR* mutations. Exclusion criteria were relevant nasal bleeding and perforation of the tympanum in general.

IV-antibiotics were administered in accordance with the current European guidelines [3] with two agents (e.g., aminoglycoside and cephalosporin or carbapenem) for 14 days. The selection of antibiotics was based on antibiotic sensitivity of pathogens cultured in sputum. NL of 20 prospectively enrolled healthy subjects served as control regarding inflammatory mediators without intervention.

Sputum samples and NL from all CF patients were collected at baseline and after approximately 14 days of treatment. Additionally, all CF patients underwent routine spirometry and biochemical blood analysis prior to therapy, according to the clinical standards in the Jena CF Center. Furthermore, patients and healthy subjects were assessed for UAW-related symptoms and health-related QoL by the Sinonasal Outcome Test 20 in its German Adapted Version (SNOT-20-GAV).

The study was approved by the Ethics Committee of the Faculty of Medicine, University of Jena, Germany (reference number: 2909/08-10). Written informed consent was obtained from each subject or their parental guardians.

2.2. Nasal Lavage. NL, using 10 mL of sterile isotonic saline (0.9% NaCl, Braun, Melsungen, Germany) per nostril, was performed as described previously [32]. Immediately after collection, NL fluid was either aliquoted with and without protease inhibitor (PI) (Protease Inhibitor Mix G, SERVA Electrophoresis GmbH, Heidelberg, Germany) or centrifuged for 7 min at 400 rpm. Supernatants were aliquoted with and without PI and frozen at −70°C. For cytological analysis, 5 mL of NL was added to 0.5 mL fetal calf serum (FCS, Biochrom AG, Berlin, Germany). The suspension was centrifuged for 7 min at 400 rpm. Supernatant was discarded leaving 1 mL for resuspension of the cell pellet. 100 μL of FCS was added.

2.3. Sputum. Sputum samples were collected from patients by spontaneous expectoration. Immediately after collection, samples were diluted with four times the sputum volume of sterile phosphate buffered saline (PBS) and homogenized. Afterwards, four times the sputum volume of freshly prepared dithiothreitol (DTT) and 0.2 mL/g sputum of DNase (Roche, Basel, Switzerland) were added, vortexed for 30 seconds, and filtered. 100 μL of FCS was added to 1 mL of the suspension for cytological analysis. The filtrated suspension was centrifuged for 7 min at 400 rpm. Supernatants were aliquoted with and without PI and frozen at −70°C [33].

2.4. Microbiology. Microbial analyses of NL and sputum collected before and after IV-antibiotic therapy were performed according to European standards. Chronic colonization was stated using the criteria published by Lee et al. [34]. The following bacteria frequently found in NL and sputum cultures were considered as part of the physiological flora of the human nasopharynx: *Neisseria* spp., alpha-hemolytic streptococci, coagulase-negative staphylococci, corynebacteria spp., stomatococci, and nonhemolytic streptococci [35, 36].

2.5. Cytology and Protein Concentrations. The analysis of total cell counts (TCC) and the automated cell differentiation were performed using fluorescence flow cytometry (Sysmex XE-5000, Sysmex Deutschland GmbH, Norderstedt, Germany) in Body Fluid Modus. For cytological differentiation (100 cells), cytospin preparations (100 ×g, 3 min) were prepared. Levels of total protein were measured using 3 μL of NL and supernatants of sputum on an LVis Plate (SPECTROstar Omega, Omega-Data Analysis, BMG Labtech, Ortenberg, Germany) at 280 nm wavelength.

2.6. Inflammatory Mediators

2.6.1. Multiplexed Immunoassays. Concentrations of MMP-9 and TIMP-1 (Milliplex MAP Kit, Millipore Corporation, Billerica, USA, Human MMP Panel 2 number HMMP2MAG-55K, Human TIMP Panel 1 number HTMP1MAG-54K) were measured by applying multiplexed immunoassays according to the manufacturers' instructions. In brief, all different antibody-coated beads were incubated with 25 μL of NL or sputum. Sputum was diluted using assay buffer (MMP-9 1 : 20, TIMP-1 1 : 4). For detection, antibodies and streptavidin were added. Samples were measured using Bio-Plex 200 System. Results were calculated by using Bio-Plex Manager 6.0.

2.6.2. ELISA. Analysis of NE, SLPI, and CTSS in NL and sputum was done in duplicate using ELISA according to the manufacturers' instructions (PMN Elastase ELISA, Milenia Biotec, Gießen, Germany, number MKEL1; SLPI ELISA,

number E91312Hu; CTSS ELISA, number E91933Hu, Uscn Life science Inc., Wuhan, China). Additionally, sputum was diluted 1 : 10 for NE and CTSS detection and 1 : 100 for SLPI with assay buffer. For washing an automated washer (SLT Typ Columbus, Labtechnologies, Austria) and for detection a spectrometer FluoStar Galaxy (BMG Labtechnologies, Offenburg, Germany) were used.

2.7. SNOT-20-GAV. The SNOT-20-GAV is a disease-specific 20-item survey on rhinological and general complaints as well as on QoL for patients with rhinosinusitis [37, 38]. Scores were assessed before and after IV treatment and range between 0 and 5 for each item, with higher scores indicating a greater health-related burden by rhinosinusitis. In this study, the SNOT overall score with all 20 items was included for evaluation.

2.8. Statistical Analysis. Data was evaluated using MS Excel, IBM SPSS 21.0, and Graph Prism 6. Longitudinal values of measured parameters were compared using Wilcoxon test. Data analysis was performed using descriptive statistics, including absolute and relative frequencies, mean and standard deviation, and median and range. Correlations between measured inflammatory markers in transverse sections and clinical or serological parameters were done using Spearman's Rho. Analyzed groups were compared performing Mann-Whitney U test. Statistical value of $P \leq 0.05$ was considered significant.

3. Results

3.1. Demographic Data. 17 CF patients (10 female/7 male, mean age 25.1 yrs, range 8–35) who attended in the Jena University Hospital CF Center, Germany, were included. Patients received either an elective routinely IV-antibiotic treatment (18/19) or an IV treatment for acute pulmonary exacerbation (APE) (1/19). Median duration between the first and second dates within the study resulted in 15 days (range 12–23 days). The 20 healthy controls (15 female/5 male) were aged 28.5 years by mean (range: 23–48 years).

5 of 17 patients fulfilled the criteria for chronic rhinosinusitis (CRS) according to EPOS 2012 criteria [46]. Sinonasal symptoms SNOT-20-GAV scores decreased significantly ($P = 0.001$) during therapy from a mean of 27.3 points (median = 26; range: 6–56 points) to 17.4 points (median = 19; range: 3–44 points) as seen in Figure 1; in contrast to CF patients prior to therapy the included healthy subjects stated a mean of 4.7 points (median = 3; range = 0–26; $P = 0.033$, $r = 0.489$).

Serological inflammation markers, for example, CRP and ESR, were determined only prior to IV therapy. No significant correlations between inflammatory mediators in sputum and NL and systemic inflammation markers were found. Further clinical and serological data of included patients are presented in Tables 1 and 2.

3.2. Microbiological Data. At inclusion date pathogenic bacteria and/or fungi were detected in 12 (63.2%) and 16 (84.2%)

Table 1: Clinical, microbiological, and serological characteristics of included patients and healthy controls.

Nominal variables	N	Absolute frequency
Cystic fibrosis patients		
Gender (female)	17	10 (58.8%)
Nasal polyps	17	5 (29.4%)
History of sinonasal surgery	17	6 (35.3%)
Chronic rhinosinusitis	17	5 (29.4%)
Allergy		
Aspergillus fumigatus	17	7 (41.2%)
House dust mite		3 (17.6%)
Grass pollen		2 (11.8%)
Pet hair (cat/dog)		2 (11.8%)/1 (5.9%)
ABPA	17	2 (11.8%)
Allergic rhinitis	17	2 (11.8%)
Diabetes mellitus	17	5 (29.4%)
IV-antibiotics		
Tobramycin	19	18 (94.7%)
Ceftazidime		9 (47.4%)
Tazobactam/piperacillin		4 (21.1%)
Colistin		1 (5.3%)
Meropenem		6 (31.6%)
Therapy		
Current azithromycin	17	12 (70.6%)
Current oral antibiotics		9 (52.9%)
Current inhalative antibiotics		16 (94.1%)
Recombinant DNAse		10 (58.8%)
Nasal topical bronchial steroids		8 (47.1%)
Current oral antimycotics		10 (58.8%)
Chronic colonization of UAW with[*1]		
P. aeruginosa permanent	17	4 (23.5%)
P. aeruginosa intermittent		4 (23.5%)
Chronic colonization of LAW with[*1]		
P. aeruginosa permanent	17	5 (29.4%)
P. aeruginosa intermittent		3 (17.6%)
UAW: detection of [*2]	19	12 (63.2%)
P. aeruginosa (mucoid)		6 (31.6%)
P. aeruginosa (nonmucoid)		4 (21.1%)
S. aureus		2 (10.5%)
MRSA		2 (10.5%)
LAW: detection of [*2]	19	16 (84.2%)
P. aeruginosa (mucoid)		9 (47.4)
P. aeruginosa (nonmucoid)		7 (36.8%)
S. aureus		3 (15.8%)
MRSA		2 (10.5%)
P. aeruginosa serum antibodies positive:	16	8 (50.0%)
Alkaline protease/borderline		8 (50%)/2 (12.5%)
Exotoxin A/borderline		9 (56.3%)/1 (6.3%)
Elastase/borderline		9 (56.3%)/1 (6.3%)
Healthy controls		
Gender (female)	20	15 (75%)
Allergic rhinitis		1 (5%)
Allergy in general		4 (20%)
Postnasal drip		4 (20%)
History of ORL surgery		5 (25%)
Snore		3 (15%)

[*1]Permanent and intermittent colonization were stated using the criteria published by Lee et al. [34]. Chronic colonization is defined if 50% or more of cultures within the last year were found positive and intermittent if less than 50% of cultures within the last year were found positive.
[*2]At inclusion date.

Table 2: Clinical and serological characteristics of included patients and healthy controls.

Metric and ordinal variables	N	Mean ± SD	Median	Range
Age (yrs)	17	25.5 ± 7.1	25.0	8–35
BMI (kg/m^2)	17	19.5 ± 3.6	19.2	14.7–29.3
FEV1 (l)/(% predicted)	17	1.9 ± 1.3 (57.9 ± 38.1)	1.3 (38.1)	0.8–5.7 (25–141)
MEF75/25 (l)/(% predicted)	13	1.4 ± 1.4 (36.9 ± 40.4)	0.7 (19.0)	0.3–4.6 (8.0–139.8)
ESR (mm/h)	18	34.3 ± 26.0	24.0	2–85
CRP (mg/l)	19	18.6 ± 30.5	5.7	0.5–108.1
Total IgG (g/l)	19	16.5 ± 4.7	17.4	9.5–27.8
Total IgE (kU/l)	18	324.3 ± 529.0	62.7	6.4–1568
SNOT-GAV-20				
Prior to therapy	19	27.3 ± 13.7	26.0	6–56
After therapy	19	17.4 ± 10.6	19.0	3–44
		Healthy controls		
Age (yrs)	20	28.2 ± 7.4	25.0	23–48
BMI (kg/m^2)		21.9 ± 3.1	21.0	17.7–28.3
SNOT-20-GAV		4.7 ± 11.8	3.0	0–26

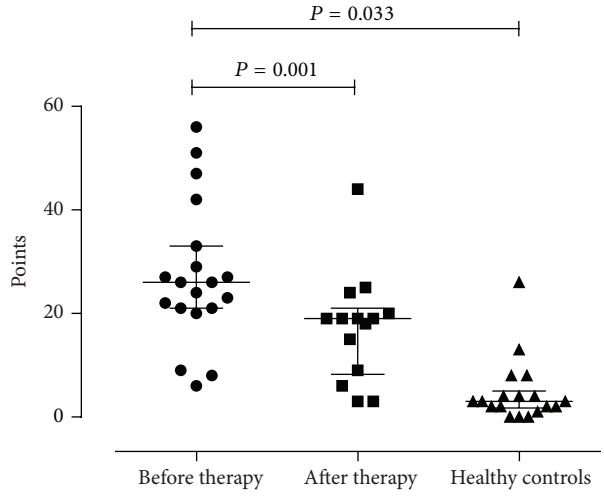


Figure 1: Comparison of SNOT-20-GAV scores before and after IV-antibiotic therapy in CF and in healthy controls. In CF a significant decrease was shown during therapy from a median of 26 points to 19 points ($P = 0.001$) which was still elevated (n.s.) compared to healthy controls (median: 3 points).

out of 19 patients for NL and sputum and at exclusion date in 10 (52.6%) and 11 patients (57.9%). *P. aeruginosa* was the most commonly cultured bacterium detected in both the upper and lower airways before therapy. 42.1% of NL and 52.6% of sputum samples revealed the pathogen prior to therapy and detection rates declined to 36.8% for both sputum and NL after therapy. Whereas *S. aureus* including *MRSA* was less frequent in sputum samples (21.1% of NL and 26.3% in sputum) before therapy, none were detectable after therapy. *E. coli* was detected in 4 patients. Culture-based microbiological findings of both patients and controls before and after therapy are displayed in Table 3.

Chronic colonization of the UAW or LAW with *P. aeruginosa* was found in 4 (23.5%) and 5 (29.4%), respectively, out of 17 patients; those who were intermittently infected were 4 (23.5%) and 3 (17.6%), respectively, patients [34]. Further data are shown in Table 1. In four patients chronic colonization status could not be determined for lack of data.

3.3. Cytological Data and Protein Concentrations. TCC was assessed for all patients before and after therapy and decreased in both NL and sputum during therapy. However, TCC in UAW did not differ significantly between CF patients and healthy controls (Figure 2(a)). Again, the decrease of the median TCC after IV-antibiotic therapy was statistically significant only in sputum ($P = 0.005$; see Figure 2(b)). Significant positive correlations were found between TCC and MMP-9 ($r = 0.805^{\mathrm{UAW1}}$, $P < 0.001$; $r = 0.620^{\mathrm{UAW2}}$, $P = 0.008$) before and after IV therapy. Changes of TCC correlated significantly with changes of protein ($r = 0.706^{\mathrm{LAW1}}$, $P = 0.013$; $r = 0.846^{\mathrm{LAW2}}$, $P = 0.001$) at both time points. Interestingly, only after IV therapy TCC correlated significantly with MMP-9 ($r = 0.620^{\mathrm{UAW2}}$, $P = 0.008$) and protein ($r = 0.586^{\mathrm{UAW2}}$, $P = 0.017$; $r = 0.846^{\mathrm{LAW2}}$, $P = 0.001$) in both airways.

A decline of the median protein concentrations during IV-antibiotic treatment was seen for both airways (Figures 2(c) and 2(d)). However, statistical significance ($P = 0.008$) was reached only for the LAW. In healthy controls protein concentrations in NL resulted to be similar to CF patients. Changes of protein concentrations and cytology in the UAW and LAW as well as the results of healthy controls are summarized in Table 4.

3.4. Standardization by TCC and Protein. For standardization of the immunological markers, we divided the measured values by concentrations of TCC and protein. Calculated values did not differ significantly when related to protein concentrations. Therefore, we can exclude protein concentrations as bias. In contrast, normalization by TCC resulted

Table 3: Culture-based detection of pathogens in UAW and LAW before and after IV-AB therapy.

	Age/gender	Site	Before therapy	After therapy
Pat. 1	35 yrs/m No chronic colonization evaluable due to lack of data	UAW	*P. aeruginosa* (mucoid) *S. aureus*	*P. aeruginosa* (mucoid) *P. aeruginosa*
		LAW	*P. aeruginosa* (mucoid) *S. aureus* *Enterococcus aureus*	*P. aeruginosa* (mucoid) Yeast
Pat. 2	32 yrs/f	UAW	*MRSA*	n.m.
		LAW	*MRSA* *Aspergillus flavus*	Culture negative
Pat. 3	30 yrs/f Permanent P.a.$^+$ UAW/LAW Intermittent S.a.$^+$ UAW/LAW (MRSA)	UAW	*P. aeruginosa* (mucoid) *MRSA* *Klebsiella oxytoca*	*P. aeruginosa* (mucoid) *P. aeruginosa*
		LAW	*P. aeruginosa* (mucoid) *P. aeruginosa* *MRSA* *Enterococcus* spp.	*P. aeruginosa* (mucoid) *P. aeruginosa* Yeast
Pat. 4	30 yrs/m*	UAW	*E. coli*	*P. aeruginosa* (mucoid) *P. aeruginosa*
		LAW	*P. aeruginosa* (mucoid) *P. aeruginosa* *S. aureus* *E. coli*	*P. aeruginosa* (mucoid) *P. aeruginosa* Yeast
Pat. 5	27 yrs/m Permanent P.a.$^+$ UAW/LAW Permanent S.a.$^+$ LAW Intermittent S.a.$^+$ UAW	UAW	*P. aeruginosa* (mucoid) *S. aureus* *Serratiamarcescens*	*Comamonas testosteroni*/*P. alcaligenes*
		LAW	*P. aeruginosa* (mucoid) *S. aureus*	*P. aeruginosa* (mucoid) Yeast
Pat. 6	25 yrs/f Intermittent P.a.$^+$ LAW	UAW	Culture negative	Culture negative
		LAW	*P. aeruginosa* (mucoid) *P. aeruginosa* *E. coli*	*P. aeruginosa* *E. coli*
Pat. 7	23 yrs/f Intermittent P.a.$^+$ UAW/LAW	UAW	Culture negative	*P. aeruginosa*
		LAW	Culture negative	Culture negative
Pat. 8	23 yrs/f Permanent S.a.$^+$ UAW/LAW	UAW	Culture negative	Culture negative
		LAW	Yeast	Yeast
Pat. 9	18 yrs/m*	UAW	n.m.	Culture negative
		LAW	*S. aureus*	Culture negative
Pat. 10	35 yrs/f Permanent P.a.$^+$ LAW Intermittent P.a.$^+$ UAW	UAW	*P. aeruginosa*	n.m.
		LAW	*P. aeruginosa* (mucoid) *P. aeruginosa* Yeast	n.m.
Pat. 11	31 yrs/f Intermittent S.a.* LAW?	UAW	*E. coli* *Proteus mirabilis*	*E. coli*
		LAW	*E. coli* *Proteus mirabilis* *Aspergillus fumigatus* Yeast	*E. coli* *Aspergillus fumigatus* *P. fluorescens*
Pat. 12	15 yrs/f Intermittent P.a.$^+$ UAW/LAW Permanent S.a.$^+$ UAW/LAW	UAW	*P. aeruginosa*	Culture negative
		LAW	*P. aeruginosa*	Culture negative
Pat. 13	25 yrs/f Permanent P.a.$^+$ UAW/LAW	UAW	*P. aeruginosa* (mucoid) *Enterococcus faecalis*	Culture negative
		LAW	*P. aeruginosa* (mucoid) *P. aeruginosa* *Aspergillus fumigatus*	Culture negative

Table 3: Continued.

	Age/gender	Site	Before therapy	After therapy
Pat. 14	30 yrs/m Permanent P.a.$^{+}$ UAW/LAW	UAW	*E. coli* *P. fluorescens*	n.m.
		LAW	*E. coli* *P. fluorescens* *S. viridans* Yeast	*Aspergillus fumigatus* *Klebsiella oxytoca* *P. putida* *Enterobacter cloacae* *Yeast*
Pat. 15	8 yrs/m Intermittent P.a.$^{+}$ UAW Permanent S.a.$^{+}$ UAW/LAW	UAW LAW	Culture negative *Haemophilus parainfluenzae*	Culture negative Culture negative
Pat. 16	22 yrs/f*			
	Course 1	UAW	*P. aeruginosa* (mucoid) *P. aeruginosa*	*P. aeruginosa* (mucoid) *P. aeruginosa*
		LAW	*P. aeruginosa* (mucoid) *P. aeruginosa* *Aspergillus fumigatus*	*P. aeruginosa* (mucoid) *P. aeruginosa*
	Course 2	UAW	n.m.	*P. aeruginosa* (mucoid) *P. aeruginosa*
		LAW	n.m.	*P. aeruginosa* *Streptococcus pneumoniae*
Pat. 17	24 yrs/m*			
	Course 1	UAW	*P. aeruginosa* (mucoid) *P. aeruginosa*	*P. aeruginosa* (mucoid) *P. aeruginosa* Enterococci
		LAW	*P. aeruginosa* (mucoid) Yeast	Yeast
	Course 2	UAW	n.m.	n.m.
		LAW	n.m.	n.m.

f = female, m = male, and n.m. = not measured.
*Chronic colonization status not evaluable due to lack of data.

Table 4: Changes of protein concentrations and cytology before and after IV-AB treatment and comparison to findings in healthy controls.

Analyte (unit)	Concentrations						P
	Median			Range			
	Controls	CF prior to therapy	CF after therapy	Controls	CF prior to therapy	CF after therapy	
NL (UAW)							
Total protein (mg/mL)	0.26	0.38	0.32	0.14–0.51	0.12–0.61	0.08–0.69	0.734* 0.073°
TCC (TCC/μL)	19	36	22	2–95	5–433	3–259	0.178* 0.078°
PMN (%)	78.5	69	88	42–100	0–90	45–100	0.011* 0.044°
MN (%)	21	31	12	0–58	10–100	0–55	0.012* 0.038°
Sputum (LAW)							
Total protein (mg/mL)		6.5	3.8		3.0–15.9	1.4–9.1	0.008*
TCC (TCC/μL)		3452	1272		400–21234	408–6788	0.005*
PMN (%)		84	88		26–95	39–96	0.636*
MN (%)		16	12		6–74	4–61	0.636*

$^{*}P$ value between CF prior to and after therapy; $^{\circ}P$ value between CF prior to therapy and healthy controls in UAW.

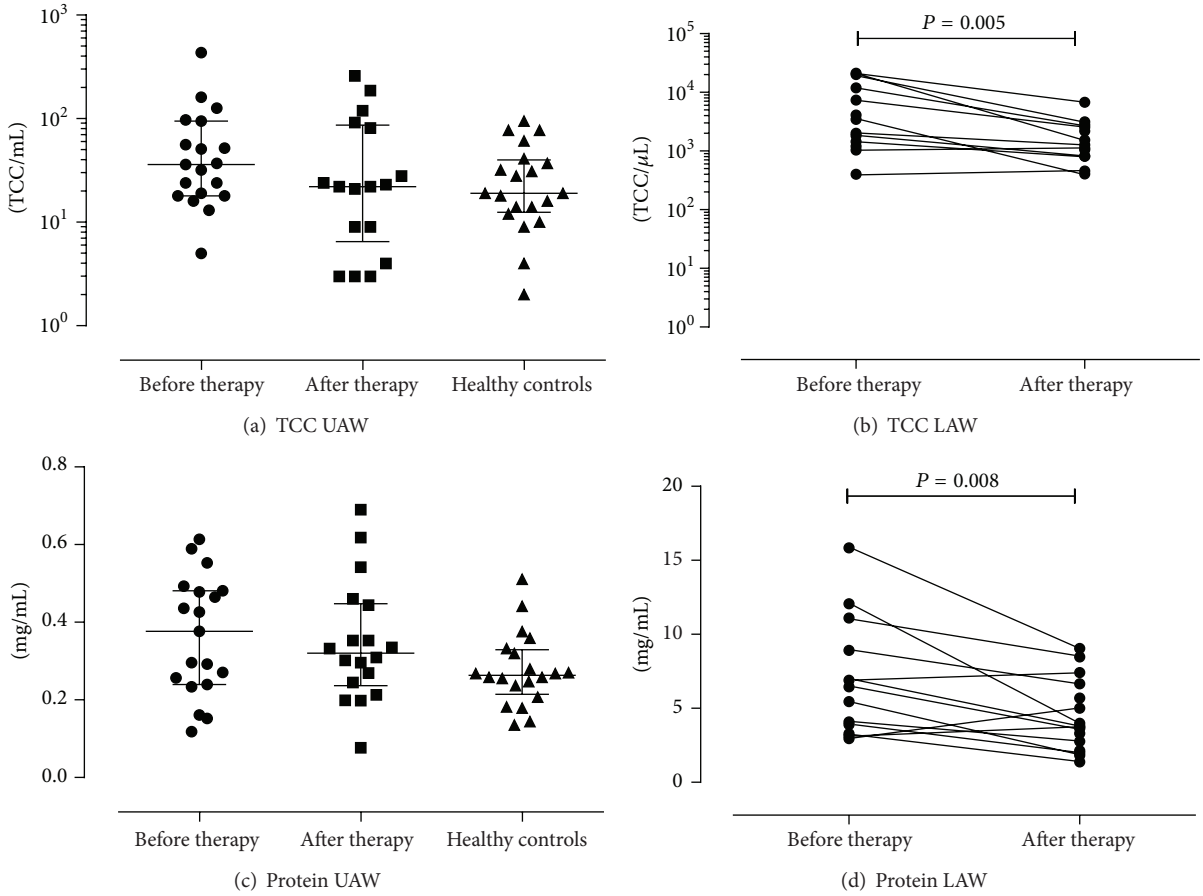


Figure 2: Changes of TCC and protein concentration. Both decreased in UAW and LAW after IV-antibiotic therapy, but only the decline in the LAW was shown to be statistically significant for TCC (b) and protein (d). Similar results were seen for CF patients' UAW and healthy controls (a + c).

in differences for all assessed parameters. Thus, cell count in secretions critically influences concentrations of inflammation markers in NL and sputum.

3.5. Analysis of Inflammation Markers in CF Patients before and after IV Antibiotic Therapy, Compared to Healthy Controls. Regularly detected inflammation markers in UAW were NE (Figure 3(a)), TIMP-1 (Figure 4(c)), and MMP-9 (Figure 4(a)). In contrast CTSS (Figure 5(b)), TIMP-1 (Figure 4(d)), and MMP-9 (Figure 4(b)) were found consistently in LAW, whereas NE (Figure 3(b)) was only detected in 61.5% before and in 81.3% after therapy. In NL of healthy controls NE was found regularly; CTSS was detected frequently in 85% of samples. In comparison to CF samples levels of NE (see Figure 3(a)) and CTSS in NL of healthy controls were significantly lower (1.66 ng/mL and 0.04 ng/mL, resp., in comparison to 73.39 ng/mL and 0.07 ng/mL, resp., $P < 0.001$ and $P < 0.001$, resp.). SLPI was hardly detected in the UAW of CF patients as well as in healthy subjects, being more often found in LAW (see Figure 5(a)). Frequencies of detection, detection limits, median, and ranges are listed in Table 5. Only TIMP-1 decreased significantly during antibiotic therapy in UAW from 1.83 ng/mL to 1.65 ng/mL ($P = 0.036$) as shown in Figure 4(c). In LAW a significant decrease of MMP-9 (1359.7 ng/mL to 1195.9 ng/mL; $P = 0.017$) was found (Figure 4(a)). The ratio of MMP-9/TIMP-1 appeared to decline as well in NL as in sputum but did not reach statistical significance (Table 5). A significant correlation was shown between NE and the MMP-9/TIMP-1 ratio in the UAW before and after therapy ($r = 0.681$, $P = 0.001$ and $r = 0.515$, $P = 0.035$, resp.). The NE/SLPI ratio was 10-fold higher in CF patients in comparison to healthy controls (Figure 6(c)); in both compartments no significant change after IV therapy was measurable due to fewer counts of ratios. Only a calculation of the SLPI/CTSS ratio for sputum samples was done as detection frequencies and values were too low in NL. Before and after treatment MMP-9 in NL correlated significantly with NE ($r = 0.587^{\text{UAW1}}$, $P = 0.008$ and $r = 0.501^{\text{UAW2}}$, $P = 0.029$). Only at inclusion a significant correlation between MMP-9 and TIMP-1 ($r = 0.605^{\text{UAW1}}$, $P = 0.006$) was detected.

4. Discussion

The airway system of CF patients is commonly infected with pathogens that cannot be effectively cleared due to the underlying ion channel defect and the resulting viscous secretions. The pathogens' virulence factors and the resulting

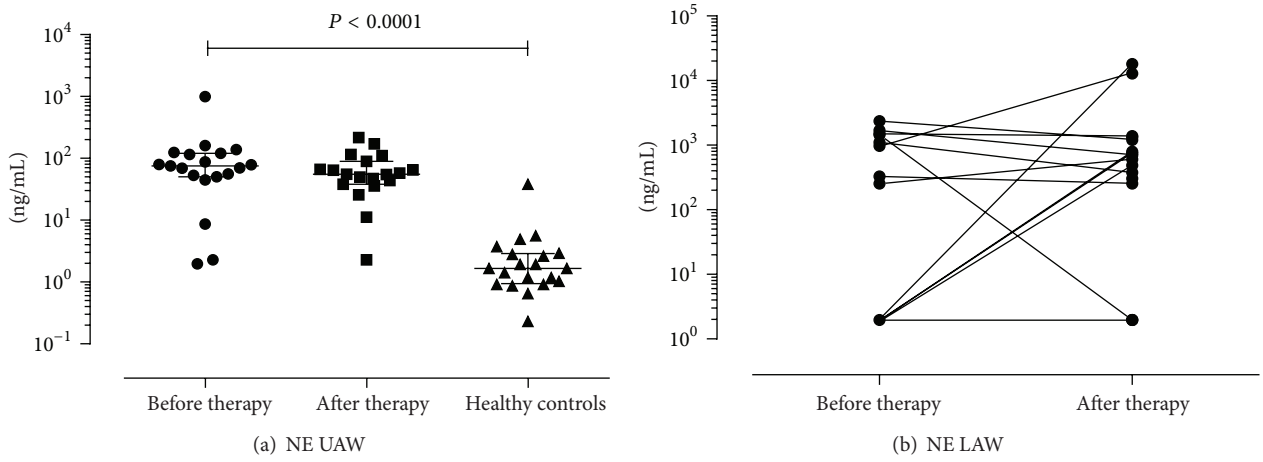


Figure 3: Changes of NE. Levels decreased in the UAW (a) after IV-antibiotic therapy. NE was statistically significantly lower in healthy controls compared to CF patients ($P < 0.0001$). In the LAW median levels increased after therapy, but five of seven matched pairs diminished after therapy (b).

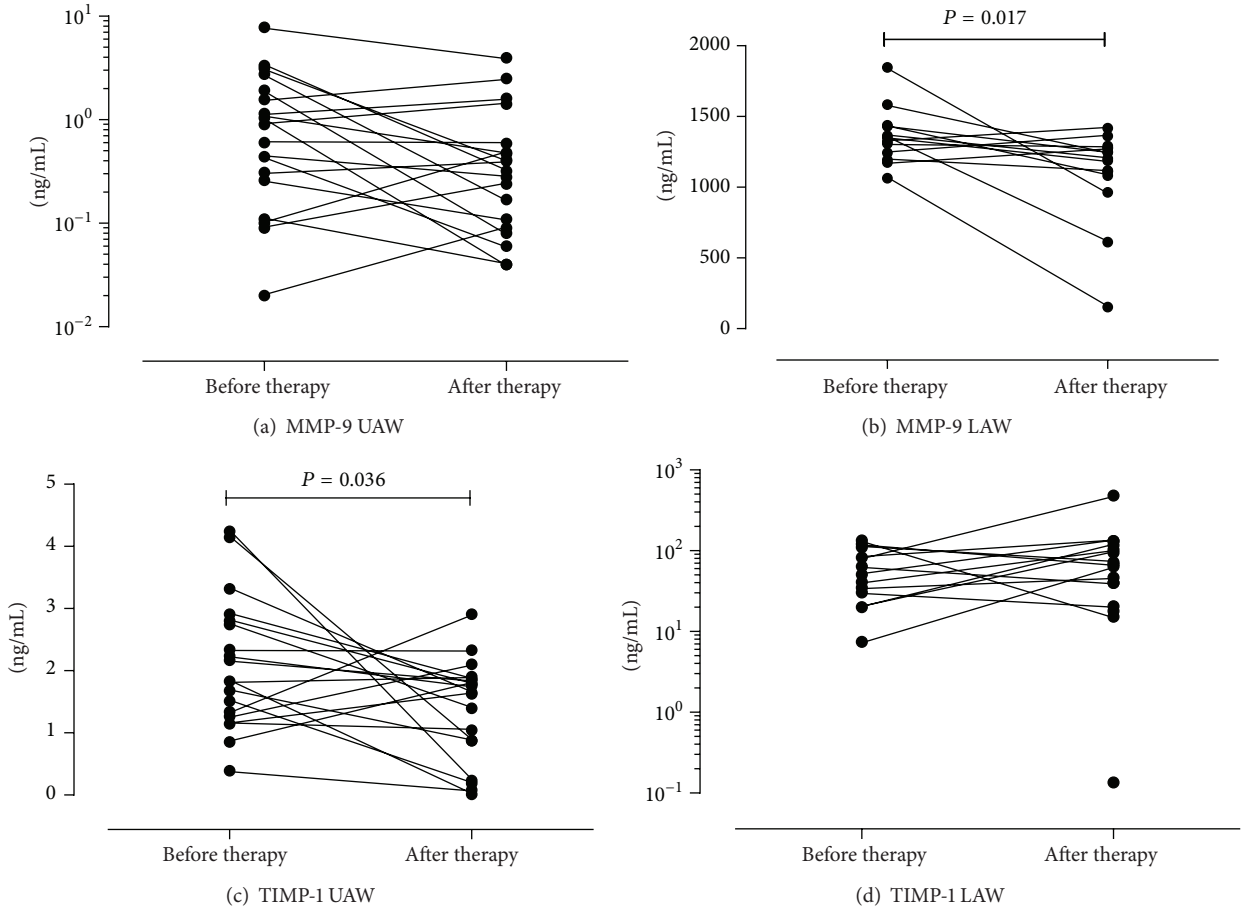
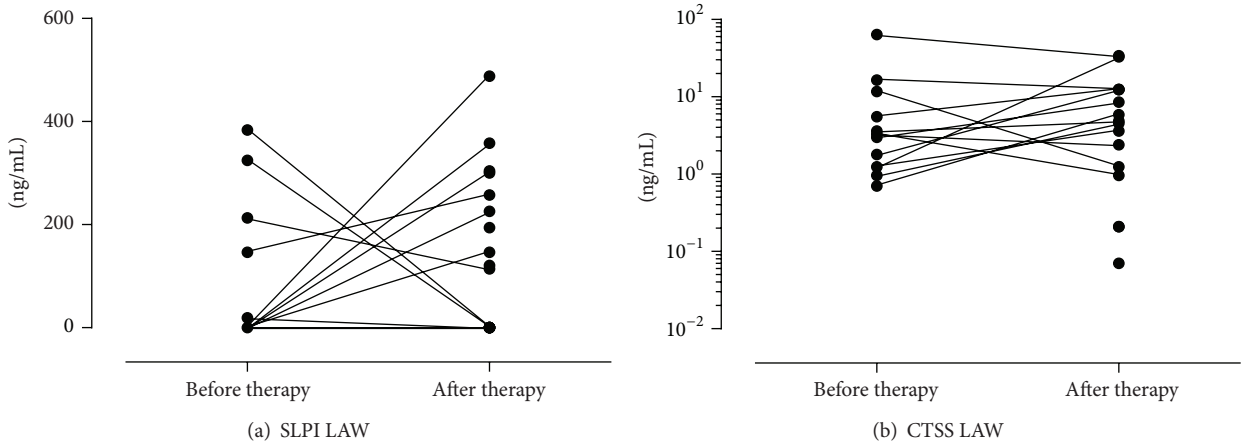


Figure 4: Changes of MMP-9 and its inhibitor TIMP-1. Levels of MMP-9 decreased in UAW (a) and LAW (b) after therapy; only changes in LAW reached statistical significance. Concentrations of TIMP-1 decreased significantly in the UAW (c) while levels increased in the LAW (d).

Table 5: Inflammation markers and ratios in controls and CF patients before and after IV-AB treatment.

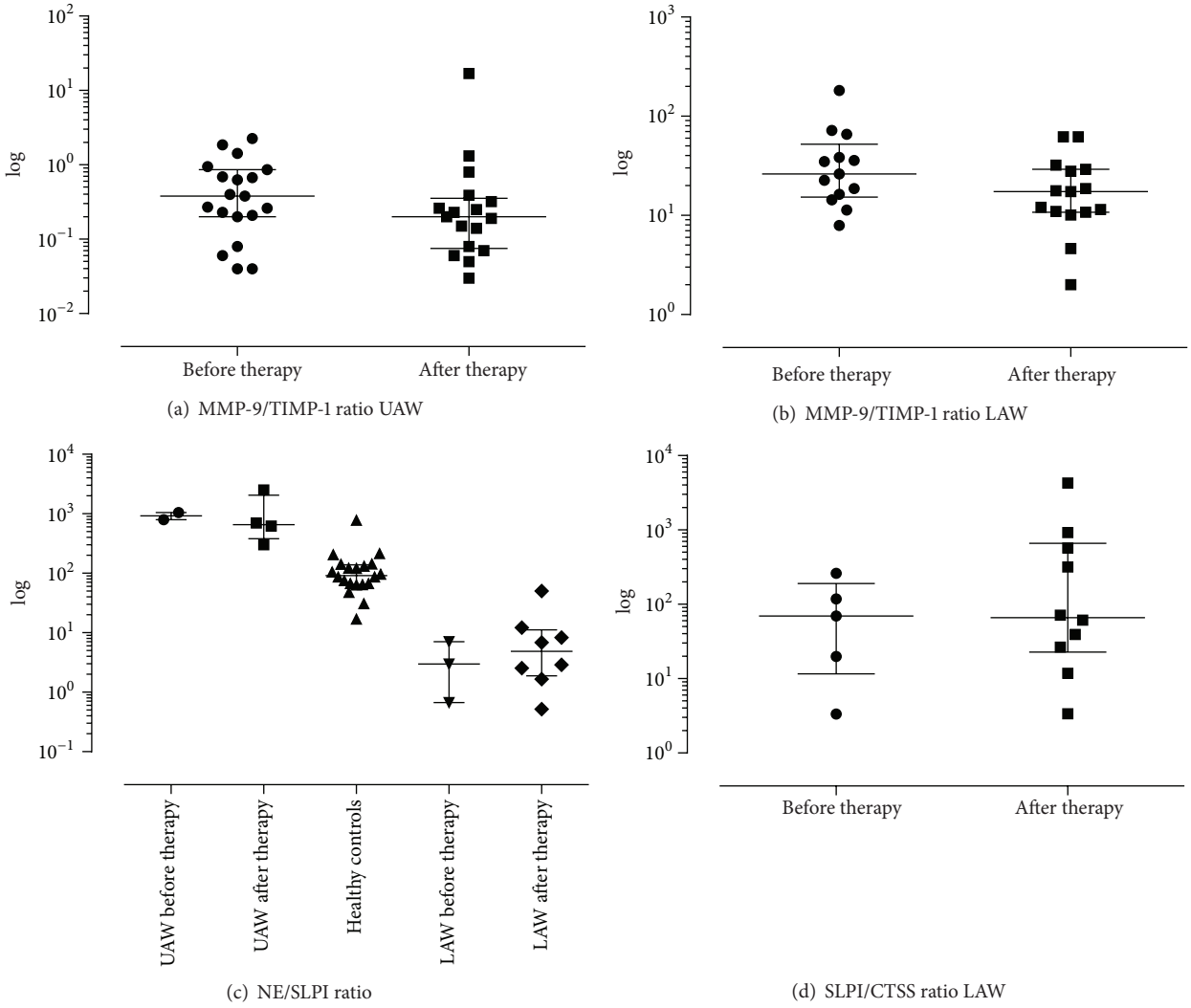
Analyte	DL	Detection frequency (%)			Inflammatory marker or ratio concentrations						P
					Median			Range			
		Controls	CF prior to therapy	CF after therapy	Controls	CF prior to therapy	CF after therapy	Controls	CF prior to therapy	CF after therapy	
NL (UAW)											
NE (ng/mL)	1.98	100.0	89.5	100.0	1.7	73.4	55.6	0.2–38.0	2.3–990.0	2.3–233.6	0.374* 0.0000°
SLPI (ng/mL)	0.01	30.0	10.5	21.1	0.01	0.01	0.01	0.01–0.06	0.01–0.17	0.01–0.15	1.000* 0.235°
CTSS (ng/mL)	0.07	85.0	5.3	21.1	0.04	0.07	0.07	0.00–0.24	0.07–0.14	0.07–1.08	0.125* 0.0004°
MMP-9 (ng/mL)	0.04		100.0	100.0		0.9	0.32		0.02–7.8	0.04–3.96	0.106*
TIMP-1 (ng/mL)	0.01		100.0	100.0		1.8	1.7		0.4–4.2	0.01–2.9	0.036*
MMP-9/TIMP1		n.m.	100.0	89.5	n.m.	0.38	0.20	n.m.	0.0–2.3	0.03–17.0	0.359*
NE/SLPI		100.0	10.5	21.1	91.7	919.1	658.1	16.9–780.1	791.0–1047.1	301.8–2510.8	n.mb.
SLPI/CTSS			—	—	—	—	—	—	—	—	—
Sputum (LAW)											
NE (ng/mL)	1.98		61.5	81.3		328.8	697.4		2.3–2366.5	2.3–17987.2	0.791*
SLPI (ng/mL)	0.01		38.5	62.5		0.01	133.4		0.01–383.6	0.01–487.9	0.432*
CTSS (ng/mL)	0.07		100.0	93.8		3.3	4.7		0.7–63.8	0.1–33.9	0.376
MMP-9 (ng/mL)	0.04		100.0	100.0		1359.7	1195.9		1063.2–1848.0	153.5–1417.5	0.017*
TIMP-1 (ng/mL)			100.0	100.0		50.7	66.0		7.5–134.2	0.14–480.8	0.191*
MMP-9/TIMP1			100.0	93.8		26.1	17.4		7.9–181.8	2.0–62.2	0.094*
NE/SLPI			23.1	50.0		3.0	4.9		0.7–7.1	0.5–50.2	n.mb.
SLPI/CTSS			38.5	62.5		60.6	37.3		3.4–117.9	3.4–71.3	1.000*

*P value between CF prior to and after therapy, °P value between CF prior to therapy and healthy controls in UAW, DL = detection limit, n.m. = not measured, and n.mb. = not measureable.



(a) SLPI LAW (b) CTSS LAW

Figure 5: Changes of SLPI and CTSS.



(a) MMP-9/TIMP-1 ratio UAW (b) MMP-9/TIMP-1 ratio LAW

(c) NE/SLPI ratio (d) SLPI/CTSS ratio LAW

Figure 6: Ratios of proteases and antiproteases: MMP-9/TIMP-1 ratio (a) was higher in the LAW (b), whereas NE/SLPI ratio (c) was higher in the UAW and in healthy controls. SLPI/CTSS (d) was only calculable in the bronchial compartment for low detection rates of both parameters in the UAW. For all of these ratios in UAW and LAW changes did not reach statistical significance. 1 = before therapy; 2 = after therapy.

inflammatory host response relevantly contribute to pulmonary destruction. The UAW are coming into the clinical and scientific focus as they were identified as a reservoir for initial and persistent airway colonization with pathogens like *S. aureus* and *P. aeruginosa,* that can be followed by LAW colonization, inflammation, and deterioration [25, 28]. Our previous studies assessed the correlation of colonization and inflammation in different airway compartments [16, 20, 47, 48]. Here, we assessed changes in pathogen colonization, proteases, antiproteases, and cells as well as symptoms after elective IV-antibiotic treatments primarily directed against *P. aeruginosa*.

In general, concentrations of detected proteases, antiproteases, and cells were lower in the UAW compared to LAW and even lower in the UAW of healthy controls. Fluid dilution, consistence, and origin of samples as well as processing of the materials may play a role in these differences. Otherwise, recent studies revealed different defense mechanisms in the upper and lower airway compartments; Kasper Aanaes showed that IgA plays a pronounced role in the UAW whereas a neutrophil-dominated host response with a strong oxidative burst is characteristic of the LAW first line host defense mechanisms [49, 50].

Reduction of TCC was found in NL and sputum, but only in sputum decreases reached statistical significance ($P = 0.005$) as reported earlier in our group [48]. Similar results were found in protein concentrations; decline was seen in both airway levels, but again, only in the pulmonary compartment, changes reached statistical significance ($\text{protein}^{\text{LAW}}$: $P = 0.008$) (see Figure 2(d)). In accordance with other studies that investigated an association of LAW inflammatory mediators with lung function, we neither found a significant correlation of MMP-9 nor TIMP-1 with FEV_1 [13, 51]. However, NE in the LAW correlated significantly with FEV_1 ($P = 0.033$, $r = 0.593$) before treatment. As we only assessed pulmonary function prior to IV-antibiotic therapy we are not able to make a statement on how lung function correlated with proteases in NL and sputum on the long run.

Altogether, chronic lung diseases and inflammation are characterized by an imbalance of protease and antiprotease. In CF patients with bronchiectasis, elevated MMP-9 levels have been reported compared to non-CF bronchiectasis patients and healthy controls [52]. Besides elevated concentrations of MMP-9 and TIMP-1, an increased MMP-9/TIMP-1 ratios have been reported from sputum and BAL [13, 17, 18]. In our study, concentrations of MMP-9 decreased in the UAW as well as in the LAW during IV-antibiotic treatment while levels of its main inhibitor TIMP-1 attenuated in NL but even rose in sputum of CF patients. Interestingly, the reduction of MMP-9 in LAW resulted in a decline of MMP-9/TIMP-1 ratio after therapy, suggesting the ratio as a good marker for therapeutic success. The trend for reduction of MMP-9 in our study points to the inhibition of inflammation throughout the antibiotic treatment and may serve as an interesting marker to assess therapeutic effects in future studies.

Coherence between NE, MMP-9, and TIMP-1 has been described previously [13, 15, 19]. Gaggar et al. demonstrated a strong correlation between NE and MMP-9 in CF patients' sputum [15]. Due to the modified balance of MMP-9 and TIMP-1, progressive damage of lung tissue mediated by increased NE levels as well as an elevated humoral inflammation and influx of inflammatory cells is the consequence [13]. Furthermore, neutrophils can release MMP-9 in response to the proinflammatory cytokine TNF, which enhances tissue degradation. In our study MMP-9 and NE correlated significantly only in the UAW prior to and after therapy ($P = 0.009$, $r = 0.582$ and $P = 0.031$, $r = 0.496$, resp.). In contrast to Jackson et al. [19] we only found a significant correlation between NE and the MMP-9/TIMP-1 ratio in the UAW, detectable for both times of assessment ($P = 0.001$, $r = 0.681$ and $P = 0.035$, $r = 0.515$, resp.). In this respect, the previously described proteolytic imbalance in the LAW also has to be regarded for the UAW, which underlines the need to look upon the CF patients' upper and lower airways as one airway system.

Our findings confirmed the chronic neutrophil-dominated pulmonary inflammation in CF resulting in higher levels of NE in the airway surface liquid not only being relevant in LAW, but also affecting the UAW [16]. In our patients, NE in NL prior to therapy was 44-fold increased when compared to healthy subjects ($P < 0.001$). This accords well with LAW data from Gaggar et al. who reported a 40-fold increased activity of NE in sputum of CF patients compared to healthy controls [15]. Within the IV-antibiotic treatment, levels of NE decreased in NL, different from our results obtained in a previous study [47]. This difference possibly is caused by a shorter observation period of 6 days in the preceding report, compared to 14 days in the present study. Unlike other publications demonstrating a significant decrease of NE in sputum after antibiotic therapy [40], median NE levels redoubled in our study. However, regarding matched values for NE before and after therapy, in five of seven patients the enzyme decreased during treatment and the huge increase in the remaining two patients cause this surprising increase of medians (see Figure 3(b)). Explanation may be that NE can be bound within neutrophil-extracellular traps (NETs), which are part of the innate immunity composed of granule and nuclear constituents, for example, DNA. NETs are regularly found in sputum of CF patients and are released by activated neutrophils [53]. Due to the routine usage of DNAse in CF patients NETs can be cleared leading to elevated levels of NE [54]. As we used DNAse in processing of sputum, more NE may be liberated and increased concentrations can be measured.

Previously Weldon et al. reported that SLPI is susceptible to proteolytic degradation by NE in chronic infection whereby it neutralizes the anti-NE capacity of SLPI [55]. The imbalance may be enhanced by high burdens of NE in ASL which can overwhelm and inactivate SLPI [8]. Additionally, SLPI as an immunomodulatory protein is capable of decreasing MMP-9 in monocytes [56]. Low detection frequencies of SLPI in all assessed materials are a limitation of the present study. As induced sputum was not taken from controls, a comparison to SLPI levels in the healthy could not be performed. However, during therapy, levels of SLPI increased in sputum whereas its concentrations in NL of CF patients as those of healthy controls remained low, if detectable.

Also CTSS has the potential to cleave and inactivate SLPI which further increases NE levels and facilitates bacterial colonization and infection [9, 57]. Lecaille et al. assessed the cleavage of surfactant protein A, which belongs to the innate immunity, system by CTSS which also facilitates infections by pathogens like *P. aeruginosa* [58]. However, in healthy lungs, cathepsins have not been detected routinely, but they may be stimulated by different mediators, such as IFN-γ or IL-13 [9]. Whereas our findings of elevated cathepsin levels in sputum confirm earlier reports, detection frequencies and levels of CTSS in NL compared to healthy controls were rather low.

Interestingly, inverse results of calculated protease/antiprotease ratios were found in both airway levels. While MMP-9/TIMP-1 ratios were higher in the LAW than in the UAW before and after therapy (65-fold: 26.1/0.4 and 87-fold: 17.4/0.2, resp.), NE/SLPI ratios were higher in the sinonasal compartment compared to the lung (306-fold: 919.1/3.0 and 134-fold: 658.1/4.9, resp.) (Figure 1). These MMP-9/TIMP-1 and NE/SLPI ratios accord well with recent findings from Hentschel et al. who additionally detected elevated SLPI/CTSS values in LAW compared to UAW (16-fold) [16]. As expected, MMP-9/TIMP-1 ratios showed a trend to decrease during systemic treatment in both airway levels (UAW: 1.9-fold, LAW: 1.5-fold, not statistically significant). In this regard, it is remarkable that even clinical stable CF patients with mild pulmonary disease revealed an imbalance of the MMP-9/TIMP-1 ratio in BAL indicating the contribution of the proteases in the chronic inflammatory process in CF lung disease [17]. While in our patients the NE/SLPI ratio in the UAW was 1.4-fold higher before therapy, inverse results were shown for the LAW where the ration was higher after therapy (1.6-fold). Compared to healthy results, NE/SLPI ratio was 10-fold elevated in the sinonasal compartment.

This paper for the first time compares changes in UAW and LAW colonization pattern after IV-antibiotic treatment. Before and after therapy *P. aeruginosa* was frequently detected in the UAW and LAW of the patients with history of chronic colonization with this pathogen (see Table 3) indicating that the IV-antibiotic treatment may reduce but not eradicate colonization and reduce the resulting inflammatory response. Further analyses assessing the upper and lower airways' microbiome by molecular methods with a comparable study design are of high scientific interest.

Only few studies examined changes of sinonasal symptoms and health-related QoL during targeted therapy in CF patients. In this regard, Mainz et al. reported a significant reduction of symptoms assessed by the SNOT-20-GAV scores after sinonasal inhalation with dornase alfa or tobramycin [59, 60]. Savastano et al. evaluated the postoperative outcome of CF patients undergoing sinonasal surgery for CRS and nasal polyposis using the SNOT-22 score and also concluded a positive impact on QoL [23]. In our study, a remarkable improvement of SNOT-20-GAV scores was found after IV-antibiotic treatment. However, scores still were significantly higher when compared to those of healthy controls (see Figure 1). In conclusion, elective IV-antibiotic treatment does not only improve LAW inflammation; it also reduces subjective symptoms of the UAW and general QoL.

5. Conclusion

The present paper for the first time demonstrates changes in UAW and LAW proteases and antiproteases (NE, SLPI, CTSS, MMP-9, and TIMP-1) and pathogen colonization after IV-antibiotic therapy. Further analyses on changes of the protease/antiprotease imbalance especially in the paranasal sinuses and its correlation to changes in the microbiome are of special interest.

Abbreviations

AB:	Antibiotics
ABPA:	Allergic bronchopulmonary aspergillosis
APE:	Acute pulmonary exacerbation
ASL:	Airway surface liquid
BMI:	Body mass index
CF:	Cystic fibrosis
CFTR:	Cystic fibrosis transmembrane conductance regulator
COPD:	Chronic obstructive lung disease
CrP:	C-reactive protein
CRS:	Chronic rhinosinusitis
CTSS:	Cathepsin S
DL:	Detection limit
DNA:	Desoxyribonucleic acid
DTT:	Dithiothreitol
ELISA:	Enzyme linked immunosorbent assay
ENT:	Ears, nose, and throat
ESR:	Erythrocyte sedimentation rate
FCS:	Fetal calf serum
FEV1:	Forced expiratory pressure in 1 second
IFN:	Interferon
Ig:	Immune globulin
IL:	Interleukin
IV:	Intravenous
LAW:	Lower airways
MEF75/25:	Mean exploratory flow
MMP:	Matrix metalloproteinase
MN:	Mononuclear leucocytes
MRSA:	Methicillin-resistant *Staphylococcus aureus*
NE:	Polymorphonuclear neutrophil elastase
NL:	Nasal lavage
P. aeruginosa, P.a.:	*Pseudomonas aeruginosa*
PBS:	Phosphate buffer saline
PCR:	Polymerase chain reaction
PI:	Protease inhibitor
PMN:	Polymorphonuclear leukocytes
S. aureus:	*Staphylococcus aureus*
SLPI:	Secretory leukocyte protease inhibitor
SNOT-20-GAV:	Sino-Nasal Outcome Test 20 German Adapted Version
TCC:	Total cell count
TIMP:	Tissue inhibitor of metalloproteinase
UAW:	Upper airways.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Julia Hentschel and Jochen G. Mainz contributed equally to this paper.

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Research Article

Multitracer Stable Isotope Quantification of Arginase and Nitric Oxide Synthase Activity in a Mouse Model of Pseudomonas Lung Infection

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Cystic fibrosis airways are deficient for L-arginine, a substrate for nitric oxide synthases (NOSs) and arginases. The rationale for this study was to quantify NOS and arginase activity in the mouse lung. Anesthetized unventilated mice received a primed constant stable isotope intravenous infusion containing labeled L-arginine, ornithine, and citrulline. The isotopic enrichment of each of the infused isotopomers and its product amino acids were measured in plasma and organ homogenates using liquid chromatography-tandem mass spectrometry. The effect of infection was studied three days after direct tracheal instillation of Pseudomonas-coated agar beads. In the infusion model, lung infection resulted in a significant (28-fold) increase in NOS activity in lung but not in trachea, kidney, liver, or plasma. Absolute rates of arginase activity in solid tissues could not be calculated in this model. In an isolated lung perfusion model used for comparison increased NOS activity in infected lungs was confirmed (28.5-fold) and lung arginase activity was increased 9.7-fold. The activity of L-arginine metabolizing enzymes can be measured using stable isotope conversion in the mouse. Accumulation of L-ornithine in the whole mouse model hindered the exact quantification of arginase activity in the lung, a problem that was overcome utilizing an isolated lung perfusion model.

1. Introduction

Nitric oxide synthases (NOS) and arginases compete for the amino acid L-arginine as substrate. The isoforms of NOS produce nitric oxide (NO) and L-citrulline, whereas arginases produce urea and L-ornithine [1]. The known interactions between the two pathways are thought to result in reciprocal regulation of the *in vivo* enzyme activities. For instance, N^{ω}-hydroxy-L-arginine (NOHA), formed as an intermediate during NO production from NOS, acts as an arginase inhibitor and arginase activity also decreases with S-nitrosylation of the enzyme [1]. Increased arginase activity can cause substrate limitation for and uncoupling of NOS, leading to reduced NO and a switch to superoxide and subsequently peroxynitrite production [2]. NOS can also be affected by products of arginase activity, as the L-ornithine derived polyamines act as NOS inhibitors [3]. Other endogenous NOS inhibitors include methylated arginine derivates such as the asymmetric dimethylarginine (ADMA) [1–6].

Increased NOS expression has been shown to occur in infection or inflammation, but quantification of NOS activity in specific organs or tissues is usually indirect often by measuring NO metabolite concentrations. However, nitrate and nitrite concentrations may not accurately reflect tissue NOS activity [7, 8] specifically in the presence of denitrifying bacteria [9]. Multitracer stable isotopes have previously been

used to measure systemic L-arginine metabolism in humans and in animals [10–12]. We here aimed to develop methods using stable isotopes in the mouse that would allow quantification of whole body and organ-specific activities of the L-arginine metabolizing enzymes arginase and nitric oxide synthase and changes in activity of these enzymes in response to *Pseudomonas aeruginosa* infection of the lung.

2. Methods

The experiments were approved by the institutional Animal Care Committee and were conducted in accordance with the guidelines of the Canadian Council for Animal Care.

2.1. Whole Mouse Infusion Model. A detailed description of the multitracer studies and analytic procedures can be found in the supplement (see Supplementary Material available online at http://dx.doi.org/10.1155/2014/323526). A stable isotope solution containing L-arginine (m+6 L-arginine U-$^{13}C_6$), L-ornithine (m+2 L-ornithine $^{15}N_2$), and L-citrulline (m+5 L-citrulline 5-^{13}C; 4,4,5,5-H_4) in normal saline (0.9%) was used similarly as previously described [10]. Pilot experiments using each of the three isotopes individually and in combinations were performed to demonstrate no interaction of the isotopes when used in the final combination. Primed constant isotope infusion with the final combination for a length of up to 60 min resulted in isotopic steady state after 30 min (see Figure 1 in supplement). In the actual experiments, infusions were delivered beginning with a bolus given within 20 seconds, followed by a constant infusion of the isotope mix at 1 mL/hr for 45 minutes (see Table 1).

To administer the infusion into the living mouse, a small incision was made in the neck of the animal and a jugular vein dissected. A 30-gauge tubing (Tygon Micro-Bore tubing, Saint Gobain Performance Plastics) was inserted into the vein through a small incision and fixated with help of a ligature thread. The distal end of the tubing was connected to a needle attached to a syringe that contained the isotope solution. The infusion was delivered by a single-syringe infusion pump (KDS 100, kdScientific, Holliston, MA) at a rate of 1 mL/hr for 45 min, while the mice were situated on a mouse warming pad at 37°C. Following the infusion, mice were euthanized with ketamine/xylazine and necropsy was performed. Isolated serum and lung, kidneys, and liver were snap-frozen in liquid nitrogen and then stored at −80°C.

2.2. Isolated Lung Perfusion Model. A previously described isolated lung perfusion model [13] was utilized to study L-arginine metabolism in the lung *ex vivo*. Lungs were ventilated with negative pressure ventilation (end-expiratory pressure -3 cm H_2O, tidal volume 10 ± 2 mL/kg, rate 92/min, and gas mixture of 5% CO_2, 30% O_2, and balanced N_2) and perfused (1 mL/min constant rate) with a blood-free perfusate containing physiological but labeled concentrations of L-arginine (m+6, 150 μM), L-ornithine (m+2, 134 μM), and L-citrulline (m+5, 90 μM) stable isotopes. The osmolality of the perfusate was adjusted to 340 mmol/L by adding NaCl. The perfusion circuit, reservoir, and mouse lungs were kept at 37°C during the experiment. The perfusion was administered in a single-pass of fresh solution and perfusate recovered from the lungs was discharged. Lungs were harvested after 45 min and stored at −80°C.

Table 1: Isotope concentrations used for primed constant infusion.

Isotopes	Prime [nmol/0.25 mL] in 20 s	Constant infusion [nmol/mL/h] for 45 min
L-arginine ($m + 6$)	850	1700
L-ornithine ($m + 2$)	425	850
L-citrulline ($m + 5$)	215	430

2.3. Liquid Chromatography-Tandem Mass Spectrometry and Calculations. Each of the infused isotopomers and its product amino acids were measured in serum and organ tissue homogenates using liquid chromatography-tandem mass spectrometry, similar to what is previously described [10, 11]. The isotopic enrichment was determined using the previously described formulas [10, 14]. Enrichment of the product of enzymatic conversion (transfer of label from precursor to product) was expressed as moles percent excess (MPE). Absolute conversion rates were calculated as the fractional conversion (enrichment of product divided by enrichment of the precursor) multiplied by the flux rate of the product (see supplement). Logically, the enrichment of the product must be equal to or less than the enrichment of the precursor; that is, the MPE ratio (or fractional conversion) should be ≤1, unless there is delayed clearance of the product from the tissue. This, however, appeared to be the case for arginase (i.e., enrichment of ornithine derived from arginine) in solid tissues of living mice in the infusion model. Tissue arginase activity in these studies is therefore expressed as product enrichment.

2.4. Mice and Infection Protocol. Eight-to-ten-week-old female C57BL/6 mice purchased from Charles River Laboratories (Charles River, Oakville, Quebec, Canada) were housed in a pathogen-free environment and received autoclaved food and water in the laboratory animal services at our institution.

Agarose beads embedded with *Pseudomonas aeruginosa* (mPAO1) were made following a published protocol [15] and modified by us. Briefly, bacteria were grown overnight in trypticase soy broth (TSB, Fisher scientific) at 37°C and 2% agar in phosphate buffered saline (PBS), pH 7.4, was mixed with the bacteria broth when bacteria were in late log phase. The agar broth mixture was added to heavy mineral oil that was equilibrated at 50–55°C, rapidly stirred for 6 min at room temperature, and then cooled over 10 min. The beads were washed with 0.5% and 0.25% sodium deoxycholate in PBS and then washed 3-4 times with PBS. Larger beads were removed by using a spectra mesh filter (Opening Ø213 M, Spectrum Laboratories); thereafter, more than 80% of beads are between 70 and 200 μm. Finally, 10-fold serial dilutions of a homogenized aliquot of the bead slurry were platted on

Table 2: Tissue L-arginine metabolizing enzyme activities after stable isotope infusion *in vivo*.

	NOS (nmol/g mouse/h)			Arginase (MPE)		
	Control	Infection	*P* value	Control	Infection	*P* value
Lung	9.0 ± 1.4	254.6 ± 36.3	0.0006	16.8 ± 1.6	23.4 ± 1.9	0.017
Trachea	8.6 ± 1.7	6.5 ± 1.3	0.362	17.2 ± 2.0	26.5 ± 3.3	0.043
Liver	1478 ± 294	2208 ± 417	0.136	7.8 ± 0.6	14.7 ± 1.5	0.001
Kidney	16.6 ± 3.6	19.9 ± 1.9	0.231	26.9 ± 3.4	19.0 ± 3.1	0.244

Trypticase Soy Agar (TSA, Becton Dickinson Company) and incubated at 37°C for 18–20h. Bacterial colony counts were then recorded as colony-forming units (CFU)/mL. Sterile control beads were prepared identically except bacteria that were absent and confirmed to be sterile by checking for bacterial growth after plating a subsample on agar before each use.

Beads were injected into the airways after intubation under direct vision as previously described [16] in anaesthetized mice (ketamine 150 mg/kg and xylazine 10 mg/kg administered intraperitoneally). A final *P. aeruginosa* dose of 2×10^6 CFU in a volume of 40–50 μL was injected into the trachea. Body weight was monitored daily, prior to, and for 3 days following the infection. Mice underwent the above described stable isotope studies on day three of the infection.

Arginase activity was also measured by conversion of L-arginine to ornithine *in vitro*, as previously described [17] and modified by us [18, 19]. The NO metabolites nitrate and nitrite were measured in lung tissue homogenate using the Griess reagent, as previously described [20]. NO metabolite (nitrate + nitrite) concentrations were expressed as nmol/mg protein. Immunoblotting was performed as previously described, and bands on imaging film were quantified by densitometry and expressed as a ratio to the corresponding GAPDH densities [18, 21]. Antibodies for immunoblotting were purchased from Santa Cruz Biotechnology (Dallas, TX).

Data were expressed as mean ± SEM. Group comparisons were made by *t*-test or Mann-Whitney test, where appropriate. A *P* value of <0.05 was considered significant. Analysis of variance (ANOVA) was used for repeated measures of body weight.

3. Results

3.1. Whole Mouse Infusion Mouse Model. The plasma MPE ratios in naïve mice ($n = 6$) were 0.015 ± 0.001 for NOS (MPE Arg > Cit/MPE Arg) and 1.05 ± 0.12 for arginase (MPE Arg > Orn/MPE Arg). Corresponding calculated plasma enzyme activities were 1.46 ± 0.19 nmol/g mouse/h for NOS and 288.1 ± 63.5 nmol/g mouse/h for arginase. In solid tissues, MPE ratios for NOS were also <1 in naïve mice. Calculated NOS activities for lung, trachea, liver, and kidney are shown in Table 2. Unlike for NOS, ornithine enrichment from arginine exceeded labeled arginine MPE in all solid tissues studied (lung, trachea, kidney, and liver), which resulted in arginase MPE ratios of greater >1, respectively. As accurate calculations of enzyme activities are imprecise using MPE ratios that largely exceed 1, we therefore expressed solid tissues arginase activity in the whole mouse infusion model as ornithine enrichment from arginine (or MPE Arg > Orn) (Table 2).

Pseudomonas infection was associated with significant weight loss and a 14% reduction in body weight on day 3 (19.2 ± 0.5 versus 16.5 ± 0.4 g, $P < 0.001$, ANOVA) of the infection. No change in weight was observed in the noninfected control animals (20.6 ± 0.8 versus 20.9 ± 0.7 g). As expected, infection resulted in a significant increase not only in protein expression of NOS 2 (0.384 ± 0.149 versus 0.006 ± 0.0016 NOS2/GAPDH, $P = 0.029$), but also in arginase 1 (1.97 ± 0.29 versus 0.71 ± 0.24 Arg 1/GAPDH, $P = 0.01$) and arginase 2 (1.11 ± 0.08 versus 0.44 ± 0.04 Arg 2/GAPDH, $P < 0.0001$) in lung ($n = 6$ per group) (Figure 1).

NO metabolite (nitrate + nitrite) concentrations were significantly increased in lung homogenates of *Pseudomonas* infected mice compared to controls (1.75 ± 0.09 versus 0.80 ± 0.19 nmol/mg protein, $P < 0.001$) ($n = 6$ per group) (Figure 2), as was *in vitro* arginase activity (32.0 ± 5.4 versus 14.3 ± 1.6 mU/mg protein, $P = 0.028$) ($n = 4$ per group) (Figure 3).

Enzyme activities measured after stable isotope infusion in *Pseudomonas* infected mice demonstrated mean plasma MPE ratios of 0.025 ± 0.003 for NOS and 0.87 ± 0.05 for arginase ($n = 7$). Calculated plasma enzyme activities after infection were 1.63 ± 0.2 nmol/g mouse/h for NOS and 242.5 ± 95.7 nmol/g mouse/h for arginase, which was not significantly different from the naïve controls ($P = 0.579$ and $P = 0.709$, resp.). Calculated tissue NOS activity in infected animals was significantly increased in lung but not in trachea, liver, or kidney (Table 2). In contrast, arginase MPE in infected animals showed a small but statistically significant increase for lung, trachea, and liver, but not for kidney (Table 2).

3.2. Isolated Lung Perfusion Model. An isolated lung perfusion model was used to confirm the findings from the whole mouse infusion model. In the isolated lung perfusion model, the mean MPE ratio in naïve mice ($n = 5$) was 0.037 ± 0.002 for NOS and 0.634 ± 0.046 for arginase. The ratio increased to 0.356±0.02 ($P < 0.0001$) for NOS in infected animals ($n = 5$). For arginase, the ratio was ≤1 in 4 out of 5 lungs and the mean was 1.183 ± 0.18 ($P = 0.0184$). Calculated NOS activity in lung increased from 0.36 ± 0.12 to 10.30 ± 1.48 μmol/g lung/h (28.5-fold) and arginase activity increased from 4.12 ± 1.49 to 39.9 ± 10.2 μmol/g lung/h (9.7-fold) (Figure 4).

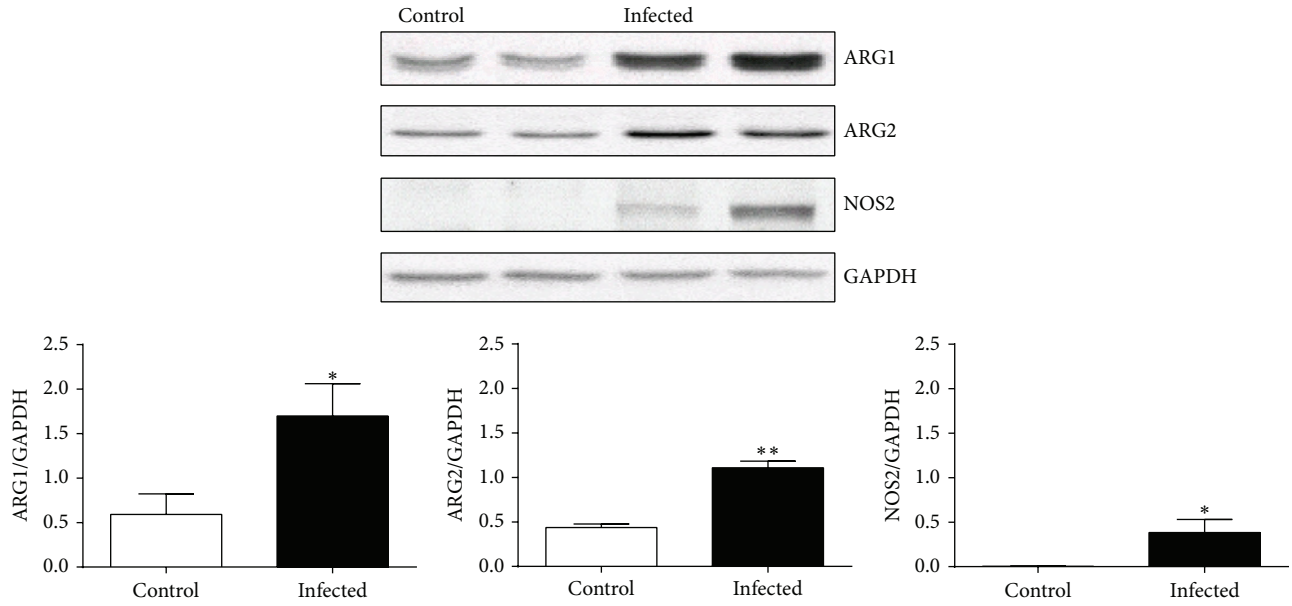

Figure 1: Expression of inducible nitric oxide synthase (NOS2) and the arginase isoform types I (ARG1) and II (ARG2) normalized to GAPDH, respectively, in lung of *Pseudomonas aeruginosa* infected mice and naïve control ($n = 6$ per group). Infection resulted in a significant increase in protein expression of all three enzymes ($^{*}P < 0.05$, $^{**}P < 0.0001$).

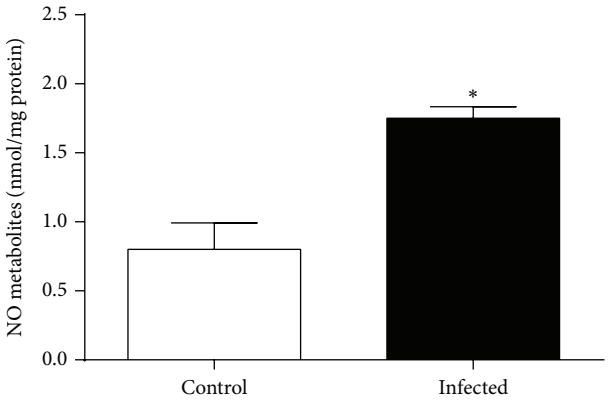

Figure 2: NO metabolite (nitrate+nitrite) concentrations in lung homogenates of mice infected with *Pseudomonas aeruginosa* and noninfected controls ($n = 6$ per group). NO metabolite concentrations were higher in the infected animals ($^{*}P < 0.001$).

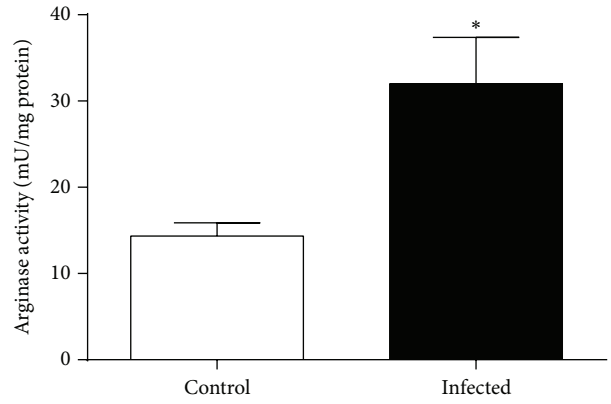

Figure 3: Arginase activity measured in lung homogenates of mice infected with *Pseudomonas aeruginosa* and noninfected controls *in vitro* ($n = 4$ per group) ($^{*}P < 0.05$).

4. Discussion

Using multitracer stable isotope techniques, we measured organ-specific L-arginine metabolism by NOS and arginase in the mouse *in vivo*. Infection of the lung with *Pseudomonas*-coated beads resulted in a significant increase in NOS activity in the lung but not in other organs. In contrast, arginase activity after lung infection was increased not only in lung, but also in trachea and liver.

The amino acid L-arginine is substrate to five groups of enzymes in mammalians (Figure 5), which include arginyl-tRNA synthetase, arginine:glycine amidinotransferase, arginine decarboxylase, arginase, and nitric oxide synthase isoforms [22]. We here aimed to quantify the activity of arginase and NOS in the lung using stable isotopes. Similar approaches have previously been used to characterize systemic L-arginine metabolism in animals and human subjects [10–12, 23–26]. To characterize NOS and arginase activity in the whole mouse (using plasma) but also in isolated solid organs, we modified a method that was recently used by members of our group to quantify the effects of arginine intake on whole body arginine metabolism in neonatal piglets. Using primed constant intravenous infusion of stable isotopes in living mice, quantification of NOS activity was unproblematic for whole body (systemic) activity as well as for all solid organs analyzed separately (lung, trachea, liver, and kidney). In contrast, the MPE for arginase (enrichment of ornithine from arginine) was greater than the precursor MPE arginine

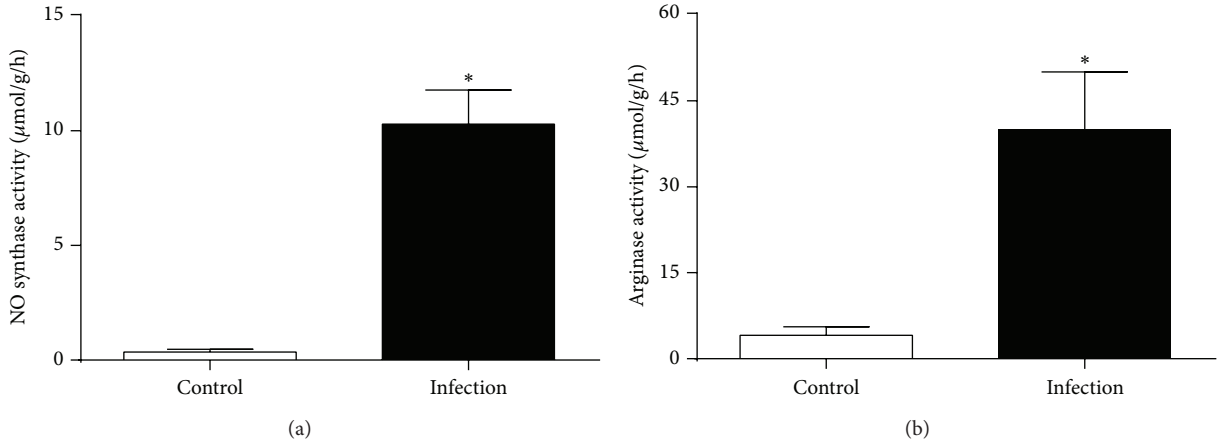

Figure 4: (a) Nitric oxide synthase (NOS) and (b) arginase activity in mouse lung calculated from stable isotope conversion in an isolated lung perfusion model. NOS and arginase activity were significantly increased three days after direct tracheal instillation of *Pseudomonas*-laden agarose beads (infection) compared to control ($^*P < 0.001$, resp.).

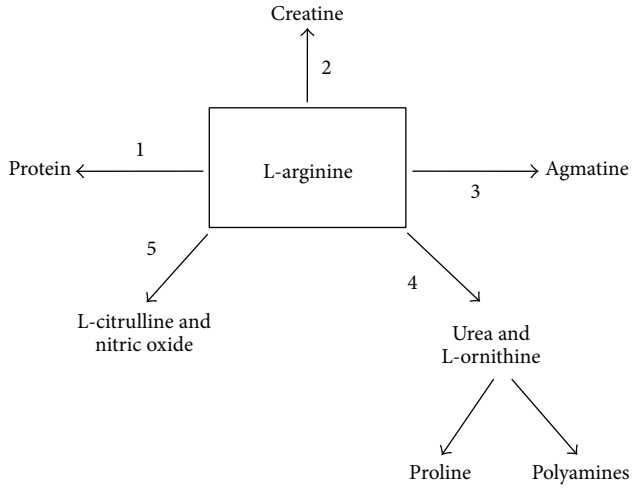

Figure 5: The arginine metabolism. Enzymes that use L-arginine as substrate are (1) arginyl-tRNA synthetase, (2) arginine:glycine amidinotransferase, (3) arginine decarboxylase, (4) arginases, and (5) NO synthases (NOS).

in solid tissues (but not in plasma) at the time point of organ harvest (45 minutes of constant infusion). While this observation may be explained by differences in the kinetics of L-arginine and L-ornithine metabolism in tissue, ratios largely exceeding 1 cannot be used to calculate absolute rates of arginase activity. We therefore used the ornithine enrichment to quantify solid tissue arginase activity in this model. However, whether the ornithine enrichment is an accurate reflection of arginase activity remains unclear.

Because of this limitation, we then utilized an *ex vivo* isolated lung perfusion model using stable isotopes in the perfusate. Different from the whole mouse infusion experiments, in this model the MPE ratios for NOS and arginase were below 1 (with the exception of arginase MPE ratio in one animal), which allowed for calculation of lung enzyme activities. Increased lung NOS and arginase activities after infection of the lung with *Pseudomonas* were confirmed in this model. Both models revealed similar increases in NOS activity following infection (28-fold in the whole mouse infusion model and 28.5-fold in the isolated lung perfusion model). Infection-induced changes in lung arginase activity were 9.7-fold in the isolated lung perfusion model suggesting that arginase MPE derived from the whole mouse infusion model underestimates true arginase activity, as the change seen in the infusion model was only 1.5 fold. Interestingly, however, changes of arginase activity in an *in vitro* enzyme activity test using excess substrate concentrations in the millimolar range were more consistent with the MPE data derived from the whole animal infusion model (2.2-fold change) suggesting that this enzymatic assay is also not reflective of true arginase activity in tissue.

One potentially important difference between infusions in intact mice and the isolated lung perfusion model was that in the latter a blood-free perfusate and an open circuit were used, which means that the perfusate recovered from the lungs was not recycled. This may potentially affect tissue concentrations of factors important for enzyme activity including cofactors, endogenous inhibitors, and amino acid concentrations that determine uptake of L-arginine via CAT and thus L-arginine availability for intracellular enzymes. Nevertheless, fold changes in lung NOS activity after the infection were very similar comparing both models. In contrast, NO metabolite concentrations in lung homogenates only doubled following infection, but NO-metabolite concentration may not represent an accurate reflection of NOS *in vivo* activity [7, 8].

One strength of the described isolated organ perfusion model is that it seems to allow for an accurate calculation of both NOS and arginase and thus it can be utilized to assess the arginase/NOS activity ratio as a measure of balance of L-arginine metabolizing enzymes. Under baseline

conditions L-arginine consumption by arginase exceeded that by NOS in plasma and in the lung. Interestingly, however, while infection did not significantly change the arginase/NOS activity ratio in plasma, there was evidence for significant arginase/NOS imbalance in lung as the activity ratio decreased from 11.4 in naïve to 3.9 following the infection. One limitation of our approach is that we quantified the L-arginine metabolism by arginase and NOS only, but L-arginine is involved in many other metabolic pathways (Figure 5). A characterization of the balance between L-arginine metabolizing enzymes beyond arginase and NOS may be helpful in future preclinical studies aiming to assess efficacy of therapeutic interventions targeting L-arginine homeostasis (such as arginase inhibitors), for instance, in animal models of cystic fibrosis (CF) lung infection [27]. Studies in CF have previously demonstrated increased arginase activity, increased concentrations of the endogenous NOS inhibitors asymmetric dimethylarginine (ADMA) and spermine, reduced L-arginine availability for NOS, and reduced NO formation in CF airways [19, 28–32].

In summary, our results support previous work that systemic and organ-specific arginine metabolism by arginase and NOS can be measured in the mouse using multitracer stable isotope methods. While NOS activity could be accurately calculated in both models, the isolated lung perfusion model allowed for a more precise calculation of arginase activity compared to the infusion model. Infection of the lung with *Pseudomonas* results in increased NOS and arginase activity in lung and isolated increases of arginase in other organs including trachea and liver. This suggests that infection-induced changes in pulmonary L-arginine metabolism lead to an imbalance of NOS and arginase not only in the lung but also in other organ systems. Whether this imbalance in L-arginine metabolism results in systemic regulatory responses to control overshooting NOS activity or contributes to associated complications such as inflammation and airway narrowing or remodeling, as suggested for asthma [33–35], needs to be further investigated. Further studies are needed to show whether multitracer stable isotope techniques could be utilized to accurately assess the L-arginine metabolism beyond the balance of NOS and arginase.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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