Mediators of Inflammation

Airway Inflammatory/Immune Responses in COPD and Cystic Fibrosis

Lead Guest Editor: Virginia De Rose
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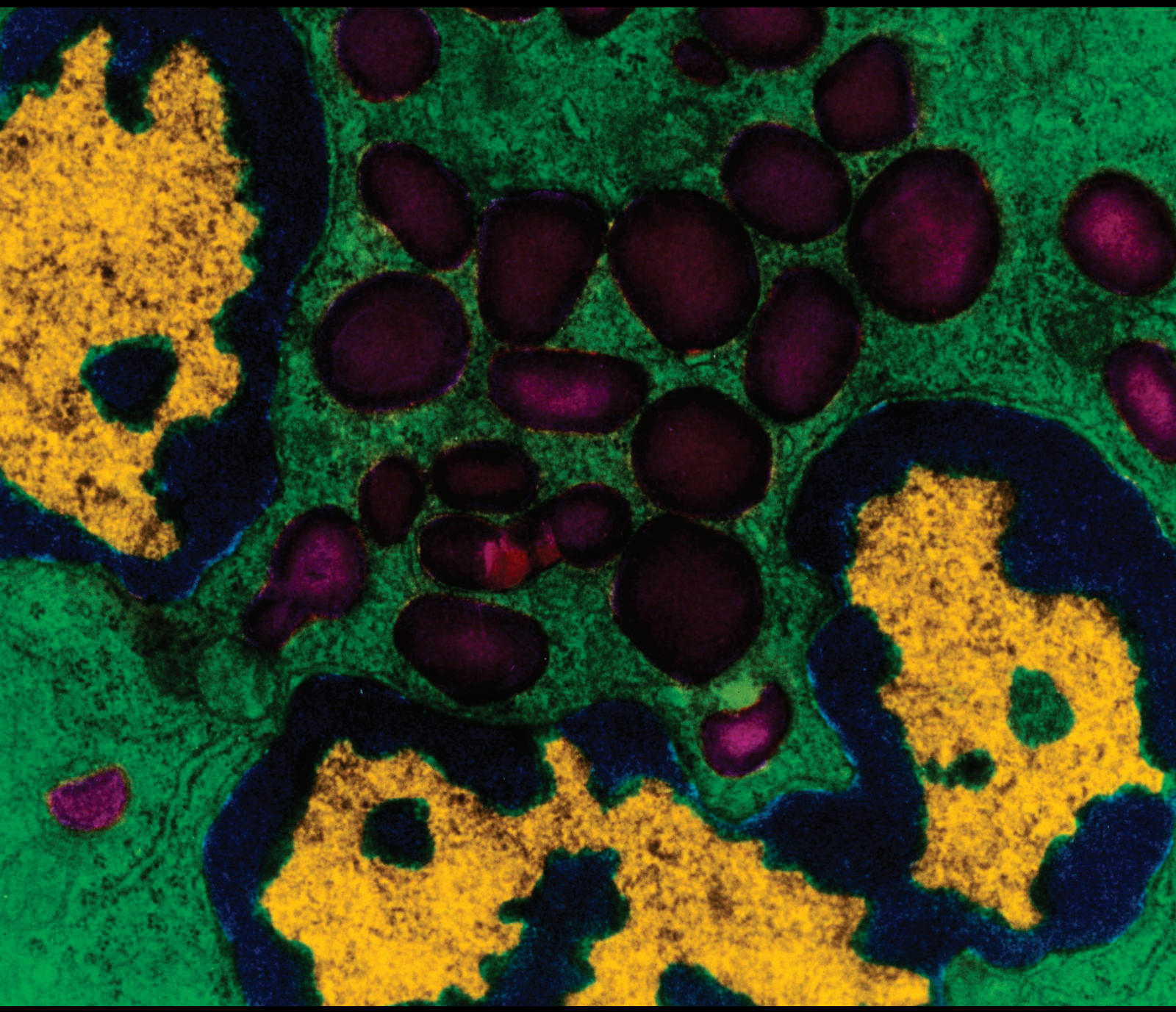

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Editorial Board

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Mediators of Inflammation
Volume 2018, Article ID 7280747, 3 pages
https://doi.org/10.1155/2018/7280747

Editorial

Airway Inflammatory/Immune Responses in COPD and Cystic Fibrosis

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Received 7 March 2018; Accepted 7 March 2018; Published 2 May 2018

Cystic fibrosis (CF) is a genetic disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene and remains one of the most common fatal hereditary disorders worldwide. Although CF is a complex multiorgan disease, morbidity and mortality are mainly determined by progressive chronic obstructive lung disease.

COPD is a major global health problem. The disease is caused by both genetic and environmental factors; among these, cigarette smoking (CS) is the main risk factor and is associated with a marked oxidative stress burden and a persistent airway inflammatory response.

Although the etiology and the pathophysiology of these diseases are different, they share key phenotypical features, including reduced mucociliary clearance, airway mucus obstruction especially in small airways, chronic neutrophilic airway inflammation, and chronic/recurrent bacterial infections. In both diseases, a persistent high-intensity inflammation, driven by continuous neutrophil recruitment, leads to permanent structural damage of the airways and progressive airflow obstruction.

Several defective inflammatory/immune responses have been linked to CFTR deficiency including innate and acquired immunity dysregulation, cell membrane lipid abnormalities, and various transcription factor-signaling defects. During the past few years, several *in vitro* and *in vivo* studies have shown that CS decreases CFTR expression and function and induces an acquired CFTR dysfunction in patients with a normal CFTR genotype. Thus, it is possible that common mechanisms may contribute to the chronic nonresolving inflammation and the altered immune responses in both CF and COPD. These mechanisms however are still poorly defined.

This special issue aims to contribute to knowledge of these mechanisms and to provide a better understanding of altered inflammatory/immune responses in both CF and COPD as well as a roadmap for potential novel therapeutic approaches relevant to both diseases. We hope this issue may be a useful reference to all readers and investigators interested in potential mechanistic links and common therapeutic targets in CF and COPD and that it may be an incentive for further investigations in this field.

Airway epithelial cells are among the first sites of contact for pathogens and other noxious environmental irritants and play a critical role in maintaining normal airway functions as well as in modulating inflammatory/immune responses in the airways. Relevant molecular and morphologic changes occur in the airway epithelium in both CF and COPD. In their review, V. De Rose et al. addressed the evidence for a critical role of dysfunctional airway epithelium in impaired local defences, altered immune responses, chronic airway

inflammation, and remodelling in CF and COPD, highlighting the common mechanisms involved in epithelial dysfunction as well as the similarities and differences in the two diseases. They discuss the *in vitro* and *in vivo* findings showing that CS induces an acquired CFTR dysfunction in patients with COPD, reducing the expression and/or function of the protein; they highlight that this CFTR dysfunction is involved in most of the pathogenetic pathways common to both COPD and CF and may represent a potential target for the development of novel therapeutic approaches in COPD.

M. Stolarczyk and B. J. Scholte reviewed the role of the EGFR/ADAM17 axis in the development of chronic lung disease in CF and COPD. They discuss the evidence suggesting that the ADAM17/EGFR axis and downstream regulatory pathways are hyperactive in both diseases. The enhanced ADAM17/EGFR signaling may contribute to inflammation, epithelial metaplasia, and fibroblast and smooth muscle activation, as well as tissue remodelling observed in CF and COPD lung disease. They also discuss a possible mechanistic link between EGFR/ADAM17 activity, CF, and COPD, suggesting that the genetic CFTR defect in CF and the CS-induced CFTR dysfunction in COPD interfere with glutathione transport in the airways, enhancing oxidative stress, which would activate the ADAM17/EGFR axis.

In the context of airway inflammation and mucosal immunity, M. Puccetti et al. have reviewed the role of the aryl hydrocarbon receptor (AhR) in COPD and CF lung disease. They discuss the effects of this receptor on the immunological status of the gastrointestinal and respiratory tracts and highlight its relevance in establishing and maintaining signaling networks which facilitate host/microbe homeostasis at the mucosal interface. They also analyze the evidence suggesting that changes in AhR expression and function may be a risk factor for COPD and other lung inflammatory diseases in smokers and that the AhR status could be dysregulated in smokers. Finally, they also discuss the possible therapeutic use of AhR ligands in cystic fibrosis.

A common feature shared by CF and COPD is the increased susceptibility to respiratory infections; recurrent infectious exacerbations significantly contribute to morbidity and mortality in both diseases. To better understand the mechanisms of recurrent exacerbations in COPD, G. Pehote et al. investigated the mechanisms of CS-induced impairment of bacterial phagocytosis in this disease and showed that an autophagy defect mediated by CS is a critical mechanism involved in the impairment of phagocytosis that may account for recurrent exacerbations in COPD. An autophagy defect has also been described in CF and suggested to underlie the increased susceptibility to infections with certain microbes in this disease; thus, the study of G. Pehote et al. suggests additional common mechanisms responsible for the increased susceptibility to respiratory infections in COPD and CF. The findings of this study also suggest the therapeutic potential of autophagy-inducing drugs, with antioxidant characteristics, in restoring CS-impaired phagocytosis in COPD and other chronic airway inflammatory diseases, such as CF.

A genuine microbiota resides in the lung, which emanates from colonization by the oropharyngeal microbiota. Changes in the oropharyngeal microbiota might be the source of dysbiosis observed in the lower airways in patients suffering from chronic airway inflammatory diseases, including COPD, asthma, and CF. S. Boutin et al. analyzed whether differences occur in the throat microbiota of children with asthma and CF in comparison to that of healthy children and reported that the microbiota in these three populations shows high levels of similarities, revealing the existence of a core microbiome. However, in the CF group, a decrease in both diversity and total bacterial load in the throat microbiota was observed in comparison to asthmatic and control children, whereas, on the contrary, a significant increase was found in typical pathogens like *Pseudomonas* and *Staphylococcus* and the atypical pathogen *Phyllobacterium*, which is consistent with the impaired host defences associated with CFTR dysfunction in the CF airways.

Toll-like receptors (TLRs) expressed on the airway epithelium respond to infection or tissue damage by sensing local microbial and host-derived factors; TLRs recognize LPS which activates intracellular molecules such as IL-1 receptor-associated kinases (IRAKs) leading to overproduction of pro-inflammatory cytokines. IRAK-M, a negative regulator of TLR-mediated NFkB activation, is expressed in both airway epithelial cells and monocytes/macrophages in healthy lungs. H. Gong et al., using IRAK-M KO mice, studied the effects of IRAK-M deficiency on CS-induced airway inflammation under acute or subacute conditions. They showed that IRAK-M has distinctive effects on airway inflammation and influences the Treg/Th17 balance and expression of costimulatory molecules by dendritic cells and macrophages, depending on the duration and intensity of the stimulus. In fact, whereas upon short-term CS exposure IRAK-M provided airway protection, it played a proinflammatory role in airway pathology upon subacute CS exposure. As TLRs largely contribute to chronic airway inflammation also in CF lung disease, it would be interesting in the future to investigate the effect of IRAK-M on CF airway inflammation.

Increasing evidence support a crucial role of long noncoding RNAs (lncRNAs) in controlling gene expression; furthermore, it has been shown that lncRNAs play important roles in biological and pathological processes and are dysregulated in various human diseases, including CF and COPD. X. Qu et al. evaluated lncRNAs and mRNA expression profiles of peripheral blood mononuclear cells (PBMCs) from healthy nonsmokers, smokers without airflow limitation, and COPD patients to determine if lncRNA differential expression may be linked to dysregulated mRNA expression relevant to COPD pathogenesis. They identified 158 differentially expressed lncRNAs in PBMCs from COPD patients compared with smokers without airflow limitation; they further analyzed the regulation network between lncRNAs and mRNAs, where the genes CXCL16, HMOX1, SLA2, and SIGLEC14 were predicted to be regulated by certain lncRNAs. Their study may provide clues for further studies targeting lncRNAs to control inflammation in COPD.

In summary, the articles presented in the present special issue constitute a contribution to the idea that COPD and CF share some clinical and pathophysiological aspects

and that progress in the understanding of rare diseases (e.g., cystic fibrosis) could contribute to the development of novel approaches for more common diseases (e.g., COPD and bronchiectasis).

Acknowledgments

We would like to thank all the people, authors, and expert reviewers, who supported us with their valuable contribution to this special issue.

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Pierre-Régis Burgel
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Hindawi
Mediators of Inflammation
Volume 2018, Article ID 1309746, 20 pages
https://doi.org/10.1155/2018/1309746

Review Article

Airway Epithelium Dysfunction in Cystic Fibrosis and COPD

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Received 21 September 2017; Revised 15 January 2018; Accepted 1 February 2018; Published 8 April 2018

Academic Editor: Maria Rosaria Catania

Cystic fibrosis is a genetic disease caused by mutations in the CFTR gene, whereas chronic obstructive pulmonary disease (COPD) is mainly caused by environmental factors (mostly cigarette smoking) on a genetically susceptible background. Although the etiology and pathogenesis of these diseases are different, both are associated with progressive airflow obstruction, airway neutrophilic inflammation, and recurrent exacerbations, suggesting common mechanisms. The airway epithelium plays a crucial role in maintaining normal airway functions. Major molecular and morphologic changes occur in the airway epithelium in both CF and COPD, and growing evidence suggests that airway epithelial dysfunction is involved in disease initiation and progression in both diseases. Structural and functional abnormalities in both airway and alveolar epithelium have a relevant impact on alteration of host defences, immune/inflammatory response, and the repair process leading to progressive lung damage and impaired lung function. In this review, we address the evidence for a critical role of dysfunctional airway epithelial cells in chronic airway inflammation and remodelling in CF and COPD, highlighting the common mechanisms involved in the epithelial dysfunction as well as the similarities and differences of the two diseases.

1. Introduction

Cystic fibrosis (CF) is the most common genetic disease in the white population and results from mutations in a single gene encoding for a 1480 residue transmembrane glycoprotein, the cystic fibrosis transmembrane conductance regulator (CFTR). Lung disease, characterized by chronic neutrophilic inflammation, progressive airflow obstruction, and airway bacterial infections, is the major cause of morbidity and mortality in patients with CF [1, 2]. Chronic obstructive pulmonary disease (COPD) is a major global health problem, and it is estimated to become the third leading cause of death worldwide by 2020; the disease is caused by both genetic and environmental factors; among these, cigarette smoking is the main risk factor for COPD and triggers an inflammatory response throughout the airways, in the alveoli, and in the pulmonary vasculature [3, 4]. The two predominant phenotypes in COPD are emphysema and chronic bronchitis; however, they often overlap in most patients. Although CF and COPD are different in many aspects, they also share key phenotypical and pathologic features suggesting potential mechanistic links (Figure 1). Both diseases are associated with progressive airflow obstruction, chronic neutrophilic inflammation in the airway lumen, and recurrent infectious exacerbations; disease

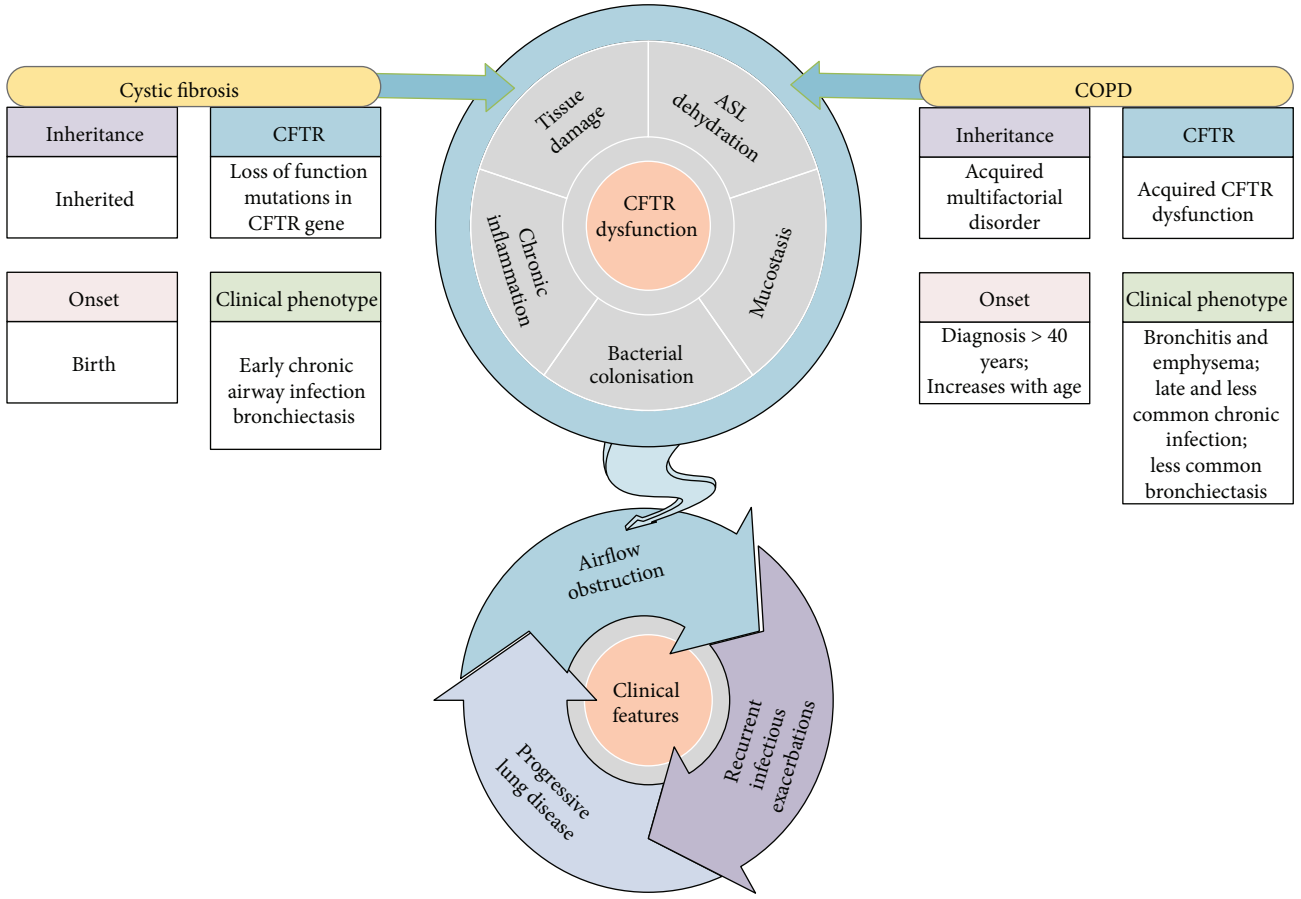

Figure 1: Similarities and differences between cystic fibrosis and COPD. Cystic fibrosis (CF) is a genetic disease caused by mutations in the CFTR gene, while COPD is a multifactorial-acquired disease of later onset predominantly associated with cigarette smoke exposure. Although the diseases are different in many aspects, they also share key phenotypical features. Recent evidence suggests that cigarette smoke induces an acquired CFTR dysfunction in COPD and that both CF and COPD have corresponding molecular phenotypes of CFTR dysfunction resulting in airway surface liquid (ASL) dehydration, mucostasis, bacterial colonization, chronic inflammation, and tissue damage. Airflow obstruction, recurrent infective exacerbations, and a progressive decline in lung function characterize both conditions.

exacerbations significantly contribute to morbidity and mortality in both diseases and have a relevant impact on patient's quality of life as well as on health care expenditures [1–4].

Airway epithelial cells (AEC) are among the first sites of contact for inhaled insults and play a crucial role in maintaining normal airway function [5, 6]. The airway epithelium represents the primary site of relevant molecular and histologic changes in both CF and COPD, and growing evidence in recent years has suggested that it plays a key role in disease initiation and progression in both diseases. Structural and functional changes in both airway and alveolar epithelium have a relevant impact on alteration of the airway milieu, host defences, and the repair processes that contribute to the airflow limitation characteristic of both diseases. The airway epithelium could therefore represent a suitable target for novel therapeutic strategies aiming to restore barrier integrity and defences against inhaled particles and pathogens. In this review, we address the evidence for a critical role of dysfunctional airway epithelium in impaired local defences, altered immune responses, chronic airway inflammation, and remodelling in CF and COPD, highlighting the common mechanisms involved in epithelial dysfunction as well as the similarities and differences of the two diseases.

2. Properties of Airway Epithelium

The airway epithelium acts as a physical barrier to prevent potential pathogens or noxious agents entering airway mucosa and reaching the bloodstream [7]. In addition, the airway epithelium controls ion transport to keep the airways hydrated and, furthermore, acts as a key regulator of innate immune responses toward invading pathogens.

2.1. Barrier Function. The crossing of one or multiple host barriers by a pathogen is critical to initiate infection. The airway epithelium provides a physical barrier to microbial invasion; it consists of a monolayer of polarised epithelial cells which maintain their integrity by apical junction complexes (AJC). These consist of occluding tight junctions (TJ), anchoring adherens junctions (AJ), desmosomes, and GAP junctions.

TJs regulate the movement of ions, macromolecules, and immune cells through the paracellular space; they are critical for ion transport and act as a barrier to regulate the access of inflammatory cells and against the entry of harmful substances such as microbial components into the airway lumen. TJs are constructed from zona occludens proteins (ZO), occludin, claudins, and junctional adhesion molecules (JAMs). AJs mediate cell–cell adhesion and signalling pathways that control cell growth, morphology, and differentiation. They are located below TJs in the lateral membrane and utilise cadherins to mediate calcium-dependent cellular adhesion by binding a cadherin of the same type on an adjacent cell [8]. Gap junctions are not part of AJCs and are pathways of intercellular communication constructed with channel proteins called connexins [9].

2.2. Mucociliary Escalator. The airway is maintained in a constant state of hydration through the coordinated actions of the CFTR channel and amiloride-sensitive epithelial sodium channel (ENaC). The airway surface liquid layer (ASL) comprises a mucus layer which functions to trap particulate matter, bacteria, and viruses, and the underlying periciliary liquid layer (PCL), which provides hydration, enabling effective mucociliary transport and clearance [10]. A finely tuned balance between chloride secretion by CFTR on the one hand and sodium absorption by ENaC on the other keeps the ASL sufficiently hydrated to permit effective and sustained mucus clearance via the mucociliary escalator; additional ion channels contribute to maintain ASL homeostasis (reviewed in Section 3). Mucus dehydration, defective mucociliary clearance of microbes, infection, and inflammation are the hallmarks of CF and COPD lung disease.

2.3. Pathogen Sensing. The airway epithelium senses pathogens using pattern recognition receptors (PRRs) including Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), Nod-like receptors (NLRs), C-type lectin receptors (CLRs), protease-activated receptors (PARs), and the bitter and sweet taste receptors, amongst others. TLRs are expressed by epithelial cells throughout the respiratory tract including tracheal, bronchial, and alveolar type II cells and respond rapidly to infection or tissue damage by sensing local microbial and host-derived factors (Figure 2(a)). The nucleic acid-sensing TLRs (TLR3, TLR7, and TLR9) and the RLRs, RIG-I, and MDA-5 provide defence against viruses invading the respiratory tract [11], by generating IL-6, CXCL8 (IL-8), and IFN-β responses. RIG-I and MDA-5 expression is increased in influenza A virus-infected nasal epithelium in a process believed to resist IAV infection [12].

Various NLRs are expressed by airway epithelial cells. NOD1 activation can reduce airway hyperresponsiveness and decrease allergen-specific T-cell proliferation in allergen-induced lung inflammation [13]; its expression is downregulated during pollen season among patients with allergic rhinitis [14]. NLRP3 mediates airway epithelial cell responses to inhaled particulate matter, for example, PM10 [15].

A major function of epithelial CLRs is to sense fungal species, for example, dectin-1, which recognises β-glucan motifs in *Aspergillus fumigatus* and house dust mite (HDM) [16] whereas nonfungal allergens, such as Derp1 and cockroach allergen, which have proteolytic properties, can elicit allergic airway inflammation via PAR-2. Bitter and sweet taste receptors (T2R and T1R, resp.) are G-protein-coupled receptors expressed in respiratory epithelia (reviewed in [17]) that are activated by quorum-sensing molecules such as homoserine lactones from *Pseudomonas aeruginosa* or sugars. Their activation can enhance mucociliary clearance [18]. In addition, new roles are emerging for other receptors expressed by airway epithelium such as fractalkine receptor CX3CR1 [19] and the short-chain fatty acid receptor GPR41 [20].

In addition to membrane-bound PRRs, in the lung, soluble forms also exist that include mannose-binding lectin (MBL) and surfactant proteins A and D. Ligands for MBL include high mannose and N-acetylglucosamine oligosaccharides that trigger the activation of the classical complement pathway [21]. Surfactant proteins (SP) B and C lower alveolar surface tension whereas SP-A and SP-D bind to macrophages and stimulate their chemotaxis [22] or Gram-negative bacteria and inhibit T-cell proliferation [23], respectively.

2.4. Innate Immunity. The airway epithelium plays a key role in the lung's innate immune responses. Together with the complement system, the antiproteases, antimicrobial peptides, and cytokines expressed by airway epithelial cells or present within the airway lumen represent major factors that function to rid the lungs of infectious or invading microbes and cope with toxic intrapulmonary insults due to pathogens or environmental pollutants.

The complement system opsonizes pathogens and generates chemotactic peptides and the membrane attack complex [24]. Alpha-1 antitrypsin (AAT), secretory leukoprotease inhibitor (SLPI) and elafin are the most abundant serine antiproteases in the lung. Each has important beneficial effects within the lung and their dysregulated activity can adversely impact on the inflammatory process (Figure 2(b)) [25–30].

Antimicrobial peptides form part of the lung's innate immune defences, and inactivation of these peptides has been implicated in airway infections (Figure 2(b)). Lactoferrin is a monomeric iron-binding glycoprotein (76–80 kDa) present in the secondary granules of neutrophils [31]. It plays a microbiostatic role in mucosal fluids [32] and also has antiviral, antifungal, and anticancer activities and can act as an immunomodulator. The primary functions of LL-37 are to eliminate pathogens, inhibit biofilm, and enhance the immune responses [33]. Human beta-defensins are small (4–6 kDa) cationic peptides. Twenty-six human *DEFB* genes have been identified but only HBD1–4 are secreted by the respiratory tract and play a role in airway mucosal defense. HBDs possess antibacterial [34], antiviral [35], and antifungal properties [36]. HBD-2 and HBD-3 also have immunomodulatory properties [37].

3. Mucus Production/ASL Regulation in CF and COPD

As previously described, the airway epithelium is covered by the ASL that comprises a mucus layer and the underlying

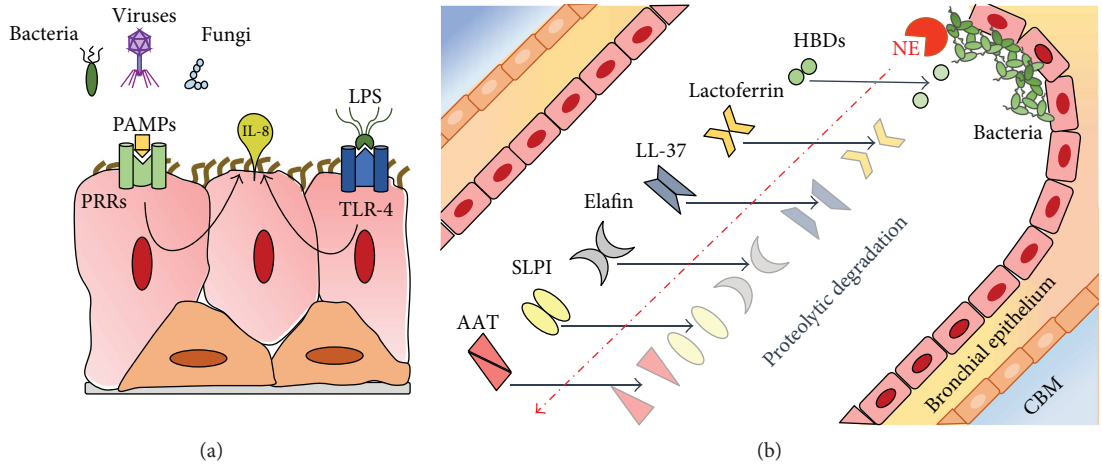

Figure 2: Airway innate immunity. (a) The major pattern recognition receptor (PRR) is the Toll-like receptor (TLR) family which interact with microbial-derived factors or pathogen-associated molecular patterns (PAMPs) such as LPS from *P. aeruginosa*. LPS-induced activation of TLR induces an IL-8 response via a NF-κB pathway. (b) Important antiproteases and antimicrobial proteins in the lung include AAT, SLPI, elafin, the cathelicidin LL-37, lactoferrin, and the human beta defensins (HBDs); these can be cleaved and inactivated by neutrophil elastase in the CF lung. CBM: capillary basement membrane.

PCL. Airway mucus is responsible for hydration of the epithelium and acts as an essential protective component of the innate defense system of the airways [38, 39]: inhaled microorganisms, noxious agents, and particles get entrapped in mucus and eliminated by mucociliary clearance (MCC) and/or cough, thus protecting the lung from airway infection and inflammation. The two main secreted airway mucins are MUC5AC and MUC5B [38, 40]; the latter but not MUC5AC seems to be required for airway defence against bacteria [41]. Properly hydrated mucus is a critical component of airway homeostasis; failure to maintain adequate mucus hydration, mucus hypersecretion, and altered mucus properties lead to impairment of mucociliary clearance and are prominent features of chronic airway diseases such as CF and COPD, contributing to airway obstruction in these diseases. Ion channels that regulate Na^+ and Cl^- transport are required for the proper hydration and composition of ASL. CFTR is the primary apical ion channel involved in Cl^- transport in the lung epithelium; in addition, it is also implicated in the regulation of ENaC that is rate limiting for Na^+ and fluid absorption from the airway surface. Additional ion channels in the airway epithelium play a role in maintaining ASL homeostasis, including the solute carrier 26A (SLC26A) family of anion exchangers and calcium-activated chloride channels [42, 43]. Alterations in ASL composition and disruption of this tightly regulated airway milieu have been linked to the pathogenesis of CF lung disease and COPD. In CF, the defect/dysfunction of the CFTR protein causes a defect in chloride and bicarbonate transport into the airway lumen that results in dehydration and acidification of ASL, production of viscous, acidic, and altered secretions, with impairment of MCC; this in turn induces airway obstruction, favouring chronic bacterial infection and inflammation (Figure 3). Recent studies showed that the ASL volume depletion causes a collapse of cilia and impairs mucus transport, further supporting the concept that ASL dehydration is a crucial mechanism in CF lung disease [42]. The role of airway surface dehydration as a disease-initiating mechanism is also supported by studies in βENaC transgenic mice and in SCL26A9 mice [44–48]. Recent biophysical studies provided novel insight on the effects of airway surface dehydration, showing that this leads to an increase of the concentration of secreted mucins and consequently of the osmotic pressure of the mucus layer; when the latter increases above a critical threshold, the PCL and the cilia get compressed leading to stasis and adhesion of mucus on the airway surface [49]. This critical mucus hyperconcentration may be induced not only by the CFTR ion transport defect but also by airway inflammation; thus, it may play an important role in the pathogenesis of chronic inflammatory diseases such as COPD [50].

In addition to airway surface dehydration, the impaired bicarbonate secretion mediated by CFTR dysfunction results in altered mucus structure, increased viscosity and abnormal mucus secretion; therefore, it may contribute to mucus plugging and the impairment of MCC in CF lung disease beyond that caused by airway dehydration alone. Several recent studies support the key role of bicarbonate secretion in regulating local pH and the airway milieu and in maintaining normal mucus properties: bicarbonate, in fact, drives ionic content and fluids on epithelial surfaces and allows mucins to unfold, and its depletion has been shown to result in dense mucus and increased ASL viscosity thus impairing mucociliary transport [51–53]. Altered ASL viscosity would be a primary CF defect that might contribute, at least in part, to the pathogenesis of CF lung disease. Subsequently, infection, inflammation, and airway remodelling, with their consequences, may further modify ASL viscosity and further enhance the defect of mucociliary transport. Loss of CFTR-mediated bicarbonate secretion has also been shown to inhibit the activity of antimicrobial peptides present in ASL [53]. Thus, dysfunction of CFTR and the subsequent lack of bicarbonate and chloride secretion create an abnormal airway milieu, with impaired innate host defences that favours chronic airway infection and inflammation leading to progressive lung

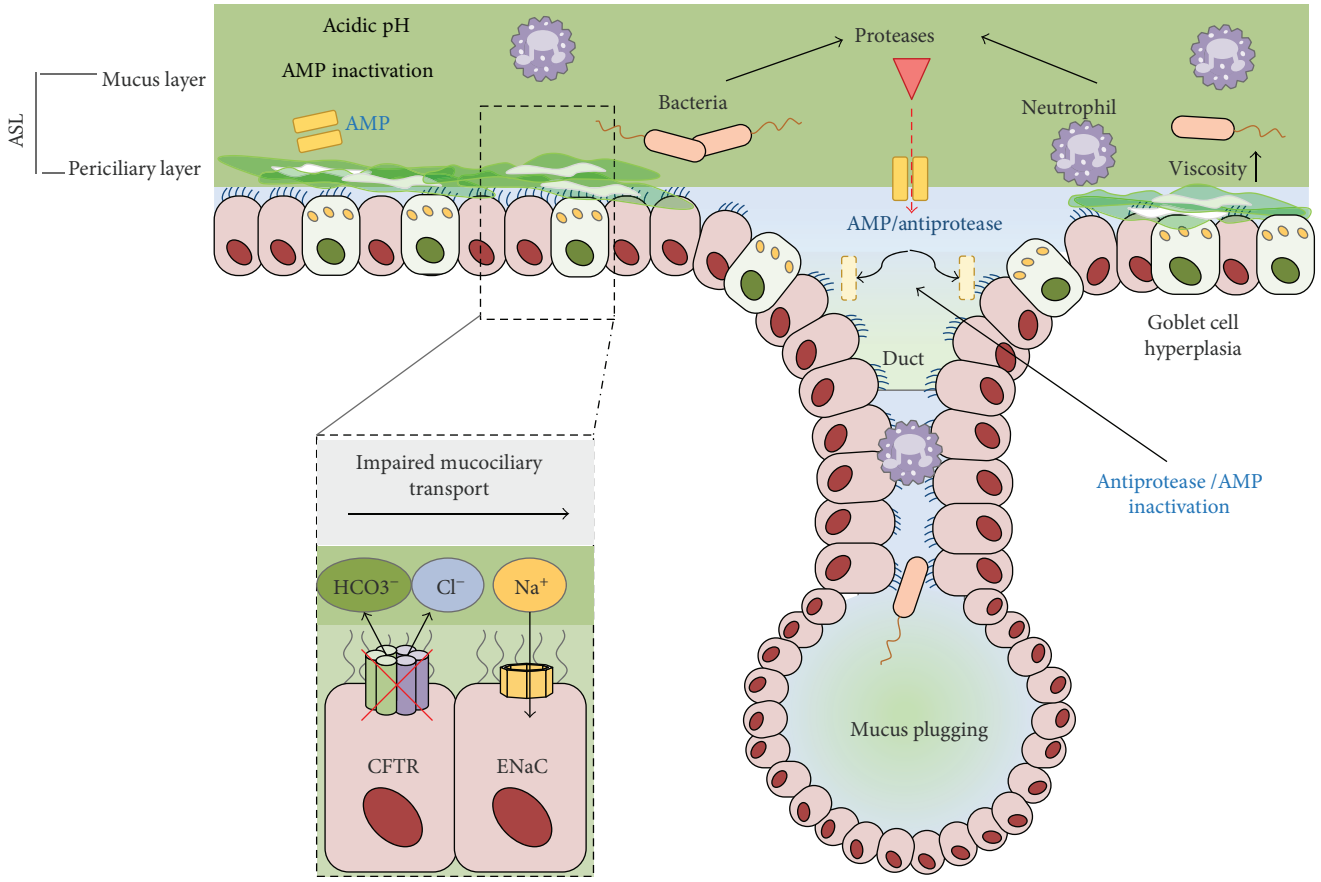

Figure 3: Pathophysiology of airway disease in cystic fibrosis. In cystic fibrosis, loss of normal CFTR function and overactivity of ENaC result in acidification (loss of $HCO3^-$ secretion) and dehydration of the airway surface liquid layer (ASL) disrupting the normal mucociliary escalator. This results in an increase in ASL viscosity, mucus plugging, bacterial colonization, and neutrophil-dominated inflammation. An overabundance of bacterial and neutrophil derived proteases degrades important antiproteases and antimicrobial peptides in the CF airways further compounding an already-overwhelmed impaired innate immune system.

injury and ultimately to respiratory failure (Figure 3). Furthermore, recently, it has been shown that the loss of CFTR leaves H^+ secretion by the nongastric H^+/K^+ adenosine triphosphatase (ATP12A) unchecked, which further decreases the ASL pH further impairing airway innate host defences [53].

Cigarette smoking is the major risk factor for COPD, a disease that shares some phenotypical features with CF, such as impaired mucociliary clearance, chronic airway inflammation, progressive airflow obstruction, and recurrent bacterial exacerbations. In particular, COPD patients with the chronic bronchitis phenotype exhibit pathologic and clinical features similar to CF, including goblet cell hyperplasia, mucin hyperexpression, and mucus accumulation and hypersecretion that contribute to bacterial infection and subsequent inflammatory responses and have been associated with lung function decline in COPD patients (Figure 1).

Increasing evidence shows that cigarette smoking induces an acquired CFTR dysfunction in patients with normal CFTR gene. Welsh firstly reported that cigarette smoke decreases chloride secretion in the airway epithelium [54]. Several subsequent studies then showed that sustained exposure to cigarette smoke and cigarette smoke extract (CSE) was able to reduce CFTR expression and function *in vivo* in smokers and in COPD patients and in airway epithelial cells *in vitro* [55–58]. Several independent mechanisms have been proposed to account for these effects of smoke, including aberrant transcript expression, direct effects of smoke metabolites on CFTR function, and accelerated internalization of CFTR Cl^- channels from the apical plasma membrane (Figure 4) [55, 56, 58–61]. Smoke-induced CFTR dysfunction results in ASL dehydration, and it was shown that ASL height decreased permanently after chronic smoke exposure due to changes in active ion transport, indicating an effect of smoke on ASL homeostasis. It has also been shown that CSE is capable of inducing a delayed mucociliary transport *in vitro* and a marked increase of mucus expression both *in vitro* and *in vivo*. CSE has also been shown to be capable of inducing MUC5AC expression and mucus hyperproduction in AEC, an effect that seems to be mediated through ROS-dependent autophagy [62–64]. Together with mucin hypersecretion, smoke-induced CFTR dysfunction will aggravate mucus hyperconcentration and plugging in COPD airways (Figure 4) [42, 65–68]. Tobacco smoke components such as acrolein and acetaldehyde have been shown to impair mucociliary clearance [69]; furthermore, acrolein and

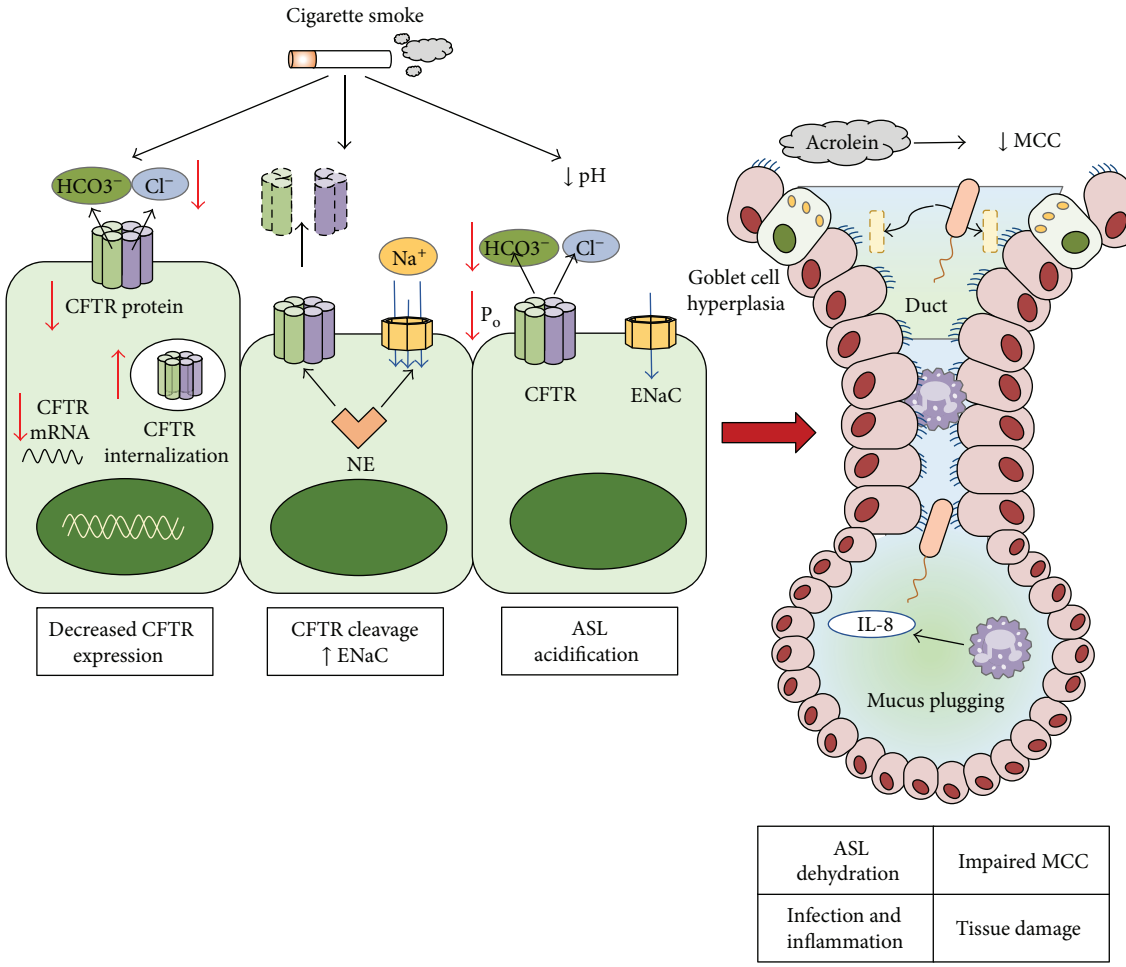

Figure 4: Effects of cigarette smoke on CFTR and pathophysiology of airway disease in COPD. Cigarette smoke components (e.g., acrolein and cadmium) can decrease expression and function of the CFTR protein by decreasing CFTR mRNA and protein levels, accelerated CFTR internalization, and decreased channel opening probability (P_o). Increased levels of neutrophil elastase in COPD may worsen CFTR dysfunction by degrading CFTR and upregulating ENaC expression. Loss of CFTR function results in ASL dehydration and acidification, mucus hypersecretion, and mucus plugging leading to reduced mucociliary clearance, chronic inflammation, impaired innate immunity, and infection.

cadmium inhibit CFTR function *in vitro* and can reach detectable levels in humans as well (Figure 4) [70, 71].

In ciliated cells from nasal brushing of smoke-exposed subjects and moderate/severe COPD patients, ciliary beat frequency was significantly reduced compared to control subjects [72, 73]. Furthermore, CS exposure has been shown to affect ciliogenesis and to cause ciliary shortening [74], effects that further contribute to the impairment of MCC.

Reduced MCC leads to accumulation and adhesion of mucus in the airways, favouring retention of irritants and noxious agents contained in cigarette smoke. This in turn triggers exaggerated inflammatory/immune responses, with recruitment of neutrophils and macrophages and excess of protease release that promotes the formation of emphysema in the lungs of smokers with COPD [60, 75]. Recent studies shows that CFTR protein expression correlates inversely with emphysema severity in lungs of COPD patients, suggesting that impaired CFTR function may also be implicated in emphysema formation in humans [76]. A role of CFTR dysfunction in the pathogenesis of emphysema is further supported by studies in βENaC transgenic mice demonstrating that CF-like airway surface dehydration does cause not only chronic mucus obstruction and airway inflammation but also emphysematous changes in these mice [44, 46, 77]. Interestingly, we demonstrated that genetic deletion and pharmacologic inhibition of PI3Kγ decreases both neutrophilic airway inflammation and structural lung damage in βENaC transgenic mice [78].

As previously discussed, defective CFTR also decrease bicarbonate secretion, that is, crucial in pH ASL regulation and in maintaining normal mucus properties. Tobacco smoke exposure decreases bicarbonate secretion presumably resulting in acidification of ASL and increased ASL viscosity (Figure 4) [42, 79, 80].

A marked decrease of CFTR function has been reported in the upper and lower airways of smokers and patients with COPD; interestingly, in COPD patients, significant correlations have been shown between clinical manifestations such

as dyspnea and symptoms of chronic bronchitis and the levels of CFTR suppression in their lower airways [56]. The marked oxidant burden and neutrophilic inflammation associated with COPD and particularly with advanced disease further aggravate the effects of CFTR dysfunction in COPD airways. Studies using cell lines expressing wild-type CFTR have shown that a prolonged exposure to oxidant stress with either t-butylhydroquinone or cigarette smoke extract was capable of inhibiting CFTR function, protein levels, and gene expression [55, 81, 82]. Furthermore, it has been reported that CS-induced CFTR dysfunction and the associated inflammatory/oxidative stress cause an impairment of autophagy that also contributes to the development of emphysema [56, 82, 83].

All these data support the concept that CFTR dysfunction caused by cigarette smoke may contribute to the pathophysiology of COPD and may represent a potential target for the development of novel therapeutic approaches.

4. Innate Immune Response in CF and COPD Epithelium

The airway epithelium contributes to host defence by a variety of mechanisms including its barrier function and mucociliary clearance, as well as by the production of antimicrobial peptides and proteins and a range of cytokines, chemokines, and growth factors that mediate leukocyte recruitment, modulation of innate and adaptive immunity, and tissue repair and remodelling [84–86]. Through these mechanisms, the airway epithelium contributes directly to host defence and augments the immune response via the recruitment of inflammatory/immune cells and the interaction with other structural cells in the airway wall.

4.1. Innate Immune Responses in CF Epithelium. A vicious cycle of infection followed by intense neutrophil-dominated inflammation, ineffective clearance of infection, and irreversible airway destruction is characteristic of CF. Airway inflammation is ultimately driven by a dysfunctional epithelium due to an impairment in innate host defence systems, secondary to mutations of CFTR [87–90]. Defective bicarbonate secretion due to CFTR dysfunction reduces ASL pH, impairing the activity of antimicrobial peptides and mucus properties. Despite the ineffectiveness of the CF airway surface to remove mucus, goblet cells are likely to continue secreting mucins [91] leading to plugging. Specific defects in many aspects of the lung's innate immune repertoire are evident in CF.

The CF airway is a TLR agonist-rich environment, and chronic inflammation in CF may for the most part be due to activation of TLRs [92]. Although TLR hyperresponsiveness is largely accepted to contribute to CF lung disease, hyporesponsiveness to TLRs can also negatively impact on pulmonary inflammation. During high circulating oestrogen (E2) states in the female CF airways, a TLR hyporesponsiveness, manifested by reduced IL-8 release, occurs in response to a range of bacterial agonists [93]. A hyporesponsive state may be problematic, leading to ineffective clearing of microorganisms.

NOD1 (or CARD4) and NOD2 (or CARD 15) detect peptidoglycan of Gram-positive or Gram-negative bacteria, and their activation causes induction of proinflammatory NF-κB-dependent cytokines [94]. IL-17 has been shown to augment expression of NOD1, NOD2, and TLR4. By upregulating these PRRs, IL-17 primes the airway to increase its inflammatory response (IL-8) to bacterial ligands. Interestingly, pretreatment of CFTRΔF508 cells with IL-17 resulted in a 10-fold increase in IL-8 synthesis following treatment with a NOD1 agonist, highlighting the importance of NOD1 as contributor to aberrant inflammatory responses in the CF lung [95].

Polymorphisms in the mannose-binding lectin 2 (MBL2) promoter can affect MBL serum levels. Relative deficiency of MBL appears to accelerate the age-related decline in lung function in CF patients as MBL-deficient patients older than 15 years of age displayed poorer lung function compared with those younger than 15 years of age [96].

Likewise, relative reduction in SP-A and SP-D has been observed in patients with CF [97] and levels inversely correlate with inflammation and bacterial burden [98]. The cause may be related to the ability of *P. aeruginosa* elastase and protease IV to degrade SP-A and SP-D in the CF lung [99, 100]. Furthermore, CFTR-dependent alterations in complement-mediated interactions between *P. aeruginosa* and monocytes may contribute to enhanced susceptibility to infection in patients with CF [101].

An inherent proinflammatory state exists in the CF airways and may precede bacterial colonization. Overproduction of the neutrophil chemokine IL-8 by CF airway epithelial cells may be a consequence of both intrinsic CFTR dysfunction [102] and infection [103]. Elevated concentrations of proinflammatory cytokines including TNF-α, IL-1, and IL-8 have been found in children with CF [104]. TNF-α concentrations in sputum are not significantly correlated with clinical status but do show a strong correlation with sputum IL-8 levels [105].

Interleukin 17 (IL-17-A/F) is a proinflammatory cytokine with roles in the immunopathogenesis of CF and COPD. Elevated IL-17A mRNA and protein in CF sputum implicate this cytokine in the persistent neutrophilic infiltration in CF lung disease [106]. IL-17 positively regulates the production of proneutrophilic mediators from CF epithelial cells by increasing IL-8 and IL-6 [107]. IL-17A is significantly higher in BALF of symptomatic patients as compared with clinically asymptomatic patients with CF, and its increased concentrations precede infection with microbes such as *P. aeruginosa* and thus have clinical relevance [108].

Antiproteases within the CF lung are susceptible to cleavage and inactivation by host proteases (Figure 2(b)). SLPI is susceptible to cleavage by cathepsins B, L, and S [109] and elafin by NE in lungs of patients colonized with *P. aeruginosa* [110]. Although levels of AAT rise in acute inflammation, in the CF lung, the inhibitory activity of AAT is overwhelmed by an excess of NE.

While lactoferrin has been reported to be present at higher concentrations in the CF lung compared with normal lungs [111], its activity may be suboptimal secondary to cleavage by *Pseudomonas* elastase and NE [112].

Cleavage of lactoferrin by cathepsins B, L, and S also results in loss of its microbicidal and antibiofilm activity, and in the CF lung, this may potentiate the biofilm forming ability of *P. aeruginosa* [113].

The effectiveness of LL-37 may be hampered in the CF lung through complexing with polymers including GAGs or DNA released from the breakdown of neutrophils and other cells [114, 115].

Finally, variant alleles (single-nucleotide polymorphisms) in the DEFB1 gene encoding HBD-1 may contribute to the colonization of *P. aeruginosa* in CF [116]. Beyond that, cysteinyl cathepsins which are present at higher than normal levels in the CF lung cleave and inactivate HBD2 and HBD3 [117].

4.2. Innate Immune Responses in COPD Epithelium. An increasing number of studies demonstrate that epithelial defence functions are altered or decreased in chronic airway diseases including COPD and that, as described in CF, defects in the innate immune system play a relevant role also in the pathogenesis and pathophysiology of COPD [5, 42, 118–123]. Cigarette smoke induces relevant alterations in the airway epithelial architecture and impairs epithelial barrier function by increasing the permeability of the airway epithelium, decreasing ciliary function, and reducing mucociliary clearance. This epithelial barrier dysfunction in turn can increase the entry of pathogens and noxious particles into the airway mucosa, further impairing the barrier function and host defences [4, 5, 39, 124, 125–130]. Cigarette smoke induces mucous cell hyperplasia and mucus hypersecretion contributing to airway obstruction; in the small airways, smoking-induced goblet cell hyperplasia is associated with loss of club cells, responsible for the production of secretoglobin, surfactant protein, and other defense factors [131]. A reduced expression of polymeric immunoglobulin receptor/secretory component (pIgR/SC) has also been reported in the COPD epithelium that impairs the transepithelial transport of secretory IgA and correlates with the severity of airflow obstruction [132]. Cigarette smoke may affect epithelial cell functions through a variety of mechanisms, including direct oxidant activity, TLR signalling, ER stress induction, and activation of the integrated stress response [133, 134]. In addition to impairing barrier function, cigarette smoke exposure increases the release of inflammatory mediators by AEC, whereas it decreases the expression and activity of ASL antimicrobial peptides (AMP). This defect, caused by smoking-induced CFTR dysfunction, is common to CF airways and contributes to the increased susceptibility to respiratory infections shared by both diseases. The antimicrobial activity of AMPs may also be affected by other conditions occurring in both CF and COPD airways such as AMP degradation by microbial and host proteases and AMP inhibition by microbial polysaccharides, F-actin, and DNA from dying cells. Cigarette smoke also inhibits the molecular pathways involved in interferon production in response to viral infections [135]. Whereas the expression and activity of epithelial AMP are impaired in COPD, the levels of neutrophil-derived peptides and LL-37 are increased as a consequence of the marked neutrophilic inflammation in this disease and further increase during disease exacerbations [136–145]. Likewise, increased levels of lysozyme and lactoferrin have been reported in BALF of patients with COPD [121, 146], whereas SLPI levels were reported to be increased in stable patients, but decreased during disease exacerbations [144, 147, 148]. However, some inconsistent findings concerning sputum AMP levels in COPD patients have been reported in previous studies [136–139, 149] that may be due to the small sample sizes of these studies and the different clinical conditions and disease stage of patients.

Exposure to cigarette smoke also leads to activation of several PRRs, either directly by components of smoke or indirectly by causing injury to epithelial cells [84, 121, 134, 150, 151]. As in CF, also in COPD, TLR4 has been suggested to play a key role in the inflammatory/immune response: TLR4-defective mice show attenuated lung inflammation after challenge with cigarette smoke [152–154]. Elevated levels of damage-associated molecular patterns (DAMPs), such as high-mobility group box 1 (HMGB1) [155], uric acid, and extracellular ATP [156], have been observed in the BALF of patients with COPD compared with smokers without COPD. Extracellular ATP has been shown to activate the NLRP3 inflammasome, regulating the expression of interleukin-1β and IL-18, [153] particularly during disease exacerbations [157, 158]. In IL-1R knock-out mice, pulmonary inflammation induced by acute exposure to cigarette smoke was attenuated and these mice were protected against emphysema after chronic smoke exposure [159].

Activation of AEC by cigarette smoke or pathogens also triggers an inflammatory response, with the release of cytokines and chemokines such as TNF-α, IL-1, IL-6, GM-CSF, and IL-8 acting on inflammatory/immune cells as well as on resident cells [5, 84, 85, 119, 121, 134, 150, 152, 158].

Similar to what occurs in CF airways, activated AEC from COPD patients release more IL-8 than cells from smoking control subjects [160] and display a proinflammatory phenotype in culture [161], although the molecular mechanisms are different in CF and COPD. Levels of IL-17 and other Th17 cytokines are also increased in sputum and airways of patients with COPD as reported in CF, further enhancing neutrophil recruitment. [162, 163]. Whereas physiologically the inflammatory process induces protective immune responses, in patients with COPD and CF, chronic airway inflammation amplifies the tissue damage and further impairs local immune defences, thus contributing to susceptibility to recurrent infections. Neutrophils and alveolar macrophages are increased and activated in COPD, as well as in the CF lung, and contribute to the oxidant burden and the protease/antiprotease imbalance that drive the development of emphysematous changes in both diseases. Furthermore, neutrophils from COPD patients show altered chemotactic responses [164] that impair their defence function whereas macrophages show reduced phagocytic ability [165–167] and impaired innate responses to respiratory pathogens. These alterations facilitate the chronic colonization of the airways and infectious exacerbations [168]. An increased number and activation of dendritic cells in the lung of patients with COPD have also been reported in some studies that seem to correlate with disease severity [169–171],

whereas other studies have found decreased numbers of these cells and an impaired maturation status [172–175]. Recent studies suggest that innate lymphoid cells (ILC), with important roles in immune homeostasis and lung immunity, may play a role in COPD [176–178]. In mice exposed to cigarette smoke, a strong type 1 response to influenza virus was observed that was associated with a transdifferentiation of ILC2 into ILC1 cells, induced by IL-12 and IL-18 [178]; in patients with COPD, an increase of ILC1 and a decrease of ILC2 cells was reported in lung tissue [178, 179]. Finally, recently, it has also been reported that pulmonary natural cytotoxicity receptor- (NCR-) ILC3 cells tend to accumulate into the lungs of COPD patients [177]; these cells produce IL-17A and IL-22 and might contribute to driving neutrophilic inflammation in the COPD airways. Interestingly, these cytokines are also crucial in the formation of lymphoid follicles [180, 181], the numbers of which are increased around the small airways and in the lung parenchyma [182–184] in severe COPD as well as in peribronchial, parenchymal, and perivascular areas in the lung of CF patients with advanced lung disease [185]. The peribronchial localization of tertiary lymphoid organs and the epithelial expression of IL17A and chemokines involved in their development suggest a role for airway epithelium in lymphoid neogenesis in these diseases. However, differently from COPD, a shift from B cell to T-cell predominance has been observed in CF lymphoid follicles, suggesting that the cellular adaptive immune response is specifically affected in CF [185]. Recently, Frija-Masson and colleagues [186] showed that peribronchial lymphoid neogenesis was induced in the lungs of mice upon persistent bacterial infection, suggesting that chronic bacterial infection contributes to the lymphoid neogenesis observed in both COPD and CF.

A growing body of evidence now suggests that dysfunction of epithelial innate immune responses and the consequent chronic airway inflammation can drive the initiation, exacerbation, and progression of chronic inflammatory disease such as COPD and CF. In this context, therapeutic targeting of dysfunctional immune responses could be an interesting strategy in the treatment of these diseases.

5. Comparison of Epithelial Remodelling in CF and COPD

Efficient epithelial regeneration following injury is crucial in tissue homeostasis and prevention of disease. Exposure to repeated noxious/inflammatory stimuli as well as airway epithelial dysfunction affects epithelial regeneration and repair pathways and the ability of epithelial cells to restore barrier functions; this leads to aberrant remodelling and structural damage that further impair epithelial functions and generate a vicious cycle favouring aggressions by potential exogenous insults.

Both CF and COPD are characterized by dysfunctional airway epithelial repair and remodelling that impair lung architecture and contribute to disease pathogenesis and progression.

In CF, progressive remodelling of the airways ultimately results in structural damage with development of bronchiectasis, emphysema, and impaired lung function. Differently from COPD, structural changes of the airway and alveolar wall in CF appear early in life. There is still debate about the sequence of events leading to remodelling and structural alterations in CF and their relationship to infection and inflammation; in particular, it is still debated whether structural changes are related to and initiated by infection/inflammation or are a result of CFTR dysfunction independent of infection and inflammation [87–90, 102–104]. Hyperplasia of goblet cells and basal cells [187–190], squamous metaplasia [187, 190, 191], increased epithelial height [187, 192], cell shedding [187–190, 193], with loss of ciliated epithelial cells, and a disorganization of tight junctions and compound cilia [192, 194–196] have been reported in the context of epithelial remodelling in CF airways; extensive structural changes of the small airway epithelia have also been observed, including epithelial shedding and altered barrier integrity [197]. Conflicting results have been reported concerning epithelial and reticular basement membrane (RBM) in adult patients that was found thickened in some studies [189, 198, 199], but significantly thinner than normal in other studies [200]. Interestingly, many of these structural changes are similar to those observed in COPD, although the molecular mechanisms initiating these changes are different.

Whereas until recently, little was known about the frequency of occurrence and the clinical relevance of emphysematous changes in CF lung disease, recent studies show that, with the remarkable increase of patient survival, emphysema is now a more prominent disease component in CF. Using quantitative CT measurements, Wielpütz and colleagues [201] showed that early onset and progressive emphysema is a characteristic feature of CF lung disease; emphysema is observed in early adolescence, increases in adult CF patients, correlates with lung function, and contributes to disease severity. Mets and colleagues [202] pathologically and radiologically confirmed that emphysema is common in advanced CF lung disease, is related to age, and in some cases approaches the changes observed in explanted lungs of COPD patients. Thus, emphysema might become an increasingly important disease component in the aging CF population. Several mechanisms mainly related to CFTR dysfunction may induce emphysema formation in CF patients; among these, chronic inflammation with protease/antiprotease imbalance and extracellular matrix proteolysis, altered ceramide metabolism, alveolar apoptosis, and defective autophagy [63, 76, 203]. Most of these mechanisms are common to those involved in the pathogenesis of smoke-induced emphysema.

Some studies have reported that CFTR plays an important role in regulating the early events of epithelial cell migration [188, 198, 200] and that CF airway epithelial cells exhibit slower migration and wound repair than non-CF cells [188, 198, 204]. The findings that the pharmacological rescue of CFTR function in CF cells significantly improved wound healing and that inhibition of CFTR expression or activity decreased proliferation and migration of non-CF airway epithelial cells [188, 198] further support the role of CFTR in modulating airway epithelial cell

migration and the concept of a dysfunctional airway epithelium in CF. Data obtained on tissues from CF fetuses (taken at autopsy) or in a humanized nude mouse xenograft model and in CF mice suggest that the epithelial remodelling starts to occur prenatally and in the absence of infection [205–207]. A recent *in vitro* study, using CF human airway epithelial cell cultures, confirmed and extended these findings, showing that CF epithelium regeneration, even in the absence of exogenous infection/inflammation, was abnormal and associated with basal cell hyperplasia and with delayed ciliated cell differentiation. The findings of this study demonstrate that the abnormal remodelling in CF epithelium was partly induced by the intrinsic hyperinflammatory phenotype of CF cells [208]. Interestingly, in this study, an increase in CF epithelium height was observed in the context of epithelium remodelling. An increased epithelium height was previously reported *in vivo* in CF lungs as compared with lungs from COPD patients [192] and in these latter patients as compared with non-COPD subjects at both the nasal and bronchial levels [209]. Similarly, basal cell hyperplasia, a finding associated with increased epithelium height, was also reported *in vivo* in CF [187, 189] and COPD lungs [210]. It has been already emphasized that chronic inflammation is a hallmark of CF; inflammation begins early in the disease even in the apparent absence of infection, and it has been postulated that it may be dysregulated in the CF lung as a consequence of defective CFTR [87–90, 102–104, 211, 212]. Interestingly, stimulation of non-CF airway epithelial cells with proinflammatory cytokines mimicked the abnormal remodelling observed in CF epithelial cells and induced an increase of epithelium height and basal cell numbers comparable to those observed in CF cultures [208]. Overall, the findings of the previous studies suggest that exogenous infection and inflammation are not the exclusive factors affecting airway epithelium remodelling in CF and that the intrinsic dysfunction of CF airway epithelial cells plays an important role in this process and in the alterations of epithelial regeneration following injury. A growing number of *in vitro* and *in vivo* studies support the notion that chronic neutrophilic inflammation is a key factor in structural lung damage and lung function decline in CF through the release of damaging neutrophil products such as NE and reactive oxidant species [211, 212]. Airway inflammation is further augmented after onset of chronic airway infection. A vicious cycle of neutrophilic inflammation, noxious mediator release, and overwhelmed defences occurs within the airways that further exacerbates epithelial injury and remodelling, leading to disease progression and irreversible lung damage. The chronic neutrophilic inflammation, heightened during acute exacerbations of the disease, is a common process in CF and COPD and may be involved in the common abnormalities observed *in vivo* in remodelled airway epithelium, as well as in the destructive alterations of airway and alveolar walls.

There is evidence of degradation of structural components of the extracellular matrix (ECM) such as elastin, collagen, and glycosaminoglycans in the CF lung; these alterations are associated with the marked and early protease/antiprotease imbalance, with the release of high quantities of matrix metalloproteases (MMP), NE, and other proteases that contribute to the tissue damage and development of bronchiectasis and emphysema [213–216]. There is increasing evidence that the high levels of free NE activity observed in sputum and BALF from CF patients not only are able to cleave a number of ECM proteins but have also other direct and indirect deleterious effects on the epithelial repair process: in fact, they significantly impact the adhesion, proliferation, and wound repair of primary AEC [217]. There is also considerable *in vivo* evidence of an imbalance of MMP and their inhibitors (tissue inhibitors of MMP, TIMPs) in the CF airways with prevalence and activation of MMP [214, 218–221]. Activation of MMP-7 has been shown to inactivate AAT, thus further augmenting the effect of free NE activity [221], whereas the release and activation of MMP12 is involved in alveolar wall destruction [63]. A recent proteomic study of CF AEC showed significantly increased secretion of ECM proteins fibronectin, laminin, perlecan, and galectin-3 [222], whereas the CFTR defect was associated with a decreased integrin $\beta 1$ signaling and a reduced wound repair [223]. These studies further support a role of mutant CFTR protein in the abnormal migration and reparative properties of the CF epithelium. Fibrotic changes associated with small airway narrowing have also been described, in particular in end-stage CF [224–226]. A recent study, using micro CT and histology documented dilatation and obstruction of distal airways and a severe reduction in the number of functional terminal bronchioles in end-stage CF lungs, confirming that, similar to COPD, obstruction and remodelling of peripheral airways are a prominent feature in CF lung disease [226].

Similar to CF, the airway epithelium represents a primary site of relevant molecular and morphologic changes in COPD; structural modifications of the epithelium in COPD resemble that observed in CF and include basal cell hyperplasia, squamous metaplasia, goblet cell hyperplasia, decreased integrity of the apical junctional barrier, and epithelial shedding [132, 222, 227]. Moreover, a reduction of cilia length and ciliary beating have been observed in COPD and upon cigarette smoke exposure [72, 127]; altered function and/or number of ciliated and goblet cells impair mucociliary clearance, contributing to the development of airway obstruction similar to what occurs in CF. Although pathologic changes in COPD lungs involve both the airways and parenchyma, studies by Hogg and colleagues clearly showed that smoking-induced changes in the small airways are crucial in the development of airway obstruction in COPD and precede destruction of the alveolar structure [228], similar to what was observed in CF. Interestingly, recently, it has been reported that smoking is able to induce a distal-to-proximal reprogramming of the small airway epithelium in COPD lungs, with a shift toward the proximal airway epithelial phenotype. These changes represent a novel feature of small airway pathology in COPD and seem to be mediated by exaggerated epidermal growth factor/epidermal growth factor receptor signaling in small airway epithelium basal cells [229]. An increased expression of epithelial growth factor receptors

has been described in AEC of COPD patients, which might contribute to squamous metaplasia and an increased risk of bronchial carcinoma [230].

Normal differentiation of epithelial cells in COPD is altered both in terms of cellular functions and of the polarity of the epithelial cells, notably through epithelial to mesenchymal transition (EMT). This leads to dysfunctional physical, chemical, and immune barrier functions; whilst aberrant repair and remodelling of the COPD epithelium further impair epithelial functions. Cigarette smoke increases epithelial permeability by disassembly of tight junction proteins via the epidermal growth factor receptor pathway [124]. This may favour viral, fungal, and bacterial translocation through the epithelium.

As described for CF AEC, Perotin et al. recently showed that wound repair is delayed in primary cultures of severe and very severe COPD [231]. The epithelial cells undergoing EMT lose their epithelial phenotype, become able to migrate along and through the basement membrane, and presumably participate to peribronchial fibrosis [232]. EMT is reported both in upper airways (in chronic sinusitis) [233, 234] and in small and large lower airways in COPD [235] and is induced by cigarette smoke and by TGF-β [236, 237]. Cigarette smoke also promotes Wnt signaling: Heijink and colleagues reported that cigarette smoke increases Wnt-5B expression in COPD AEC cultures and favours a mesenchymal phenotype through a mechanism involving TGF-β/Smad-3 [238]. More recently, Baarsma and colleagues showed an enhanced expression of noncanonical Wnt-5A in experimental models of COPD and in human COPD that functionally impaired canonical Wnt signal-driven alveolar epithelial cell repair [239]. Overall, these findings suggest that alterations in Wnt signalling may contribute to abnormal repair and remodelling in COPD. As already highlighted, a reduced expression of the epithelial pIgR has also been reported in COPD that impairs the transepithelial transport of secretory IgA [132, 240, 241]. Interestingly, a recent study shows that pIgR-deficient mice develop COPD-like airway and parenchymal remodelling, resulting from persistent activation of inflammatory signalling by an altered lung microbiome, thus suggesting that S-IgA deficiency may play a role in small airway remodelling and disease progression in COPD [242] These findings are consistent with the results of previous studies showing that reduced pIgR expression in COPD epithelium correlates with the severity of airflow obstruction in this disease [241].

The dedifferentiation of the COPD epithelium through aberrant regenerating mechanisms is responsible for an altered physical, chemical, and immune response to inhaled particles and microbes. Furthermore, epithelial cell activation by cigarette smoke and other inhaled irritants in COPD induces the release of inflammatory mediators, further enhancing chronic airway inflammation. All these events can increase airway epithelial damage and promote an excess of extracellular matrix deposition and airway fibrosis, driving disease progression in COPD [158]. Restoration of the epithelial integrity by targeting signalling pathways involved in aberrant repair and remodelling is promising but will need further research [243, 244].

6. Conclusions and Therapeutic Perspectives

Cystic fibrosis is a genetic disease caused by mutations in the CFTR gene, whereas COPD is mainly caused by environmental factors—mostly cigarette smoking—on a genetically susceptible background [1–4]. Although the etiology and pathophysiology of these diseases are different, they share key phenotypical features such as chronic neutrophilic inflammation, progressive airflow obstruction, and recurrent infectious exacerbations, suggesting the possibility of common mechanisms. In particular, COPD patients with the chronic bronchitis phenotype exhibit pathologic and clinical features similar to CF, including goblet cell hyperplasia, mucin hyperexpression, mucus accumulation, and hypersecretion that contribute to bacterial infection and subsequent inflammatory responses, and have been associated with lung function decline in COPD patients. Growing evidence suggests that cigarette smoking induces an acquired CFTR dysfunction in patients with COPD, reducing the expression and/or function of the protein, and that this CFTR dysfunction is involved in most of the pathogenetic pathways common to both COPD and CF.

Airway epithelial cells are among the first sites of contact for pathogens and other noxious environmental irritants and play a critical role in maintaining normal airway functions and the homeostasis of the airway milieu. Relevant molecular and morphologic changes occur in the airway epithelium in both CF and COPD, similar in some aspects, and a growing body of evidence suggests that airway epithelial dysfunction is involved in disease initiation and progression in both diseases. Structural and functional abnormalities in both airway and alveolar epithelium have a relevant impact on alteration of host defences, immune/inflammatory responses, and the repair processes, leading to progressive lung damage and facilitating chronic and recurrent airway infections in both CF and COPD.

An increasing number of studies show that CFTR dysfunction affects several physiological processes relevant to airway epithelial cell functions, including chloride and bicarbonate transport, mucin secretion and MCC, host defences, and inflammatory/immune responses. Thus, CFTR dysfunction represents a potential target for the development of novel therapeutic approaches. An improved understanding of the common mechanisms affected by CFTR dysfunction in CF and COPD and the relevance of CFTR dysfunction in the different COPD phenotypes would provide new insights into disease pathogenesis and will be important for developing novel therapeutic strategies relevant to both diseases. Recently, innovative and specific therapies targeting the CFTR defect have been developed; in the context of the increasing evidence about the role of CFTR dysfunction in COPD, the possibility that therapies designed to correct the CFTR dysfunction may benefit not only CF patients but also patients with COPD is attractive and need further studies.

Conflicts of Interest

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

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Hindawi
Mediators of Inflammation
Volume 2018, Article ID 7501851, 14 pages
https://doi.org/10.1155/2018/7501851

Research Article

Long Noncoding RNAs and mRNA Regulation in Peripheral Blood Mononuclear Cells of Patients with Chronic Obstructive Pulmonary Disease

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Received 9 August 2017; Revised 8 November 2017; Accepted 27 November 2017; Published 13 March 2018

Academic Editor: Catherine Greene

Background. Inflammation plays a pivotal role in the pathogenesis of chronic obstructive pulmonary disease (COPD). We evaluated the lncRNA and mRNA expression profile of peripheral blood mononuclear cells (PBMCs) from healthy nonsmokers, smokers without airflow limitation, and COPD patients. *Methods*. lncRNA and mRNA profiling of PBMCs from 17 smokers and 14 COPD subjects was detected by high-throughput microarray. The expression of dysregulated lncRNAs was validated by qPCR. The lncRNA targets in dysregulated mRNAs were predicted and the GO enrichment was analyzed. The regulatory role of lncRNA *ENST00000502883.1* on *CXCL16* expression and consequently the effect on PBMC recruitment were investigated by siRNA knockdown and chemotaxis analysis. *Results*. We identified 158 differentially expressed lncRNAs in PBMCs from COPD subjects compared with smokers. The dysregulated expression of 5 selected lncRNAs *NR_026891.1 (FLJ10038)*, *ENST00000502883.1 (RP11-499E18.1)*, *HIT000648516*, *XR_429541.1*, and *ENST00000597550.1 (CTD-2245F17.3)*, was validated. The GO enrichment showed that leukocyte migration, immune response, and apoptosis are the main enriched processes that previously reported to be involved in the pathogenesis of COPD. The regulatory role of *ENST00000502883.1* on *CXCL16* expression and consequently the effect on PBMC recruitment was confirmed. *Conclusion*. This study may provide clues for further studies targeting lncRNAs to control inflammation in COPD.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by progressive airflow obstruction that is only partly reversible, inflammation in the airways, and other systemic effects [1]. So far there is no satisfactory therapy to treat individuals once the disease is established. The mechanism of the disease generally involves aberrant, chronic inflammation coupled with the loss of lung structural cells due to heightened apoptosis.

Long noncoding RNAs (lncRNAs) are mRNA-like transcripts longer than 200 nucleotides without protein-coding functions [2]. According to the genomic proximity to protein-coding genes, lncRNA was generally classified into five types: (1) sense, (2) antisense, (3) bidirectional, (4) intronic, and (5) intergenic [3]. lncRNAs have been shown to play important roles in diverse biological and pathological processes and are dysregulated in various human diseases [4]. However, few studies concerning the dysregulation and regulatory role of lncRNA in lung diseases have been reported. Several hundred lncRNAs were identified in the developing mouse lung by sequencing polyadenylated RNAs in embryonic and adult lung tissue. *NANCI* and *LL34* were found to regulate expression of hundreds of genes in mouse airway

epithelial cell culture, but their role in lung diseases remains unclear [5]. A recent study showed that lncRNAs including *RP11-46A10.4*, *LINC00883*, *BCYRN1*, and *LINC00882* act as miRNA "sponges" to regulate the growth of airway smooth muscle cells [6]. Bi et al. [7] analyzed lncRNA expression in the lung tissue of nonsmokers, smokers without COPD, and smokers with COPD; *RNA44121|UCSC-2000-3182* and *RNA43510|UCSC-1260-3754* were found the most over- and underregulated lncRNA, respectively.

Inflammation plays a pivotal role in the pathogenesis of COPD, where $CD8^+$ T lymphocytes, neutrophils, and macrophages are the main type of immune cells of local inflammatory milieu of COPD [8]. Different immunoregulatory properties of T cells and monocytes have been demonstrated in COPD patients [9]. In the previous study, we have analyzed the expression profile of miRNAs as well as regulation network of dysregulated miRNAs and mRNAs in PBMCs of COPD patients [10]. Several studies on lncRNA expression of peripheral blood mononuclear cells (PBMC) in other diseases have been reported [11, 12]. However, the lncRNA expression profile of PBMCs in COPD patients remains undone.

In this study, we sought to determine if lncRNAs were differentially expressed in PBMC of patients with COPD and if lncRNA expression may be linked to dysregulated mRNA expression relevant to the pathogenesis of COPD. We analyzed lncRNA and mRNA expression profiles in PBMC from COPD patients versus nonsmokers and smokers without airflow limitation. The lncRNA targets in dysregulated mRNAs were predicted.

2. Methods

2.1. Subjects. Peripheral venous blood was taken in heparin-coated tubes from 20 healthy nonsmokers, 17 smokers without airflow limitation, and 14 COPD patients at the Department of Respiration, The First Affiliated Hospital, Xi'an Jiaotong University, Xi'an, China. The COPD patients were eligible for this study if they met the following criteria: age ≥ 50 and ≤ 76 years; smoking history (≥ 20 pack years); postbronchodilator $FEV_1 \geq 25\%$ of predicted value and postbronchodilator FEV_1/forced vital capacity (FVC) ≤ 0.70; and no history of asthma, atopy (as assessed by an allergy skin prick test during screening) or any other active lung disease. Patients on home oxygen or with raised carbon dioxide tension (>44 mmHg), α_1-antitrypsin deficiency, recent exacerbation (in the last 4 weeks), an uncontrolled medical condition, or hypersensitivity to inhaled corticosteroids and bronchodilators were not eligible for the study. All nonsmokers and smokers without airflow limitation met the following criteria: age ≥ 42 and ≤ 75 years, post-BD FEV1% predicted > 80, no diagnostic cancer, diabetes, cardiovascular disease and hypertension, no use of inhaled or oral corticosteroids in the previous 6 months, no atopy, and no respiratory tract infection 1 month prior to the study. Patient characteristics are in Table 1. The experimental procedures were carried out in accordance with the approved guidelines. All experimental protocols were approved by the Research Ethics Boards of The First Affiliated Hospital, Xi'an Jiaotong University (2015-015) and the informed consent was obtained from all subjects.

Table 1: Clinical characteristics of nonsmokers, smokers without airflow limitation, and COPD patients.

	Healthy nonsmokers	Smokers without COPD	COPD
Number	20	17	14
Age	61 ± 14	56 ± 17	69 ± 8
Male/female	12/8	17/0	13/1
Current/ex-smokers	—	14/3	8/6
Post-BD FEV1% predicted	101.0 ± 8.6	92.9 ± 9.0	29.0 ± 10.6
Gold stage			
I		—	0
II		—	6
III-IV		—	8

Data are presented as mean ± SD. BD: bronchodilator; FEV1, forced expiratory volume in 1 s.

2.2. PBMC Isolation and RNA Extraction. PBMCs were isolated from venous blood by density gradient centrifugation over Ficoll-Paque PLUS reagent (GE Healthcare, Uppsala, Sweden) and suspended in QIAzol Lysis Reagent (Qiagen, Dusseldorf, Germany). Total RNA was extracted using miRNeasy Mini Kit (Qiagen) according to the manufacturer's procedure. RNA integrity was determined by formaldehyde-denaturing gel electrophoresis. We followed the methods of the previous study [10].

2.3. lncRNA and mRNA Microarray. Equal amount of RNA sample from each nonsmokers ($N = 20$), smokers ($N = 17$), or COPD patients ($N = 14$) was pooled, respectively, for lncRNA and mRNA profiling assay using Agilent Human lncRNA + mRNA Array v4.0 system by CapitalBiotech Company, Beijing, China. Each array contained a probe set comprising 40,000 human lncRNA transcripts and 34,000 human mRNAs. These lncRNA and mRNA target sequences were merged from multiple databases including Refseq, UCSC, H-InvDB, Human lincRNA catalog, NRED, lncRNAdb, and RNAdb, and 848 were from the Chen Ruisheng Lab (Institute of Biophysics, Chinese Academy of Science). Briefly, first-strand cDNAs were synthesized from 1 μg of total RNA using random or poly T primers carrying the T7 promoter sequence and the CbcScript II reverse transcriptase. Second strand cDNAs were then synthesized using RNaseH and DNA polymerase. The double-stranded cDNA was column-purified and used as a template to amplify cRNA by in vitro transcription reaction. The amplified cRNAs were purified and reverse transcribed into first-strand cDNA using CbcScript II reverse transcriptase. The second cDNA strand was then synthesized using Klenow enzyme, random primers, regular dNTP, and Cy3- or Cy5-labeled dCTP. The labeled cDNAs were hybridized with an array of analysis.

Table 2: The sequence of primers for real-time PCR.

Gene	Sequence (5′-3′)	Direction
NR_026891.1	GGACCACATCTCCTGTACCA	Forward
	CCTCATGACGTGCACTTTACC	Reverse
ENST00000502883.1	GTGTCCATGTAACTAACTCCTGGT	Forward
	GTCAGCGGGAAGGAAGACAG	Reverse
HIT000648516	ATAGAAGCGATCTACCCTCACAG	Forward
	TGCTGGGCTCGTTCGT	Reverse
XR_429541.1	TGGTCACTTTCCAGTTTCCACA	Forward
	AATTCAGATCCCACATCAGCCT	Reverse
ENST00000597550.1	GCCCTCCACCCCAGATTAAC	Forward
	CTCTTTCTTCTCTTCTGGACTTCT	Reverse
CXCL16	CTGAGAGCTTACCATCGGTGT	Forward
	TCAAGACAGCTCATCAATTCCT	Reverse
HMOX1	GGCCAGCAACAAAGTGCAAG	Forward
	ATGGCATAAAGCCCTACAGCA	Reverse
SLA2	GACATCTGCTGCCTACTCAAGG	Forward
	TGTGGCAGCTTCAGAAAACAGG	Reverse
SIGLEC14	CTGCACAGTTGACAGCAACC	Forward
	GTCTGGGAAGGATTGAGGGC	Reverse
β-Actin	TACCTCATGAAGATCCTCACC	Forward
	TTTCGTGGATGCCACAGGAC	Reverse

Hybridized slides were then washed and scanned with Agilent Microarray Scanner System (G2565CA).

2.4. Data Analysis. The lncRNA and mRNA array data were analyzed for data summarization, normalization, and quality control using GeneSpring V11.5 software (Agilent). To select differentially expressed genes, we used threshold values of ≥2-fold change, and a Benjamini–Hochberg-corrected p value of 0.05 performed on technically duplicated dots for each lncRNA. The data were Log2 transformed and median centered by genes using the Adjust Data function of CLUSTER 3.0 software. Further analysis was performed by hierarchical clustering with average linkages. Finally, we performed tree visualization using Java TreeView (Stanford University School of Medicine, Stanford, CA, USA).

2.5. Quantitative Reverse Transcription PCR Validation. Independent assays were performed using quantitative reverse transcription PCR (qRT-PCR) on all patient samples for individual lncRNA (*NR_026891.1*, *ENST00000502883.1*, *HIT000648516*, *XR_429541.1*, and *ENST00000597550.1*) and mRNAs (*CXCL16*, *HMOX1*, *SLA2*, and *SIGLEC14*) predicted to be regulated by lncRNAs. Total RNA was extracted using miRNeasy Mini Kit (Qiagen) according to the manufacturer's procedure. Quality control and RNA concentrations were determined by spectrophotometer (IMPLEN, Munich, Germany). The reverse transcription was performed on 500 ng of total RNA by using the iScript™ cDNA Synthesis kit (Bio-Rad). The cDNA was then amplified by using the CFX Connect™ Real-Time PCR Detection System (Bio-Rad) with SYBR Green (Bio-Rad) and the primers listed in Table 2. In addition, the expression of the aforementioned lncRNA was detected on the isolated different cell types including $CD4^+$ T cells, $CD8^+$ T cells, $CD14^+$ monocytes, and $CD20^+$ B cells from PBMCs in some smokers and COPD patients by positive selection (Anti-PE MicroBeads UltraPure, Miltenyi Biotec, Teterow, Germany). Data were presented relative to β-actin for lncRNAs and mRNAs based on calculations of $2^{-\Delta\Delta Ct}$. Statistical significance was defined as $P < 0.05$ as measured by the t-test using GraphPad Prism 5 software (GraphPad, San Diego, CA, USA).

2.6. lncRNA Target Prediction and Gene Ontology Analysis. The targets of differentially expressed lncRNAs were identified via cis or transregulatory effects. The validated differentially expressed lncRNAs were selected for target prediction. The genes transcribed within a 10 kb upstream or downstream of lncRNAs were considered as cis target genes. lncRNAs and potential cis target genes were paired and visualized using UCSC genome browser. The transtarget genes were chosen by BLAST software according to the impact of lncRNA binding on complete mRNA molecules. The gene ontology (GO) enrichment of dysregulated mRNAs predicted to be regulated by lncRNAs was analyzed by an integrated functional link enrichment of Gene Ontology or gene sets (http://lego.blueowl.cn/).

2.7. Stimulation of Cigarette Smoke Extract. The T lymphocyte cell line 6T-CEM (ATCC, Manassas, VA, USA) was stimulated with 5% cigarette smoke extract (CSE) [13] for 24 h, and the mRNA expression of ENST00000502883.1 and CXCL16 was examined by qRT-PCR.

2.8. lncRNA ENST00000502883.1 Knockdown by Small Interfering RNA (siRNA) Transfection. 6T-CEM cells were cultured in 24-well plates with a 5~7*10^5/ml density in RPMI1640 medium containing 10% FBS. The cells were then transfected with siRNA targeting lncRNA *ENST00000502883.1* (sequence: 5′-CAAACGUUCAUGUG AAAGATT-3′; 5′-UCUUUCACAUGAACGUUUGTT-3′) and synthesized by GenePharma, Shanghai, China, using the siRNA transfection reagent (Roche, Penzberg, Germany) for 48 h. The scrambled siRNA (GenePharma) was used as a negative control. Six hours before the end of the experiment, 80 nM of PMA, 1 μg/mL of ionomycin and10 μg/mL of Brefeldin A was added to the culture. The efficiency of knockdown was examined by qRT-PCR. The expression of target gene *CXCL16* was detected by qRT-PCR and Western blot.

2.9. Western Blot. The protein samples of 6T-CEM cells were loaded (5 μg) on a 10% acrylamide SDS-PAGE gel (Bio-Rad, Hercules, CA, USA) for protein separation, followed by transfer to PVDF membranes (Bio-Rad). The blots were then blocked with 1% BSA in 0.1% Tween 20/PBS for 1 h at room temperature and then incubated overnight at 4°C with antibodies specific for CXCL16 (PeproTech, Rocky Hill, CT). After washing with 0.1% Tween 20 in PBS, the membranes were incubated with a 1 : 3000 dilution of goat anti-rabbit IgG HRP (Calbiochem) in 1% solution of powdered milk in PBS/0.1% Tween 20. The membranes were exposed to ECL solution (Bio-Rad) and imaged by chemiluminescence (Clinx Science Instrument, Shanghai, China).

2.10. Chemotaxis Analysis. 6T-CEM cells were transfected with lncRNA *ENST00000502883.1* siRNA or scrambled siRNA for 48 h, the supernatant was collected for chemotaxis analysis. The PBMCs were isolated and suspended at a concentration of 10^6 cells/ml in chemotaxis buffer (RPMI 1640 containing 25 mM Hepes and 1% (*v*/*w*) endotoxin-free bovine serum albumin). The chemotaxis protocol was performed using a 48-well microchemotaxis Boyden chamber (Neuro Probe, Cabin John, MD) with 5 μm pore polycarbonate filters (Neuro Probe). The inferior wells were loaded with cell culture supernatants pretreated at 37°C for 30 min with neutralizing Ab against CXCL16 (R&D systems, Minneapolis, MN, USA) or goat IgG isotype control (R&D systems), chemotaxis buffer, and CXCL12 (PeproTech) at 10^{-7} M were used as negative and positive controls, respectively. 20 ng/ml of CXCL16 (PeproTech) pretreated with neutralizing Ab against CXCL16 (R&D systems) or goat IgG isotype control (R&D systems) was loaded into the inferior wells as well. The chemotaxis system was conducted for 2 h 30 min at 37°C in 5% CO_2. Each condition was performed in triplicate. Cells having migrated through the filter were counted in the inferior well, and results were expressed as index of chemotaxis compared with chemotaxis buffer.

2.11. Statistical Analysis. Statistical analysis for expression of lncRNA and mRNA by qRT-PCR in PBMCs was performed by Mann-Whitney *U* test. The paired *t*-test was performed for cell culture experiments and chemotaxis analysis. Probability values of $P < 0.05$ were considered significant. Data analysis was performed by using the GraphPad prism 5 software (GraphPad, San Diego, CA, USA).

3. Results

3.1. lncRNA Microarray. We firstly examined the lncRNA profiling in PBMCs from 20 nonsmokers, 17 smokers without airflow limitation, and 14 COPD patients. We compared the lncRNA expression between all paired groups (smokers versus nonsmokers, COPD versus nonsmokers, and COPD versus smokers) (Figures 1(a), 1(b), and 1(e)). Compared with nonsmokers, 27 lncRNAs were upregulated and 62 were downregulated in smokers (Table 3), while 165 lncRNAs were upregulated and 81 downregulated in COPD patients (Table 4). When comparing the lncRNA expression between smokers and COPD patients, there were 110 upregulated and 48 downregulated in COPD patients (Table 5).

3.2. mRNAs Microarry. We performed the parallel mRNA microarray on pooled RNA samples to compare the mRNA expression profiling in PBMCs from 20 nonsmokers, 17 smokers without airflow limitation, and 14 COPD patients. Eighty-two upregulated and 83 downregulated mRNAs were found in smokers compared with nonsmokers; 190 mRNA were upregulated and 156 downregulated in COPD patients compared with nonsmokers and 135 upregulated and 112 downregulated in COPD patients compared with smokers (Figures 1(c)–1(e), part of the results was published in the previous study [10]). Table 6 shows the top 10 upregulated and 10 downregulated genes (part of results was published in the previous study [10]).

3.3. Target Prediction. We further predicted the potential cis- and transtarget genes for the dysregulated lncRNAs between COPD patients and smokers within the dysregulated mRNAs. The predicted regulation network was shown in Figure 2. The GO enrichment analysis showed that the biological processes and molecular functions including leukocyte migration, immune response, and apoptosis are the main enriched GOs in the dysregulated mRNAs predicted to be regulated by lncRNAs (Table 7). According to the role of target mRNAs in the pathogenesis of COPD, the 5 lncRNAs *NR_026891.1*, *ENST00000502883.1*, *HIT000648516*, *XR_429541.1*, and *ENST00000597550.1* were selected for the further validation (Tables 8 and 9).

3.4. qPCR Validation of Dysregulated lncRNAs and mRNAs in PBMCs. The upregulated expression of 5 selected lncRNA (*NR_026891.1*, *ENST00000502883.1*, *HIT000648516*, *XR_429541.1*, and *ENST00000597550.1*) in COPD patients compared with smokers was further validated by qRT-PCR (Figure 3(a)). In PBMCs of smokers, *XR_429541.1* was the most highly expressed lncRNA. In PBMCs of COPD patients, *ENST00000502883.1*, *HIT000648516*, and *XR_429541.1* had the higher expression (Figure 3(b)). To analyze the relationship between lncRNA expression and lung function of subjects, the correlation analysis between lncRNA expression and FEV1% predicted was performed. The significant negative relevance appeared in the expression of these lncRNAs (Figure 3(c)). However, there is no difference in lncRNA sets

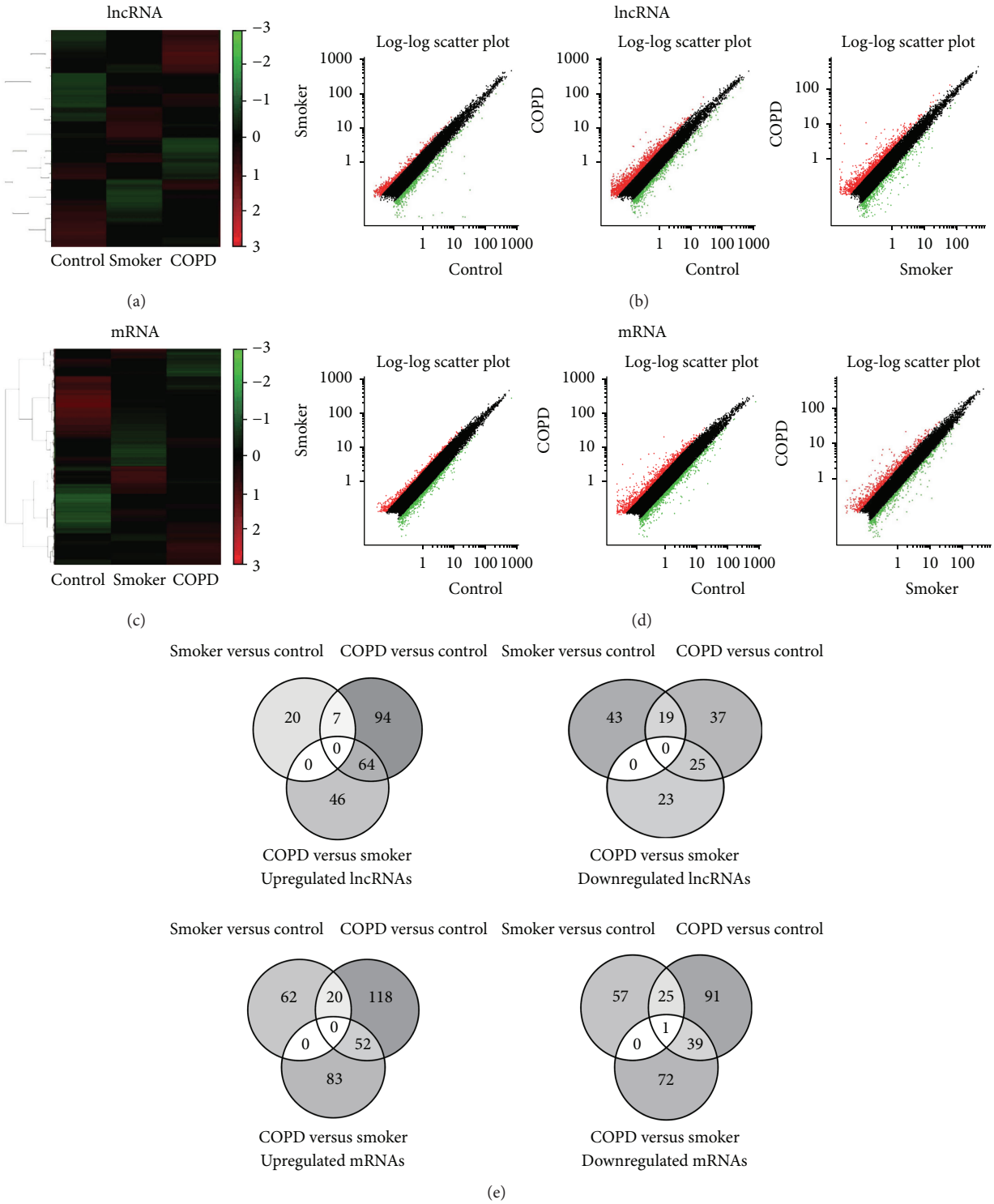

Figure 1: Hierarchical clustering, scatter plot result, and Venn diagram of differentially expressed lncRNAs and mRNAs in PBMCs from nonsmokers, smokers, and COPD patients. (a) Hierarchical clustering image of lncRNA expression of pooled RNA samples from PBMCs. (b) Scatter plot of lncRNA expression of PBMCs. (c) Hierarchical clustering image of mRNA expression of pooled RNA samples from PBMCs. (d) Scatter plot of mRNA expression of PBMCs. Red and green colored dots represent up- and downregulated miRNAs in scatter plot, respectively. (e) Venn diagram of differentially expressed lncRNAs and mRNAs. Figure reproduced from Dang et al. [10], under the Creative Commons Attribution License/public domain.

Table 3: Top 10 upregulated and downregulated lncRNAs in smokers compared with nonsmokers.

lncRNA	Fold change	*P* value
Upregulation		
ENST00000594469.1	4.132	0.0049
TCONS_00017656	2.708	0.0073
ENST00000452347.1	2.610	0.0076
ENST00000445076.1	2.523	0.0080
ENST00000517658.1	2.495	0.0081
ENST00000379928.4	2.488	0.0082
ENST00000425031.1	2.469	0.0083
ENST00000571404.1	2.398	0.0086
ENST00000417071.1	2.392	0.0087
ENST00000447643.1	2.372	0.0088
Downregulation		
ENST00000602863.1	0.004	0.0028
ENST00000445814.1	0.018	0.0029
TCONS_00017343	0.047	0.0031
ENST00000602587.1	0.059	0.0031
ENST00000553269.1	0.154	0.0040
uc.173	0.181	0.0044
ENST00000420213.1	0.207	0.0047
TCONS_00002106	0.220	0.0049
ENST00000502883.1	0.221	0.0049
ENST00000434051.1	0.226	0.0050

Table 4: Top 10 upregulated and downregulated lncRNAs in COPD patients compared with nonsmokers.

lncRNA	Fold change	*P* value
Upregulation		
ENST00000594469.1	20.223	0.0049
ENST00000416105.1	19.604	0.0030
XR_428545.1	18.063	0.0030
NR_103548.1	8.289	0.0034
TCONS_00010984	7.562	0.0035
TCONS_00010403	7.522	0.0035
ENST00000416758.1	7.113	0.0035
HIT000064697	5.478	0.0038
ENST00000513492.1	5.292	0.0039
ENST00000607854.1	4.622	0.0041
Downregulation		
TCONS_00009962	0.133	0.0041
ENST00000456917.1	0.134	0.0041
ENST00000609385.1	0.136	0.0041
ENST00000517983.1	0.156	0.0045
ENST00000420213.1	0.199	0.0053
TCONS_00005314	0.209	0.0055
ENST00000584923.1	0.212	0.0055
TCONS_00008360	0.221	0.0057
ENST00000445814.1	0.238	0.0062
ENST00000602813.1	0.239	0.0062

Table 5: Top 10 upregulated and downregulated lncRNAs in COPD patients compared with smokers.

lncRNA	Fold change	*P* value
Upregulation		
ENST00000602863.1	79.144	0.0028
ENST00000446595.1	19.715	0.0030
TCONS_00016340	17.902	0.0030
XR_428545.1	14.426	0.0031
TCONS_00017343	13.416	0.0031
ENST00000416105.1	13.057	0.0032
ENST00000445814.1	12.971	0.0032
TCONS_00009234	7.267	0.0036
ENST00000434051.1	6.920	0.0036
ENST00000416758.1	6.476	0.0037
Downregulation		
TCONS_00028904	0.056	0.0032
ENST00000517983.1	0.092	0.0035
ENST00000456917.1	0.105	0.0037
ENST00000609385.1	0.115	0.0038
TCONS_00005314	0.187	0.0048
HIT000648524_02	0.211	0.0053
TCONS_00009962	0.216	0.0053
TCONS_00008360	0.223	0.0055
XR_429946.1	0.264	0.0065
ENST00000548760.2	0.268	0.0066

between ex and current smokers in the smokers and COPD groups (data not shown).

3.5. qPCR Validation of Dysregulated lncRNAs in Different Cell Types of PBMCs. PBMCs consist mainly of T lymphocytes, B lymphocytes, and monocytes. We therefore analyzed the expression of dysregulated lncRNAs in the isolated different cell types including $CD4^+$ T cells, $CD8^+$ T cells, $CD14^+$ monocytes, and $CD20^+$ B cells from PBMCs of smokers (Figure 4(a)) and COPD patients (Figure 4(b)). *NR_026891.1* was highly expressed in monocytes and B cells of smokers, while it was expressed mainly in $CD8^+$ T cells and B cells of COPD patients, suggesting $CD8^+$ T cells contributed to the increased expression in COPD. *ENST00000502883.1* was consistently expressed higher in B cells and $CD4^+$ T cells. In COPD patients, the expression of *HIT000648516* in monocytes was decreased compared with smokers, suggesting the expression in other cells was increased in COPD patients. *XR_429541.1* was mainly expressed in $CD4^+$ and $CD8^+$ T cells in both smokers and COPD patients. $CD8^+$ T cells mainly contributed to the increased expression of *ENST00000597550.1* in COPD patients.

3.6. qPCR Validation of Dysregulated Predicted Target mRNAs in PBMCs. We further examined the expression of 4 predicted target genes known to be involved in the pathogenesis of COPD in PBMCs from smokers and COPD patients. Consistent with the results of microarray assay,

Table 6: Top 10 upregulated and downregulated mRNAs in COPD patients compared with smokers.

Gene symbol	Gene name	Fold change Smokers versus nonsmokers (*P* value)	Fold change COPD versus nonsmokers (*P* value)	Fold change COPD versus smokers (*P* value)	Function
Upregulation					
CD177	CD177 molecule	3.48 (0.0056)	78.70 (0.0029)	22.59 (0.0030)	Leukocyte migration
MUC17	Mucin 17,cell surface associated	1.08 (0.2270)	23.22 (0.0030)	21.54 (0.0030)	Extracellular matrix constituent
IL1R2	Interleukin 1 receptor, type II	0.92 (0.7332)	10.08 (0.0033)	10.96 (0.0033)	Decoy receptor, inhibits the activity of IL-1
SARDH	Sarcosine dehydrogenase	0.19 (0.0045)	1.83 (0.0100)	9.52 (0.0033)	Mitochondrial matrix
EGR3	Early growth response 3	1.12 (0.1549)	7.57 (0.0035)	6.75 (0.0036)	Positive regulation of endothelial cell proliferation
AREG	Amphiregulin	0.65 (0.0319)	4.01 (0.0071)	6.16 (0.0048)	EGF family, promote the growth of normal epithelial cells
SLC6A2	Solute carrier family 6 (neurotransmitter transporter, noradrenalin), member 2	0.39 (0.0093)	1.84 (0.0099)	4.70 (0.0042)	Sodium symporter
TMEM167A	Transmembrane protein 167A	0.79 (0.8343)	3.68 (0.0435)	4.55 (0.0077)	Golgi apparatus
KCNJ15	Potassium inwardly-rectifying channel, subfamily J, member 15	0.78 (0.5713)	3.45 (0.0049)	4.42 (0.0043)	Potassium channel activity
FCHO1	FCH domain only 1	0.26 (0.0057)	1.15 (0.0381)	4.39 (0.0043)	Clathrin-mediated endocytosis
Downregulation					
IL1A	Interleukin 1, alpha	2.03 (0.0117)	0.05 (0.0031)	0.02 (0.0029)	Immune response
IL6	Interleukin 6 (interferon, beta 2)	1.59 (0.0111)	0.10 (0.0044)	0.06 (0.0032)	Proinflammatory and anti-inflammatory role
CXCL10	Chemokine (C-X-C motif) ligand 10	1.06 (0.2735)	0.07 (0.0033)	0.07 (0.0033)	Leukocyte chemotaxis
TNF	Tumor necrosis factor	0.43 (0.0112)	0.04 (0.0030)	0.085 (0.0035)	Inflammation, cause apoptosis
CCL20	Chemokine (C-C motif) ligand 20	1.06 (0.2724)	0.13 (0.0041)	0.13 (0.0039)	Lymphocytes chemotaxis
CCL4	Chemokine (C-C motif) ligand 4	0.87 (0.3686)	0.14 (0.0045)	0.16 (0.0047)	Leukocyte chemotaxis
CCL3L3	Chemokine (C-C motif) ligand 3-like 3	0.89 (0.5755)	0.17 (0.0066)	0.19 (0.0063)	Leukocyte chemotaxis
C9orf7	Chromosome 9 open reading frame 7	1.40 (0.0126)	0.27 (0.0070)	0.19 (0.0049)	Calcium channel activity
IL1RN	Interleukin 1 receptor antagonist	0.90 (0.5833)	0.19 (0.0051)	0.21 (0.0053)	Inhibition of the activities of IL-1
RNF19B	Ring finger protein 19B	2.21 (0.0099)	0.53 (0.0362)	0.24 (0.0059)	Cytotoxic effects of natural killer (NK) cells

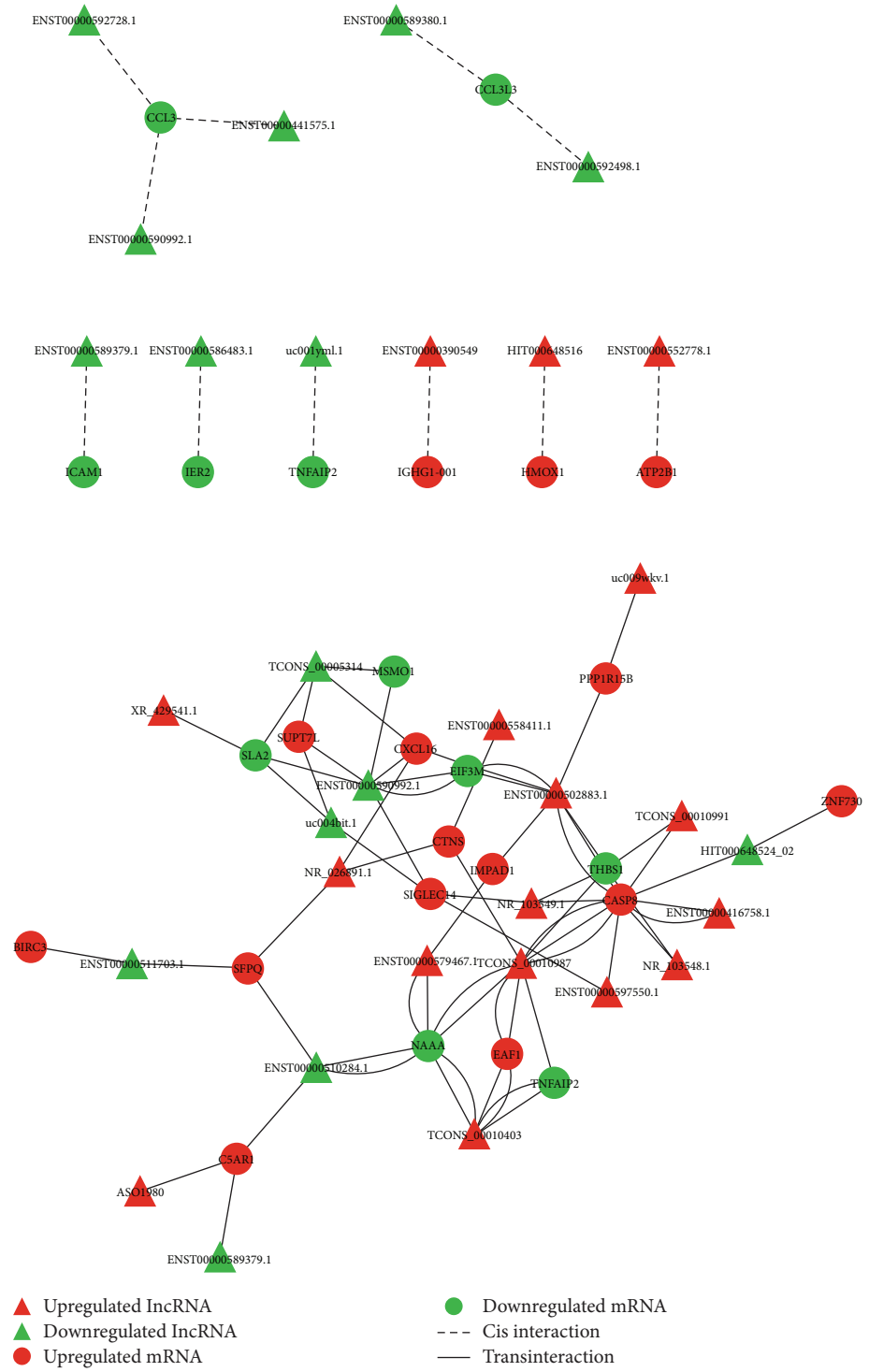

Figure 2: Regulation network between lncRNAs and mRNAs. The regulation of lncRNA on dysregulated mRNAs was predicted and the regulation network was drawn by using Cytoscape software. Red and green color represents up- and downregulated genes, and triangle and circle shape represents cis- and transinteraction, respectively.

the increased expression of *HMOX1*, *SIGLEC14*, and *CXCL16* and decreased expression of *SLA2* was observed in COPD patients compared with smokers (Figure 5).

3.7. Validation of ENST00000502883.1-Regulated CXCL16 Expression in T Cell Line. We examined the regulatory role of *ENST00000502883.1* on *CXCL16* expression. Firstly, 5% of cigarette smoke extract could significantly enhance the expression of *ENST00000502883.1* and *CXCL16* on T cells (Figure 6(a)). *ENST00000502883.1* knockdown with siRNA consequently decreased the expression of *CXCL16* at both mRNA (Figure 6(b)) and protein (Figure 6(c)) level,

Table 7: Top 10 enriched GOs of targeted genes predicted to be regulated by dysregulated lncRNAs in COPD patients compared to smokers.

GO	*P* value	Enrich score	Gene list
GO:0034612 response to tumor necrosis factor	0.000275	26.766	*CCL3*, *THBS1*, *CXCL16*, and *CASP8*
GO:0002685 regulation of leukocyte migration	0.00241	22.613	*HMOX1*, *CCL3*, and *THBS1*
GO:1,902,041 regulation of extrinsic apoptotic signaling pathway via death domain receptors	0.00252	41.244	*THBS1* and *HMOX1*
GO:2,001,236 regulation of extrinsic apoptotic signaling pathway	0.00494	15.411	*HMOX1*, *THBS1*, and *CASP8*
GO:0002443 leukocyte-mediated immunity	0.00494	14.489	*SLA2*, *IGHG1–001*, and *CCL3*
GO:0002757 immune response-activating signal transduction	0.00494	9.249	*SLA2*, *C5AR1*, *IGHG1–001*, *CASP8*, and *BIRC3*
GO:0002687 positive regulation of leukocyte migration	0.00494	23.266	*CCL3* and *THBS1*
GO:2,001,233 regulation of apoptotic signaling pathway	0.00494	8.922	*HMOX1*, *SFPQ*, *THBS1*, and *CASP8*
GO:0071356 cellular response to tumor necrosis factor	0.00494	20.39	*CCL3* and *THBS1*
GO:0010035 response to inorganic substance	0.00494	8.309	*HMOX1*, *PPP1R15B*, *THBS1*, and *CASP8*

Table 8: Selected dysregulated lncRNAs in COPD patients compared with smokers.

lncRNA	Fold change Smokers versus nonsmokers	Fold change COPD versus nonsmokers	Fold change COPD versus smokers
NR_026891.1	1.15	2.76	2.39
ENST00000502883.1	0.22	0.78	3.51
HIT000648516	0.51	1.14	2.25
XR_429541.1	1.13	2.78	2.46
ENST00000597550.1	0.79	1.61	2.05

suggesting *ENST00000502883.1* could regulate *CXCL16* expression. We then analyzed the chemotactic effect of T cell supernatant on PBMCs. The pretreatment with CXCL16 neutralizing antibody significantly decreased the chemotactic effect of NC control culture medium, suggesting the CXCL16 secreted by T cells play a good biological chemotactic function. Meanwhile, the supernatant of T cells with siRNA transfection had the reduced effect compared with NC control, which indicted lncRNA *ENST00000502883.1* could regulate *CXCL16* expression and furtherl regulate the recruitment of inflammatory cells.

4. Discussion

Increasing evidence has confirmed lncRNAs to be one of the most important factors controlling gene expression [14–16]. Therefore, for the first time, we evaluated the lncRNA and mRNA expression profile of PBMCs from healthy nonsmokers, smokers without airflow limitation, and COPD patients. We identified 158 differentially expressed lncRNAs in PBMCs from COPD subjects compared with smokers without airflow limitation. The dysregulated expression of 5 selected lncRNA including *NR_026891.1* (*FLJ10038*), *ENST00000502883.1* (*RP11-499E18.1*), *HIT000648516*, *XR_429541.1*, and *ENST00000597550.1* (*CTD-2245F17.3*) were validated by qPCR. The GO enrichment analysis for the dysregulated mRNAs predicted to be regulated by lncRNAs showed that leukocyte migration, immune response, and apoptosis are the main enriched processes that previously reported to be involved in the pathogenesis of COPD. We further validated the dysregulation of target genes *CXCL16*, *HMOX1*, *SLA2*, and *SIGLEC14* in PBMCs of COPD patients. It is noted that the predicted target genes discussed in the present study did not overlay the ones that we predicted in the study of dysregulated miRNAs of COPD patients [10]. This may suggest that the regulation profile of lncRNA and miRNA on target genes is different in PBMCs of COPD patients.

For the economic consideration, the equal amount of RNA sample from each smoker and COPD patients was pooled, respectively, for lncRNA profiling assay. And the expression of selected lncRNAs in each individual was further validated by qRT-PCR. The similar approach was previously used on lncRNA microarray assays [17–19].

CXCL16 was reported as one of the systemic inflammatory markers for COPD in a large cohort of COPD patients and controls [20]. In this study, increased CXCL16 expression was also found in PBMCs of COPD patients versus smokers. CXCL16 is expressed by dendritic cells, macrophages, T cells, and B cells and can act as a chemoattractant for Th1 cells, which implies its relevance to COPD [21, 22]. *CXCL16* was predicted to be regulated by *NR_026891.1* (*FLJ10038*) and *ENST00000502883.1* (*RP11-499E18.1*) through a transregulation manner. Few studies have been reported on the two lncRNAs except for the upregulation of *NR_026891.1* (*FLJ10038*) in an experimental model of Alzheimer's disease [23]. In this study, we confirmed the regulatory role of *ENST00000502883.1* on *CXCL16* expression and consequently the effect on PBMC recruitment.

The lncRNA *HIT000648516* was predicted to target the *HMOX1* gene by a sense manner. *HMOX1* gene encodes heme oxygenase (HO)-1, which is induced under physiological conditions such as inflammation and oxidative stress and protects against inflammatory- and oxidant-mediated cellular injury [24]. HO-1 may also play a vital function in maintaining cellular homeostasis [25]. Moreover, the increased expression of HO-1 was found in sputum samples obtained at the onset of a severe COPD exacerbation [26] and in the peripheral blood monocytes during acute inflammatory

Table 9: The selected predicted regulation of dysregulated lncRNAs on mRNAs in COPD patients compared to smokers.

lncRNA	Gene symbol	Target gene	Microarray analyze fold change	Function
NR_026891.1	*FLJ10038*	*CXCL16*	2.29	Induced by the inflammatory cytokines IFN-gamma and TNF-alpha; cytokine-cytokine receptor interaction, organism-specific biosystem
ENST00000502883.1	*RP11-499E18.1*	*CXCL16*	2.29	Induced by the inflammatory cytokines IFN-gamma and TNF-alpha; cytokine-cytokine receptor interaction, organism-specific biosystem
HIT000648516	—	*HMOX1*	2.71	Keap1-Nrf2 pathway, organism-specific biosystem
XR_429541.1	—	*SLA2*	0.46	Negative regulation of B cell activation; regulation of immune response TCR signaling in naive $CD4^+$ T cells
ENST00000597550.1	*CTD-2245F17.3*	*SIGLEC14*	2.57	Innate immune system Cell adhesion

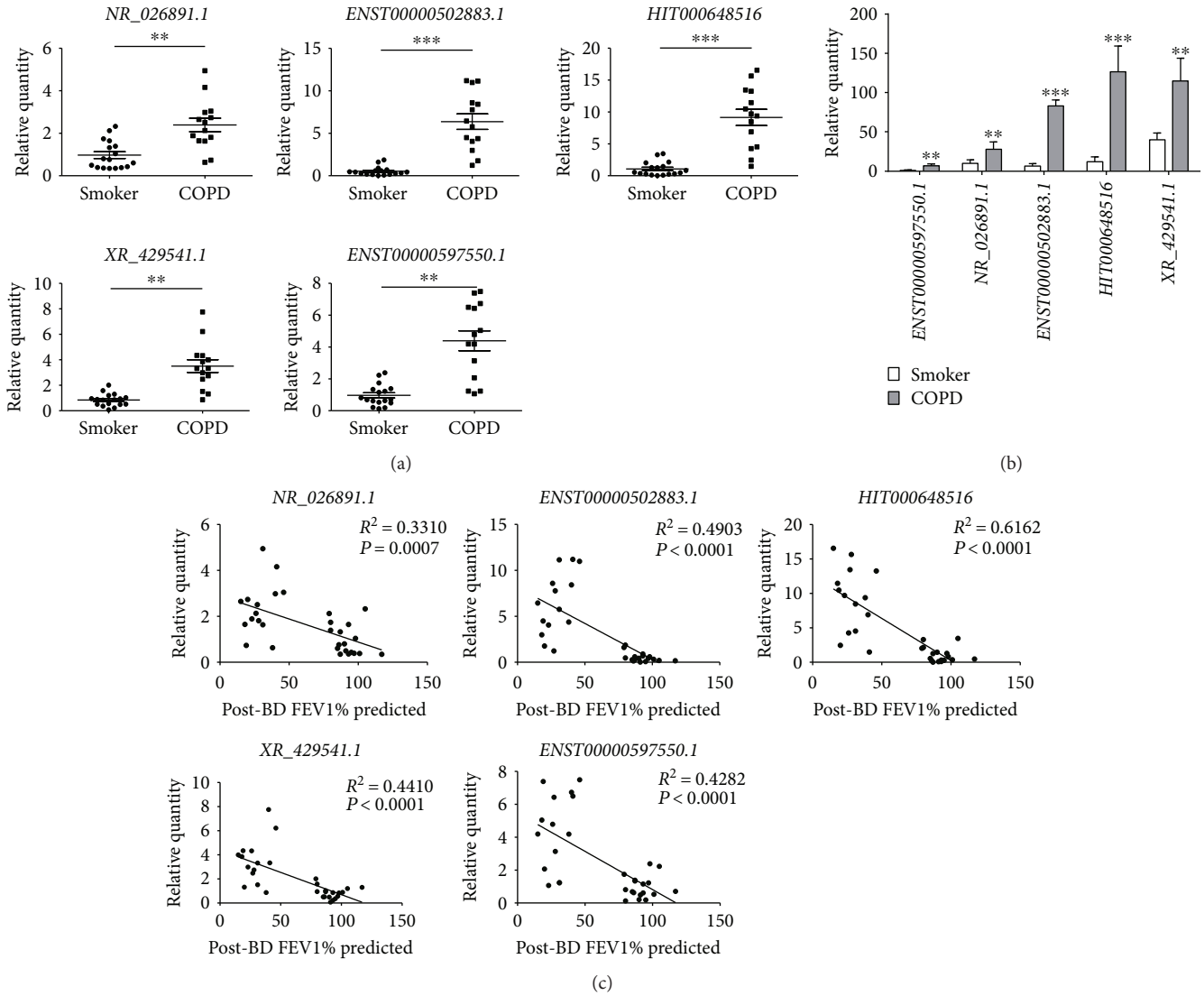

Figure 3: Validation of differentially expressed lncRNAs. (a) qRT-PCR was performed on the same RNA samples (17 smokers and 14 COPD patients) by individual lncRNA for *NR_026891.1*, *ENST00000502883.1*, *HIT000648516*, *XR_429541.1*, and *ENST00000597550.1*. Data are presented as $2^{-\Delta\Delta Ct}$ relative to β-actin. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with smokers. (b) Relative abundance of differentially expressed lncRNAs in PBMCs of smokers and COPD patients. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with smokers. (c) Correlation analysis between lncRNA expression and post-BD FEV1% predicted.

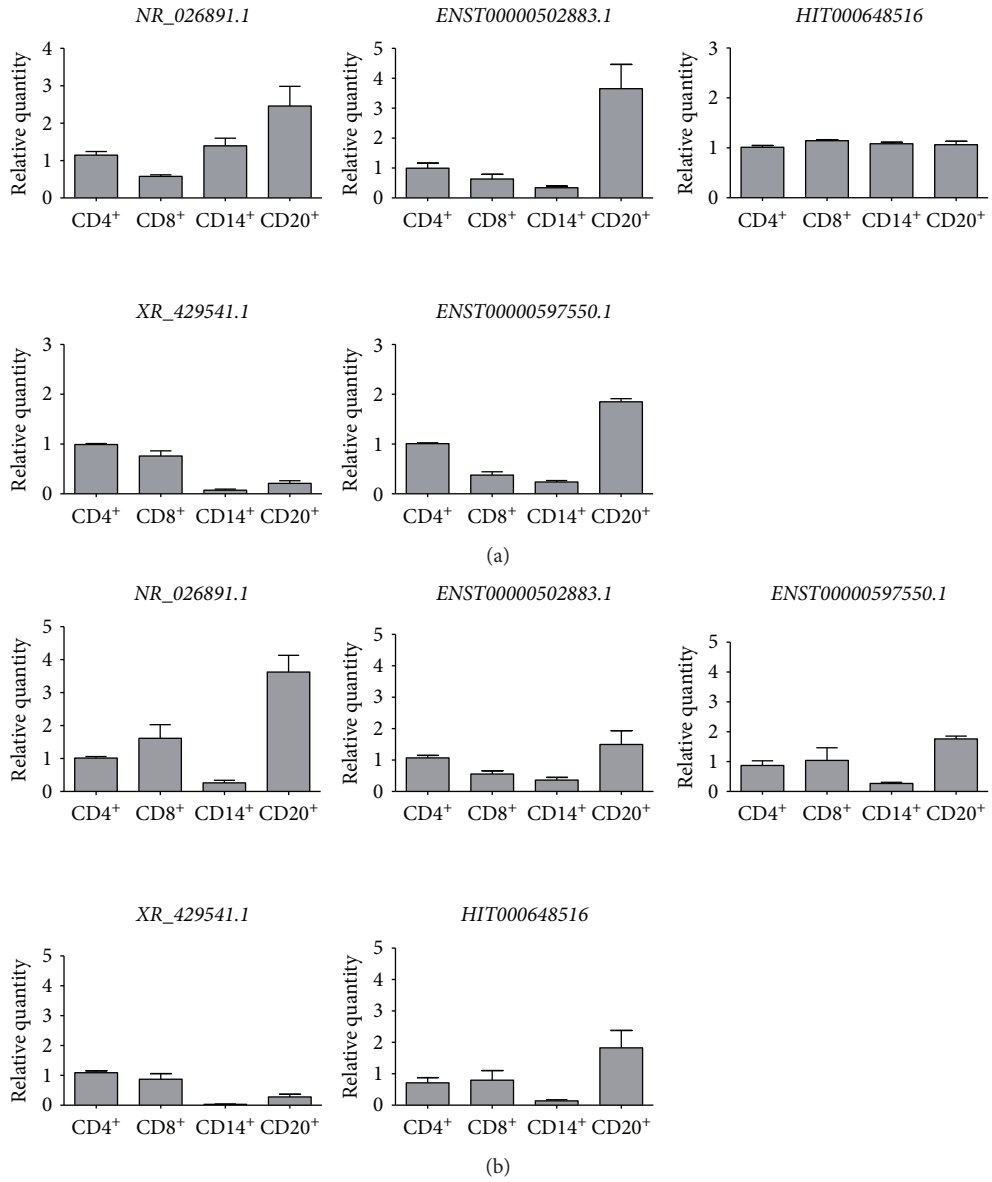

Figure 4: Expression of lncRNAs in the isolated different cell types of PBMCs from smokers (a) and COPD patients (b). The expression of *NR_026891.1*, *ENST00000502883.1*, *HIT000648516*, *XR_429541.1*, and *ENST00000597550.1* was examined by qRT-PCR on $CD4^+$ T lymphocytes, $CD8^+$ T lymphocytes, $CD14^+$ monocytes, and $CD20^+$ B lymphocytes from smokers and COPD patients. Data are presented as $2^{-\Delta\Delta Ct}$ relative to β-actin.

illnesses of children [27]. Thus, the increased expression of HO-1 in PBMCs may reflect a protective response in COPD patients under the environment of inflammation and oxidative stress.

The expression of *SLA2* gene was decreased in PBMCs of COPD patients compared with smokers, and lncRNA *XR_429541.1* was predicted to regulate this gene by transregulation. The *SLA2* gene encodes Src-like adaptor protein-2 (SLAP-2), which shares 36% sequence similarity with *SLAP*. SLAP-2 is predominantly expressed in hematopoietic cells and plays an inhibitory role in the activation of T cells [28]. In COPD, once activated, T cells are present in the lung and exert their effector functions by attracting other inflammatory cells like neutrophils and macrophages and enhancing their inflammatory functions [8]. Therefore, the downregulation of *SLAP-2* in PBMCs seen in this study may be related with inflammation in COPD patients.

The *SIGLEC14* gene expression was predicted to be regulated by *ENST00000597550.1* (*CTD-2245F17.3*). Siglecs are a family of sialic acid-binding lectins expressed mainly on innate immune cells [29]. Siglec-14, a Siglec family member with an activating signaling property, is expressed on granulocytes and monocytes. Siglec-14 serum concentration can serve as a useful marker for COPD exacerbation susceptibility and consequential decline in pulmonary function [30]. Patients with COPD who are homozygous null for this allele had fewer inflammatory exacerbations than patients expressing the wild-type allele, which suggests that Siglec-14

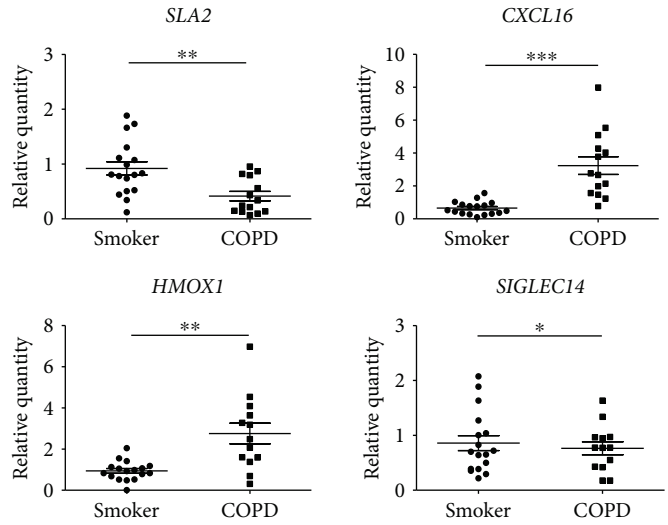

Figure 5: Validation of differentially expressed mRNAs. qRT-PCR was performed on the same RNA samples (17 smokers and 14 COPD patients) by individual mRNA for *CXCL16*, *HMOX1*, *SLA2*, and *SIGLEC14*. Data are presented as $2^{-\Delta\Delta Ct}$ relative to β-actin. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with smokers.

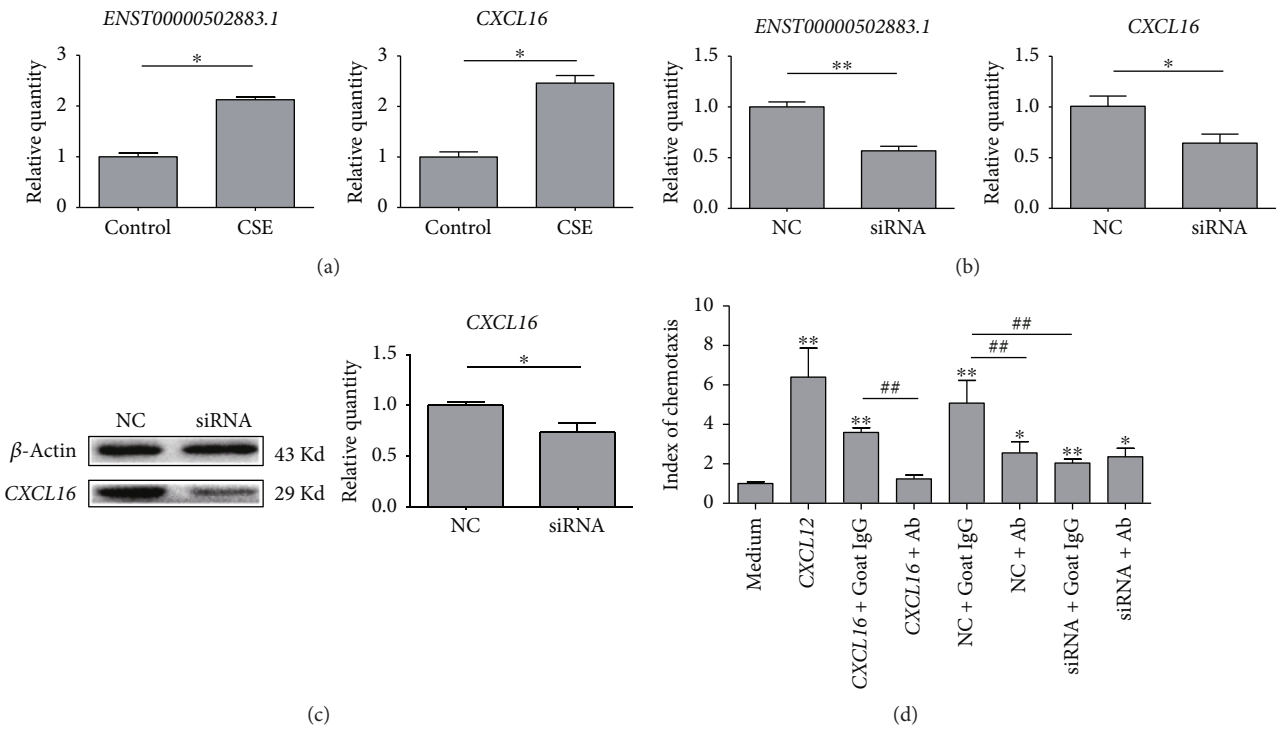

Figure 6: Regulatory role of *ENST00000502883.1* on *CXCL16* expression. (a) Expression of *ENST00000502883.1* on *CXCL16* of 6T-CEM cells under the stimulation of 5% cigarette smoke extract (CSE). Data are presented as $2^{-\Delta\Delta Ct}$ relative to β-actin. $^{*}P < 0.05$. (b) Expression of *CXCL16* on mRNA level of 6T-CEM cells with *ENST00000502883.1* siRNA transfection. Scramble siRNA was used as negative control (NC). Data are presented as $2^{-\Delta\Delta Ct}$ relative to β-actin. $^{*}P < 0.05$. (c) Expression of *CXCL16* on protein level of 6T-CEM cells with *ENST00000502883.1* siRNA transfection. CXCL16 expression was examined by Western blotting. $^{*}P < 0.05$. (d) The regulatory role of *ENST00000502883.1* on the partially CXCL16-dependent chemotactic effect of 6T-CEM cell supernatant. 6T-CEM cells were transfected with *ENST00000502883.1* siRNA or negative control (NC), and the chemotactic effect of cell culture supernatant on PBMCs was evaluated by a microchemotaxis Boyden chamber. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with medium control. $^{\#\#}P < 0.01$ when comparing the two groups with line marks.

may promote inflammatory sequelae caused by neutrophils [30]. Furthermore, inhaled corticosteroids may exert negative effects on treatment through increased Siglec-14 expression [31].

Overall, through lncRNA and mRNA expression profiling in nonsmokers, smokers, and COPD patients, we identified the dysregulated lncRNAs and mRNAs in PBMCs from COPD patients compared to smokers. We further analyzed the regulation network between lncRNAs and mRNAs, where the genes *CXCL16*, *HMOX1*, *SLA2*, and *SIGLEC14* were predicted to be regulated by certain lncRNAs through sense or miRNA regulation. This study may provide clues for further studies targeting lncRNAs to control inflammation in COPD.

Conflicts of Interest

There is no competing financial interest.

Authors' Contributions

Xiaoyan Qu isolated the PBMCs, performed qRT-PCR and cell culture, and analyzed the data. Xiaomin Dang and Dong Shang collected blood samples. Weijia Wang isolated part of the PBMCs. Ying Li performed part of cell culture. Dan Xu coordinated the collection of blood samples. Ying Chang designed the study and drafted the manuscript. All authors reviewed the manuscript.

Acknowledgments

This study was supported by the National Natural Science Foundation of China (31501044), International Cooperation and Exchange Program of Science and Technology of Shaanxi Province (2017KW-044, 2016KW-026), and the Scientific Research Foundation for the Returned Overseas Chinese Scholars, State Education Ministry. This work was supported in part by Project 985 of Xi'an Jiaotong University. The authors acknowledge Dr. Yong-Ping Shao for his critical review of the manuscript.

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Hindawi
Mediators of Inflammation
Volume 2018, Article ID 1601486, 7 pages
https://doi.org/10.1155/2018/1601486

Review Article

Towards Targeting the Aryl Hydrocarbon Receptor in Cystic Fibrosis

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Received 27 June 2017; Accepted 5 December 2017; Published 18 February 2018

Academic Editor: Virginia De Rose

Tryptophan (trp) metabolism is an important regulatory component of gut mucosal homeostasis and the microbiome. Metabolic pathways targeting the trp can lead to a myriad of metabolites, of both host and microbial origins, some of which act as endogenous low-affinity ligands for the aryl hydrocarbon receptor (AhR), a cytosolic, ligand-operated transcription factor that is involved in many biological processes, including development, cellular differentiation and proliferation, xenobiotic metabolism, and the immune response. Low-level activation of AhR by endogenous ligands is beneficial in the maintenance of immune health and intestinal homeostasis. We have defined a functional node whereby certain bacteria species contribute to host/microbial symbiosis and mucosal homeostasis. A microbial trp metabolic pathway leading to the production of indole-3-aldehyde (3-IAld) by lactobacilli provided epithelial protection while inducing antifungal resistance via the AhR/IL-22 axis. In this review, we highlight the role of AhR in inflammatory lung diseases and discuss the possible therapeutic use of AhR ligands in cystic fibrosis.

1. Inflammation in Cystic Fibrosis

The inflammatory response is complex and involves a variety of mechanisms to defend against pathogens and repair tissue. In the lung, inflammation is usually caused by pathogens or by exposure to toxins, pollutants, irritants, and allergens. Acute inflammation plays a key role in the innate host defence mechanism yet, when unchecked, may result in tissue destruction and disease. The anti-inflammatory cascade restrains the intensity of the innate response but, when unrestrained, may contribute to chronic inflammation characterized by abnormal wound repair and development of fibrotic disease. So, a delicate balance between inflammation and anti-inflammation is fundamental for lung homeostasis, and understanding cellular and molecular mechanisms behind this balance will enhance diagnosis and treatment of inflammatory and fibrotic lung diseases.

Pulmonary diseases are characterized by the abnormal accumulation and persistence of cells of the acute—such as pneumonia and cystic fibrosis (CF)—or chronic—such as asthma and chronic obstructive pulmonary disease (COPD)—inflammatory response, suggesting that the failure of homeostatic control can contribute to initiation and progression of disease. Inflammation, either secondary to chronic infection or primarily due to cystic fibrosis transmembrane conductance regulator (CFTR) mutations [1], significantly contributes to disease progression, and its control is crucial for improving patient outcomes [2]. Persistent high-intensity inflammation is not only ineffective at clearing pathogens but also leads to permanent structural damage of the airways and impaired lung function. Several defective inflammatory responses have been linked to CFTR deficiency including innate and acquired immunity dysregulation [3]. The inflammation of the CF lung is dominated by neutrophils that release oxidants and proteases, particularly elastase that correlates with lung function deterioration and respiratory exacerbations. Anti-inflammatory therapies are therefore of particular interest for CF lung disease, but

abrogating neutrophil and inflammatory response may bear the inherent risk of unleashing bacterial and fungal infections [4]. Thus, despite a plethora of proinflammatory innate immune pathways having been studied and determined as playing a significant role in CF lung disease, therapeutic exploitation of these pathomechanisms remains scarce and therapeutic interventions to dampen inflammation in CF remain an appealing yet challenging approach [4].

2. The Aryl Hydrocarbon Receptor

The aryl hydrocarbon receptor (AhR) is highly conserved through evolution [5] and is expressed in the majority of immune cell types and human tissues [6, 7]. AhR is a basic helix-loop-helix (bHLH) transcription factor that has profound effects upon the immunological status of the gastrointestinal and respiratory tracts, establishing and maintaining signaling networks, which facilitate host/microbe homeostasis at the mucosal interface, via regulation of epithelial barrier integrity, of bacterial phyla, and protection from pathogenic insults [6]. Ligand binding to the AhR results in chaperone shedding and translocation to the nucleus where it dimerizes with AhR nuclear translocator (ARNT) and binds to a DNA enhancer sequence, known as dioxin response elements (DREs), within an array of target genes encoding for phase I and phase II metabolizing enzymes (e.g., CYP1A1), as well as the AhR repressor protein (AhRR). The AhRR competes with AhR for binding to ARNT, and this AhRR/ARNT heterodimer binds to DRE and represses transcription. Finally, after ligand-binding nuclear translocation-transcriptional activation, AhR is rapidly degraded [6].

Many genes that participate in immune responses have DRE sequences in their promoters and are responsive to AhR ligands [6]. Thus, AhR is a crucial regulator of the immune system. It is expressed in cells involved in innate and adaptive immunity, such as antigen-presenting cells, mast cells, and RORγt+ innate lymphoid cells (ILC), the development and function of which is dependent on AhR [8]. Furthermore, the AhR contributes to Th cell differentiation, including FoxP3+ regulatory T cells (Tregs) and IL-10-producing Tr1 cells, Th17 and Th22 cells [9, 10]. Thus, the AhR serves as a sensor that responds to signals, both from the outside environment or internal milieu, to modulate an immune response. This is of evolutionary benefit for individuals exposed to pollution or other toxins, given the ability of AhR to modulate antimicrobial activity via Th17 cell activation, epithelial cell repair, and protection via IL-22 production, and control of inflammation via activation of Tregs. In addition, activation of the AhR causes an upregulation of cytochrome P450 enzymes that metabolize the harmful toxicants. Thus, the AhR has multitasking activities that include antimicrobial defence, tissue protection, repair, and toxin clearance. This creates numerous exciting opportunities to harness the immunomodulatory action of AhR to adapt host responses to infection.

Table 1: List of major ligands of aryl hydrocarbon receptor.

Source	Activity	Examples
Agonists		
		Halogenated aromatic hydrocarbons
		2,3,7,8-Tetrachlorodibenzo-*p*-dioxin
		Dibenzofurans
		Biphenyls
		Polyaromatic hydrocarbons
		3-Methylchlolanthrene
Xenobiotics	Strong	Benzo(a)pyrene
		Benzanthracenes
		Benzoflavones
		Pharmaceuticals
		Tranilast
		Leflunomide
		Omeprazole
		Flavonoids
		Quercetin
Dietary	Weak	Indole-3-carbinol
		3,3′-Diindolylmethane
		Indolo[3,2b]carbazole
		Tryptophan metabolites
		Kynurenic acid
		Kynurenine
		Tryptamine
		6-Formylindolo[3,2b]carbazole
		Indoxyl sulfate
		Microbiota
Endogenous	Weak	3-Methylindole
		Tryptanthrin
		1,4-Dihydroxy-2-naphtoic acid
		Indole-3-aldehyde
		Indole-3-acetate
		Phenazines
		Indirubin
		Malassezin
Antagonists		
Xenobiotic	Weak	3,4-Dimethoxy-a-naphthoflavone
		6-Methoxy-1,3,8-trichlorodibenzofuran
	Strong	CH-223191
		StemRegenin 1
Dietary	Weak	Resveratrol
SAHRM (Agonists/Antagonists)		
Xenobiotic	Weak	3,4-Dimethoxy-a-naphthoflavone
		6-Methoxy-1,3,8-trichlorodibenzofuran
Endogenous/Dietary	Weak	Indole
		Quercetin
		Apigenin

SAHRM: selective AhR modulator.

In this regard, numerous naturally occurring endogenous and exogenous AhR ligands have been identified [11–13], with the toxicant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin being the prototypic AhR ligand (Table 1)). These low molecular weight compounds include indoles, tetrapyrroles, arachidonic acid metabolites, and trp metabolites such as FICZ (6-formylindolo[3,2b]-carbazole), ITE (2-(1H-indol-3-ylcarbonyl)-4-thiazol carboxylic acid methyl ester), indirubin, I3C (indole-3-carbinol), and BaP (benzo(a)pyrene) [11, 13–15]. Among these, ITE was isolated from the porcine lung [16]. After binding to the AhR, these ligands promote the activation of various signaling cascades [17, 18]. This may explain why different ligands are able to either ameliorate or conversely aggravate inflammation or autoimmunity [19]. Thus, depending on the nature of ligands, the extracellular and intracellular environments, and the model of pathology considered, the AhR biology is both context- and disease-dependent [10, 20, 21]. This implicates that we must differentiate between the dose- and cell-specific potential triggered by toxic ligands and the physiological effects triggered by endogenous ligands.

3. AhR and Lung Inflammation

The recognition of AhR as a master regulator of mucosal barrier function suggests that the respiratory tract is sensitive to AhR signalling and function [22]. The lung is sensitive to AhR ligands, and AhR modulates the immune response in various respiratory diseases [22]. Therefore, AhR ligands (e.g., dietary substances, tryptophan photoproducts, and environmental pollutants) have the potential to be involved both as tools for comprehending the role of the AhR in lung inflammation and as therapeutics for the treatment of various inflammatory lung diseases.

AhR ligands have proven to be beneficial in asthma. TCDD, curcumin (an AhR ligand rich in Indian spice), and quercetin (a plant-derived flavonoid with AhR agonistic activity) all suppress allergic airway inflammation in rodents—reviewed in [22]. The I3C (a dietary compound produced through the breakdown of cruciferous vegetables) attenuated allergic asthma in mice by switching the cytokine response away from Th2 towards a Th1 type of response [22]. However, although Th2 cells are a major source of allergy-promoting cytokines, type 2 ILC and Th17 cells also lead to disease pathology [23–27]. In particular, RORγt+ ILC and type 2 ILC express AhR [28–30], and levels of both AhR mRNA and IL-22 protein are markedly boosted in asthmatic patients [22]. However, the functions of the AhR in lung ILCs is still poorly defined. Similarly, albeit TCDD and other AhR ligands ameliorated allergic inflammation in murine lung diseases, details about the effects on pulmonary Th cell differentiation are still to be clarified [22]. Taken together, AhR ligands may play an important role as modulators of immunological responses that contribute to allergic events. However, differences in metabolism, AhR binding, and downstream gene activation indicate the need to better analyze the role played by AhR in allergic asthma.

Given that COPD has been associated with chronic exposure to lung irritants from cigarettes, biomass burning, air pollution, and dust [31], an immunomodulatory activity of the AhR is plausible. Not surprisingly, AhR signaling both promoted and attenuated the expression of inflammatory cytokines and matrix metalloproteases in murine lung by acting on innate immune cells variably involved in COPD, such as macrophages, neutrophils, mast cells, epithelial cells, and fibroblasts—reviewed in [22]. For instance, mast cells produce IL-17 in response to AhR stimulation and AhR/IL-17 double-positive mast cells are increased in the bronchial lamina propria of COPD patients [32]. In murine cigarette smoke-induced COPD, the COPD pathogenesis was markedly enhanced in the absence of AhR via mitochondrial dysfunction, decreased levels of antioxidant regulating proteins (i.e., MnSOD and CuZn-SOD) [33], and increased Th1, Th2, and Th17 cells [22]. Recently, smoking was found to be associated with relevant alterations in methylation, especially at the AhRR [34]. These evidences suggest that changes in AhR expression and function may be a risk factor for COPD and other lung inflammatory diseases in smokers and that the AhR status in smokers could be dysregulated. Of interest, the activity of the indoleamine 2,3-dioxygenase (IDO)1 enzyme and the IL-10/IL-17 ratio were both decreased in COPD patients [35], a finding further pointing to AhR, known to promote IDO1 activity [36], as a master regulator of inflammation and tolerance in the lung.

The ability of the AhR pathway to crosstalk with other pathways such as hypoxia signaling pathway [37] would suggest a biological function in CF in which hypoxemia has been described at the tissue and cellular levels [38]. Low tissue oxygen levels induce expression of hypoxia-inducible factor (HIF), a nucleoprotein consisting of the oxygen-regulated HIF-1α and the constitutively expressed ARNT or HIF-1β [39]. HIF-1α is continuously synthesized but under normoxic conditions is targeted for ubiquitination and proteasomal degradation. In contrast, under hypoxic conditions, HIF-1α is stabilized, dimerizes with HIF-1β, binds coactivators of hypoxia response elements, and regulates the transcription of hypoxia-regulated genes that mediates adaptive responses to ensure cellular survival under hypoxic conditions but also dysregulated inflammation. Indeed, the increased HIF-1α in CF correlated with increased activation of the inflammatory receptor of advanced glycation end products (RAGE), an important mediator of airway inflammation in CF and other lung diseases [38, 40]. The important role of ARNT in both the AhR and HIF-1α signaling pathways establishes a meaningful foundation for a possible crosstalk between these two vitally important signaling pathways. This crosstalk might lead to interference between the two signaling pathways and thus might play a role in the variety of cellular responses after exposure to AhR ligands and reduced oxygen availability.

Collectively, these studies highlight the potential differences that can occur in the lung following AhR activation by disparate agonists and further emphasize the need for extensive evaluation of the different AhR ligands.

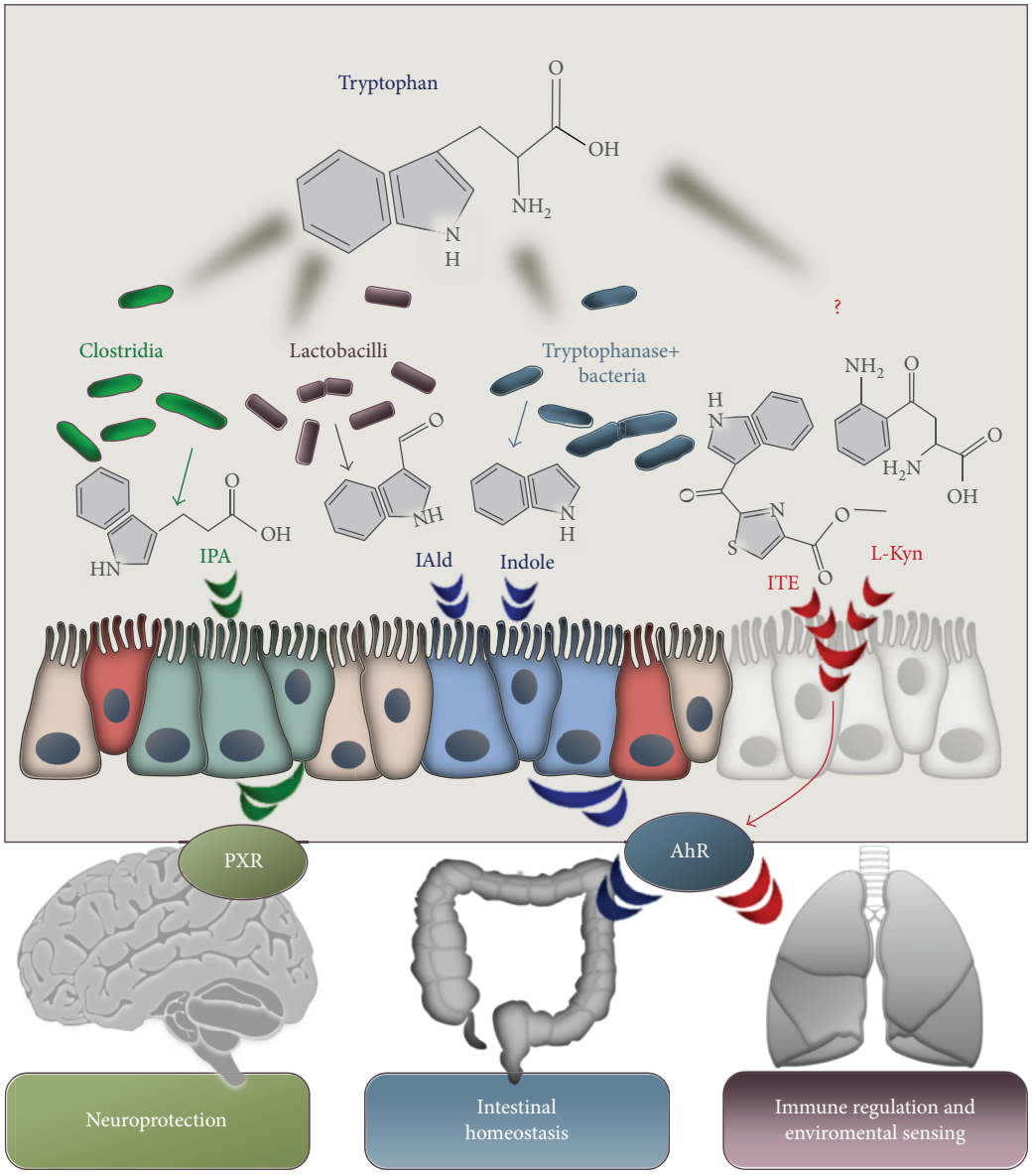

Figure 1: Tryptophan degradation pathways. The image shows how different microbes are involved in several pathways of tryptophan degradation leading to the production of immunoreactive metabolites acting locally and systemically as agonists of PXR and AhR. IPA: indole-3-pyruvic acid; IAld: indole-3-aldheyde; ITE: 2-(1H-indol-3-ylcarbonyl)-4-thiazol carboxylic acid methyl ester; L-Kyn: L-Kynurenine; PXR: pregnane X receptor; AhR: aryl hydrocarbon receptor.

4. Microbial-Derived Indoles as Therapeutic AhR Ligands

Ligands required to activate intestinal AhR derive from ingestion of plant-derived dietary ligands such as polyphenolic flavonoids (e.g., quercetin) or glucobrassicin-derived gastric acid condensation products (e.g., indolo-[3,2b]carbazole) or endogenously produced. For example, kynurenic acid and kyneurenine, products of tryptophan IDO1 and tryptophan pyrolase metabolic pathways, have been established as AhR agonists [11, 15]. Additional examples of microbial AhR agonist production occur at other barrier tissues, such as the skin where indirubin and malassezin produced by the yeast *Malassezia* are both potent AhR activators [41] and the lung where AhR sensing of the bacterial pigments phenazines regulates antibacterial defence [42].

As already mentioned, a number of studies have highlighted the capacity of AhR to respond to indolyl metabolites, including indoxyl-3-sulfate, 6-formylindolo[3,2b]carbazole, kyneurenine, kynurenic acid, tryptamine, indole-3-acetate, and dietary indoles (indole-3-carbinol and 3,3′-diindolylmethane from the Cruciferous vegetables), thus positioning the AhR as a candidate indole receptor [11, 15, 43, 44]. The aromatic bicyclic indole—made of benzene fused to a pyrrole ring—is abundantly present in nature as a metabolic derivative and as an indolyl moiety present in biological molecules utilized by plants, animals, and microbes. Functionally, indole characterizes

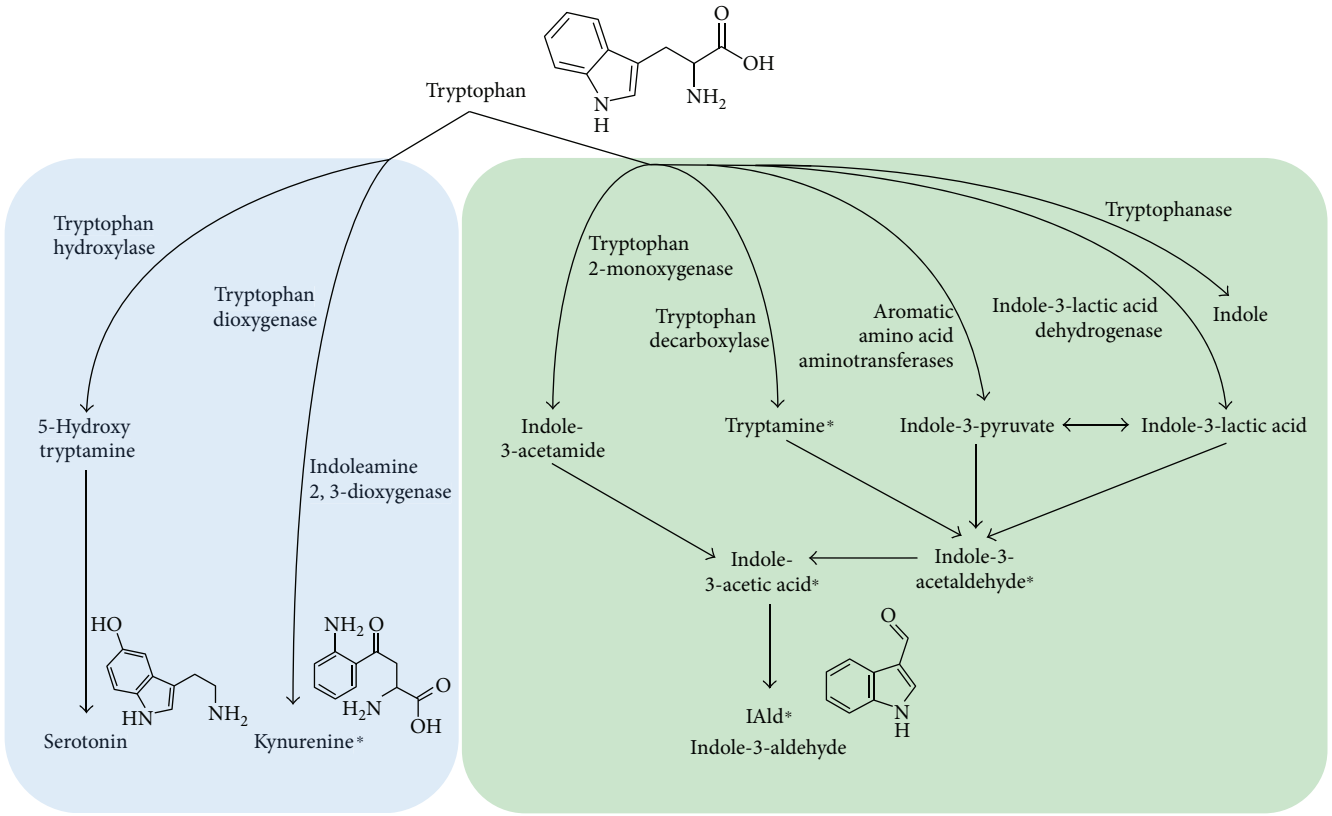

Figure 2: Tryptophan metabolic pathways. The image shows enzymes involved in the host's pathways (on the left) and microbial pathways (on the right) of tryptophan metabolism. Asterisks indicate metabolites with AhR agonistic activity.

the essential amino acid trp and is an important chemical constituent of the hormone melatonin and of auxin and indigo that are a plant signaling molecule and a natural pigment, respectively. Soils and the gastrointestinal tracts of animals contain high levels of indole, and it is now evident that indole and its derivatives are considerably more important as interkingdom signals than was originally believed. As an intercellular and interspecies bacterial signaling molecule, indole plays many roles in bacterial pathogenesis [45, 46]. Bacterial indole and indolyl compounds behave as quorum-sensing molecules among bacterial populations and are implicated in motility, virulence, biofilm formation, and antibiotic resistance [45, 46]. The gastrointestinal tract harbors numerous species (e.g., *E. coli*) which enable to synthesize indole through the tryptophanase- (TnaA-) dependent metabolism of tryptophan [47], so high micromolar concentrations of indole are detected in the gut lumen and feces reviewed in [45]. New findings have highlighted that microbial-derived indoles also mediate signaling between intestinal microbiota and the host via AhR, leading to mucosal protection and regulation of local and distant inflammation [48] (Figure 1).

The AhR ligand ITE, found in the mammalian lung [16] exhibited important immunomodulatory properties both locally and at distant sites [49], apparently by enhancing Treg activity and reducing Th17 cell function. Therefore, AhR ligands are promising therapeutic compounds for lung diseases. However, more research is required to understand the multifaceted role of AhR in the context of inflammatory lung diseases and to define AhR ligands with safe pharmacological profiles in clinical practice. By adopting a "top-down strategy" to screen host/biofluids/tissues for component of microbial origin, a microbial tryptophan metabolic pathway leading to the production of indole-3-aldehyde (3-IAld, Figure 2) has recently been identified that preserves immune physiology at mucosal surfaces while inducing anticandidal resistance via AhR [50]. Much like probiotics, 3-IAld fulfilled the requirement of protecting and maintaining mucosal integrity during fungal infections or chemical damage [51]. Our preliminary results suggest that 3-IAld may protect from respiratory allergy in murine CF and exerts potent antimicrobial activity against gram+ and gram− bacteria (data not shown). Thus, the 3-IAld/AhR axis could be therapeutically exploited for tissue immune homeostasis, microbial symbiosis, and pathogen resistance in CF and other respiratory diseases.

5. Conclusion

It is clear that the AhR and its ligands have important immunomodulatory properties that fine-tune the respiratory immune response. A deeper knowledge of AhR ligands and AhR-signaling properties relative to the development of therapeutic and preventive approaches to inflammation and fibrosis is required. In this regard, the well-known anti-inflammatory activity of lipoxin, a known AhR ligand [52],

in CF [53] points to the drugability of the AhR pathway. In addition, considering the epigenetic regulation of the CFTR transcription [54], this suggests that the therapeutic targeting of AhR will encompass epigenetic mechanisms of CFTR regulation. We are only beginning to understand the effects of indole and indole-producing bacteria on human health. Despite the efforts made to elucidate the mechanisms of action of indoles, the genetic and molecular mechanisms of indole signaling remain unclear. Hence, more research is required to understand the multifaceted role of the AhR in the context of inflammatory lung diseases, as well as the specificity, pharmacology, and dose effect of indoles in the different inflammatory lung diseases. It is clear that an efficacious AhR ligand may not exhibit a "one-size-fits-all" role for limiting inflammation.

Conflicts of Interest

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

Acknowledgments

This study was supported by the Italian Grant funded by the Italian Cystic Fibrosis Research Foundation (Research Project no. FFC#22/2014 to LR) and by the Specific Targeted Research Project FunMeta (ERC-2011-AdG-293714 to LR).

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Hindawi
Mediators of Inflammation
Volume 2018, Article ID 1067134, 22 pages
https://doi.org/10.1155/2018/1067134

Review Article

The EGFR-ADAM17 Axis in Chronic Obstructive Pulmonary Disease and Cystic Fibrosis Lung Pathology

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Received 2 June 2017; Revised 9 October 2017; Accepted 23 October 2017; Published 9 January 2018

Academic Editor: Virginia De Rose

Chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF) share molecular mechanisms that cause the pathological symptoms they have in common. Here, we review evidence suggesting that hyperactivity of the EGFR/ADAM17 axis plays a role in the development of chronic lung disease in both CF and COPD. The ubiquitous transmembrane protease A disintegrin and metalloprotease 17 (ADAM17) forms a functional unit with the EGF receptor (EGFR), in a feedback loop interaction labeled the ADAM17/EGFR axis. In airway epithelial cells, ADAM17 sheds multiple soluble signaling proteins by proteolysis, including EGFR ligands such as amphiregulin (AREG), and proinflammatory mediators such as the interleukin 6 coreceptor (IL-6R). This activity can be enhanced by injury, toxins, and receptor-mediated external triggers. In addition to intracellular kinases, the extracellular glutathione-dependent redox potential controls ADAM17 shedding. Thus, the epithelial ADAM17/EGFR axis serves as a receptor of incoming luminal stress signals, relaying these to neighboring and underlying cells, which plays an important role in the resolution of lung injury and inflammation. We review evidence that congenital CFTR deficiency in CF and reduced CFTR activity in chronic COPD may cause enhanced ADAM17/EGFR signaling through a defect in glutathione secretion. In future studies, these complex interactions and the options for pharmaceutical interventions will be further investigated.

1. Introduction

Airway epithelium, apart from providing a structural barrier against microbes and inhaled particles, also plays an active role in the first line of inflammatory responses [1], and thus emerged as a therapeutic target in chronic lung disease [2]. Airway epithelial cells respond dynamically to bacterial and viral infections [3–5] or inhaled noxious particles by transducing inflammatory signals [6, 7]. They produce cytokines, growth factors, and other inflammatory mediators to orchestrate epithelial repair and adaptation and recruit a range of inflammatory cells including neutrophils and macrophages [1, 8, 9]. Importantly, airway epithelial cells also crosstalk with the underlying connective tissue [10, 11].

This process normally leads to full resolution of inflammation and tissue damage. However, in chronic lung disease, exaggerated trans-signaling can lead to permanent changes of tissue structure and loss of lung function, chronic inflammation, and mucous metaplasia.

Despite their different etiology, cystic fibrosis (CF), a congenital abnormality caused by mutations in a chloride transporter, and chronic obstructive pulmonary disease (COPD), caused by chronic exposure to airborne irritants, share many common features in their pathology detailed in the paragraphs below (Figure 1) [12–14]. Both diseases are characterized by progressive and essentially irreversible lung damage, chronic bronchitis, bacterial colonization, reduced mucociliary clearance, and mucus plugging.

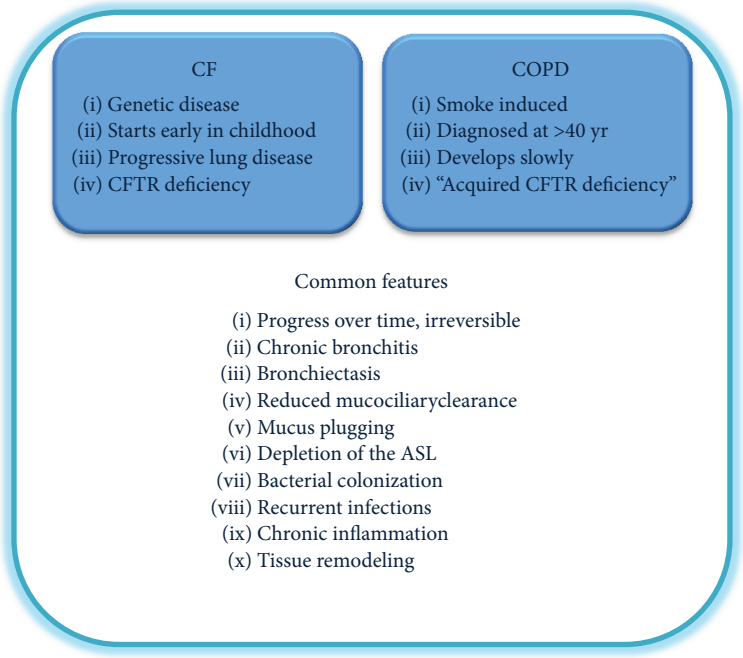

Figure 1: Common features of CF and COPD. CF and COPD lung disease share many common clinical manifestations despite the differences with respect to etiology. Mutations in the CFTR gene determine CF lung disease. Recently, CS has been shown to affect CFTR channel activity and this is recognized as a factor that may contribute to a CF-like phenotype in COPD. Thus, COPD has been recently described as "acquired CF."

This leads to the question whether these diseases, while quite different in many aspects, share common molecular mechanisms and therapeutic targets (Figure 1) [15–17]. In this review, we focus on a signaling pathway called the EGFR/ADAM17 axis and its potential role in chronic lung disease, in particular CF and COPD. Similarly, but outside the scope of this review, other forms of chronic inflammatory lung disease, including asthma, can be added to this list.

A disintegrin and metalloprotease 17 (ADAM17), a ubiquitous sheddase formerly known as TACE (Figure 2), that releases a broad spectrum of soluble biologically active ligands from airway epithelial and myeloid cells is recognized as an important transducer of airway epithelial cis- and trans-signaling [18]. As detailed below, ADAM17 interacts with the EGF receptor (EGFR), mainly through the shedding of EGFR ligands, and conversely, EGFR is required for ADAM17 activity, in a feedback signaling cascade labelled the EGFR/ADAM17 axis. This system is highly sensitive to various extracellular triggers, including cigarette smoke and bacterial toxins, resulting in the enhanced shedding of a large repertoire of growth factors, cytokines, and cytokine receptors that are substrates of ADAM17. This in turn leads to paracrine and autocrine receptor activation, which plays an important role in the resolution of airway inflammation and tissue damage.

In this review, we focus on the role of the epithelial EGFR/ADAM17 signaling pathway that transmits signals from luminal receptors towards underlying tissue (paracrine) and epithelial cells (autocrine) (Figure 3), affecting inflammation and remodeling. However, EGFR and ADAM17 are ubiquitously expressed, and also the expression of ADAM17 substrates is not limited to epithelial cells. Therefore, the complete signaling cascade also includes fibroblasts, smooth muscle cells, and myeloid cells.

The relative contribution of the different cell populations in the EGFR/ADAM17 signaling pathway in humans is still under investigation. Current knowledge is largely based on studies with conditional mutant murine models, in which ADAM17 can be selectively ablated in specific cell types. Knocking out ADAM17 in mesenchymal cells did not produce a detectable developmental phenotype, whereas epithelial ablation resulted in severe abnormalities [19]. Horiuchi et al. showed that the epithelial EGFR/ADAM17 axis plays a major role in tissue regeneration during colonic inflammation and that loss of myeloid expression does not have a major effect. However, myeloid ablation of ADAM17 does reduce the lethality of endotoxin shock induced by intraperitoneal injection [20]. While these studies give important insight in the basic architecture and activity of this system, due caution is required when applied to human tissues. In addition to obvious differences in architecture and function of human and mouse lungs, substrate specificity and expression patterns of members of the ADAM sheddase families and ligand interactions with receptors will differ.

We propose, based on evidence in the literature discussed below, that the molecular mechanism underlying the development of CF and COPD lung disease may involve abnormal activation of the epithelial ADAM17/EGFR axis. To put this in perspective, we first review CF and COPD pathology and the unmet need in clinical intervention. Next, we discuss the role of the EGFR/ADAM17 axis in lung

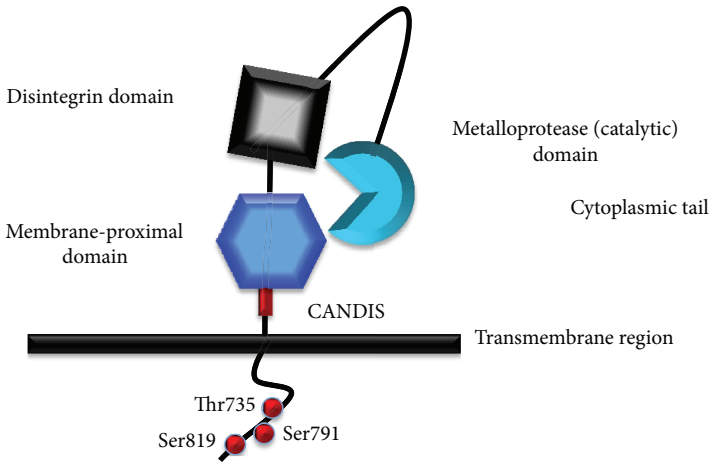

Figure 2: ADAM17 domain structure. ADAM17 is an atypical member of the ADAM family. It has additional disulfide bonds in the metalloprotease domain, and it lacks two calcium binding sites in the disintegrin domain. The membrane proximal domain (MPD), replacing the cysteine-rich and EGF-like domains, with a novel alpha/beta fold has a shorter cysteine-rich segment. The MPD has cysteine residues determining the ADAM17 conformation (open/closed) and ADAM17 protease activity (active/inactive switch). The MPD is in close proximity to the active site likely due to a C-shaped conformation of the extracellular part of mature ADAM17 [99]. ADAM17 lacks an EGF-like domain, so the MPD is followed by the juxtamembrane region "conserved ADAM17 dynamic interaction sequence" (CANDIS) involved in substrate recognition [162]. The trans-membrane region ends with a cytoplasmic tail with phosphorylation sites, which are likely important for ADAM17 activity and trafficking [97–101].

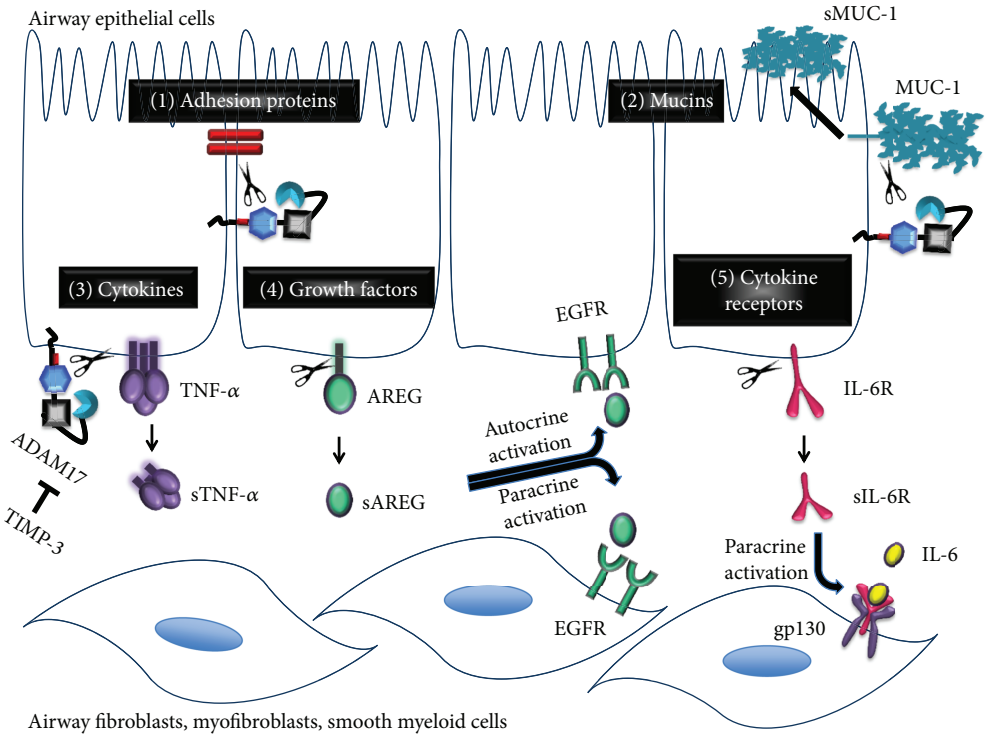

Figure 3: ADAM17 dependent paracrine and autocrine signaling. A disintegrin and metalloproteinase 17 (ADAM17), also known as a tumor necrosis factor-α converting enzyme (TACE), is involved in the immune defense mechanisms mediated by epithelial cells. ADAM17 releases extracellular domains of transmembrane proteins to produce soluble bioactive signaling proteins taking part in autocrine (activation of receptors within the same epithelial cell layer) and paracrine signaling (activation of cellular receptors on underlying neighboring cells, also termed transactivation). Among the ADAM17 substrates are (1) adhesion proteins (L-selectin, ICAM), (2) transmembrane mucins (MUC-1), (3) membrane-bound cytokines (TNF-α), (4) growth factors (AREG) and other ligands of EGFR (TGF-α, EREG, HB-EGF, and epigen), and (5) cytokine receptors (IL-6R, TNF-R). Ectodomain shedding provides the mechanism for autocrine and paracrine signaling. IL-6R shed from epithelial cells transactivates gp130 on the underlying myofibroblasts, whereas AREG shed from epithelial cells activates EGFR on epithelial cells or on the underlying fibroblasts. The ADAM17 inhibitor TIMP-3 inhibits the ADAM17 proteolytic activity.

pathology, the molecular mechanisms involved, and potential therapeutic strategies.

2. Cystic Fibrosis: A Congenital Lung Disease with an Early Onset

CF is an autosomal recessive lung disease caused by more than 2000 different mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene [21] with approximately 80,000 patients worldwide. The most common CF mutation is the deletion of a phenylalanine at amino acid position 508 in the NBD1 domain of CFTR protein (F508del) [22]. The CFTR gene encodes a chloride channel mainly expressed in the apical membrane of secretory epithelial cells [23]. CFTR has a key role in maintaining ion and water homeostasis in secretory epithelia [23]. Mutations in CFTR cause a multiorgan CF disease affecting the lungs, intestine, pancreas, liver, sinuses, and reproductive organs [24]. However, under present treatment, the main morbidity and mortality among CF patients is due to lung malfunction; consequently, most available and experimental treatments aim to prevent the progression of CF lung disease [25]. CFTR is also involved in transport of other anions, for instance bicarbonate [26] and glutathione [27, 28]. Bicarbonate is crucial to normal expression of mucins that in CF remain aggregated and poorly solubilized [29]. Glutathione, as a natural antioxidant, reduces oxidative cellular stress [30].

2.1. CF Lung Disease: An Early Disorder of Distal Airways. Advances in imaging and monitoring of the respiratory system in infant patients have revealed that severe CF lung pathology starts early in childhood and progresses irreversibly over time [31–33]. This early onset of lung abnormalities includes bronchiectasis, diagnosed thickening and dilation of the bronchial walls, air trapping, and atelectasis (partial collapse of the lung) [34, 35]. These symptoms occur simultaneously with reduced mucociliary clearance and mucus plugging [36]. Quantitative and standardized tracking of early lung disease progression in infants with CT scans is pursued to advance the comparative analysis and provide the evaluation of the treatment [37, 38].

In a recent micro CT and histological study of end-stage CF lungs, the dilatation and obstruction of distal airways and a severe reduction in the number of functional terminal bronchioles were clearly documented for the first time. This confirms that obstruction and remodeling of peripheral airways is a prominent feature in CF lung disease and therefore are prime targets of experimental therapy [14]. CF mouse models [39] and large CF animal models, like pig [40] and ferret [41], are helpful in the investigation of the mechanism involved in this early onset of CF lung disease and intervention therapy, but all have considerable limitations due to species variation. In addition to state-of-the-art clinical and biomarker studies, there is an urgent need for the development of organotypic and personalized cell culture models in which the complex molecular and cellular interactions involved in CF lung pathology can be studied.

2.2. Mucociliary Transport. In healthy subjects, mucociliary clearance requires CFTR-dependent balanced fluid and proper mucus secretion from surface cells and subepithelial glands. However, high mucus viscosity in CF is caused by reduced CFTR-dependent bicarbonate secretion, required for proper expansion of secreted mucus molecules [42, 43]. Furthermore, CF patients have intrinsically impaired ciliary beat frequency (CBF), which is not only dependent on CFTR-mediated bicarbonate transport but also regulated by soluble adenyl cyclase (sAC) [44]. Together, this impairs effective clearance of bacteria and inhaled particles in CF lung [45, 46]. Importantly, reduced mucociliary clearance is also a feature of COPD, which may be in part related to reduced CFTR activity in smokers' lungs [47].

2.3. Bacterial Infection and Inflammation. Airway epithelial cell cultures from CF patients have reduced air-surface liquid (ASL) height, presumably due to defective CFTR-dependent fluid secretion [48]. Additionally, as a consequence of reduced bicarbonate secretion, CFTR deficiency abnormally acidifies ASL [49] which impairs bacterial killing [50, 51], inhibits the activity of ASL antimicrobials [52], and increases ASL viscosity of newborn CF piglets [45].

Impaired bacterial killing and mucociliary clearance in CF lungs together facilitate colonization with opportunistic pathogens generally harmless in normal individuals [53]. Despite activation of inflammatory responses mediated by the innate and cellular immune system, eradication of bacterial infection is impaired in CF lungs. Instead, bacterial infections induce a massive and chronic recruitment of neutrophils, which ultimately contributes to irreversible airway remodeling, observed as air trapping, bronchiolar obstruction, bronchiectasis, and loss of function [14].

2.4. Sterile Lung Inflammation in CF. While chronic infection is undoubtedly a major issue in CF pathology, it remains contested whether inflammation in CF only results from bacterial infection or is an intrinsic property of CFTR-deficient mucosa. Lung disease and inflammation is already observed in CF infants before bacterial colonization, suggesting that inflammation may precede infection in CF lung. Several reports argue that bacterial infections are indispensable to inflammatory responses, in CF humans [54] or CF pig [55]. However, unchallenged CF mutant mice show inflammation [56]. Also, in the CF ferret model, inflammation and tissue remodeling do not require previous bacterial infections [57]. Such sterile inflammation may be caused by functional abnormalities in CF myeloid cells, in particular macrophages [58, 59], dendritic cells [56], and neutrophils [60] or may be primarily related to abnormal cytokine signaling by CFTR-deficient airway epithelial cells. Several studies suggest that CFTR malfunction leads to overexpression of growth factors and proinflammatory cytokines as a cell-autonomous defect [61–63]

Due to the early onset of CF lung disease and its irreversible nature, it is clear that CF patients require early intervention therapy [32]. Excessive lung inflammation and tissue remodeling observed in CF may be an inherent property of CFTR-deficient lung mucosa. Therefore, it is

important to establish the mechanisms involved in CFTR-related inflammatory responses and whether alleviation of inflammatory responses is beneficial in the management of CF lung disease [64].

3. Strategies of CF Therapy

CF lung disease is still the main cause of morbidity and mortality in CF despite intensive treatment [14]. Indirect pharmacological management focuses on anti-inflammatory agents [65, 66], antibiotics [67], and mucolytic agents [68]. Direct pharmacological management of CF disease intends to restore the functional expression of mutated CFTR at the plasma membrane by correcting its folding and gating defect [69]. In 2015, the FDA approved ORKAMBI® for homozygous F508del CFTR patients, comprising fifty percent of the CF population, the combination of CFTR corrector lumacaftor (VX-809) and potentiator ivacaftor (VX-770) in one pill. This therapy improves lung function in patients homozygous for the F508del mutation, although modestly and not in all patients [25, 70]. Therefore, further investigations are in progress to find more effective compounds [71, 72]. So far, the effects of correctors and potentiators on infections and the release of inflammatory mediators have not been broadly investigated in clinical studies. Rowe et al. showed that ivacaftor reduces *P. aeruginosa* isolated from CF patients carrying a CFTR gating mutation after 6 months treatment. However, the free neutrophil elastase and other inflammation markers like IL-1β, IL-6, and CXCL8 (IL-8) in sputum samples remained unchanged [73]. Therefore, anti-inflammatory, antibacterial, and other additional therapies are still important targets of investigation [74].

In summary, recent breakthroughs in the development of small-molecule compounds targeting the mutant CFTR protein have raised hope to find a cure for CF. However, the presently available CFTR correctors and potentiators are not sufficiently effective in a majority of CF patients, and the search for new compounds and additional therapies is still highly relevant. Though CF is considered monogenetic, the downstream responses to CFTR deficiency and responses to therapeutic intervention are highly variable in the population. The perfect corrector and potentiator combination tailored to the individual patient, supported by additional anti-inflammatory medication (personalized medicine), would likely be the best solution.

4. COPD Is Acquired CF?

Chronic obstructive pulmonary disease (COPD) is the 5th ranking cause of death worldwide. Usually, COPD is characterized by chronic bronchitis and emphysema [75]. Similar to CF, bronchiectasis [76] and peripheral airway thickening are also observed, similar to early and advanced CF lung disease [77]. Some individuals develop lung disease dominated by emphysema, while others exhibit chronic bronchitis. This heterogeneous phenotype likely reflects the contribution of multiple pathogenic mechanisms and the genetic heterogeneity of the population. Once COPD starts to develop, it tends to worsen over time, and so far its progress cannot be controlled effectively in most patients. The most prominent etiological factor leading to COPD is cigarette smoke, and also exposure to fumes, chemicals, and dust [78]. Although COPD and CF differ in primary cause, the spectra of the pathological events overlap considerably (Figure 1). Chronic bacterial infections with frequent exacerbations are observed, including colonization with the opportunistic pathogen *Pseudomonas aeruginosa*, which are also a hallmark of CF lung disease. However, the strains adapted to COPD lungs appear to differ from CF, suggesting that although similar mechanisms are involved, the luminal milieu in COPD differs from that in CF [79]. Both diseases are characterized by excessive mucus production and insufficient clearance, leading to lower airway obstruction and chronic neutrophilic infiltration. In CF and COPD, airway surface liquid (ASL) dehydration and viscous mucus secretion impair mucociliary clearance, causing chronic inflammation and facilitating recurrent infections [80]. A wide variety of proinflammatory mediators in COPD (like CXCL8, IL-6, and CCL18) overlap with CF-related mediators [74, 81]. There are also parallels on the cellular level which include goblet cell metaplasia, hyperplasia of myoblasts, and extensive extracellular matrix production [17].

The broad spectrum of common features and events observed in CF and COPD encouraged researchers to seek common factors for these diseases. Cigarette smoke decreases CFTR mRNA expression and reduces CFTR protein level through accelerated degradation, leading to impaired mucociliary clearance and depleted ASL in vitro and in vivo [82–84]. Mice treated with cigarette smoke show reduced CFTR activity that can be corrected by the cAMP agonist and phosphodiesterase inhibitor roflumilast, restoring CFTR activity [85]. Furthermore, macrophage phagocytosis and CFTR activity are impaired by cigarette smoke [86]. Similarly, CFTR-deficient macrophages are abnormal [59]. These are short-term and transient effects in normal cells, which do not explain the progressive inflammatory lung disease of COPD patients that stopped smoking. However, several reports show that the abnormal characteristics of COPD epithelial cells, including reduced CFTR expression in situ, persist in culture [87], probably due to a combination of genetic factors and epigenetic imprinting. Furthermore, COPD patients, smokers, and former smokers show signs of persistent reduced CFTR function in upper and lower airways, which may contribute to chronic lung disease [80]. Consequently, several authors described COPD as an "acquired CF" through e reduction of CFTR activity, suggesting that these diseases with different etiology have common therapeutics options [16, 17, 80, 85].

5. Strategies of Therapy in COPD

Based on the previous arguments, CFTR potentiators and correctors, such as those developed to enhance the activity of mutant CFTR, could be useful to treat COPD. However, small molecules designed to target mutant CFTR trafficking and gating may actually reduce activity of normal CFTR upon chronic treatment [88]. Nevertheless, further screening and testing of novel compounds in a personalized setting

may lead to improvement of CFTR function and reduction of lung disease in COPD patients.

Chronic inflammation is recognized as the major pathophysiological mechanism of COPD progression, with molecular targets overlapping those of CF (e.g., IL-6, CXCL8, and CCL18) [74]. Thus, chronic airway inflammation remains an important therapeutic target in COPD management. It often persists after cessation of smoking, suggesting that apart from the role of CFTR also epigenetic changes in the resolution of inflammation likely play a role [89, 90]. Presently, several compounds targeting inflammatory responses in COPD are under investigation [91, 92], though none of these have been shown to be beneficial in COPD patients as yet.

Therefore, as in CF, COPD is a complex multifactorial disease, with large variation in the patient population due to largely undefined genetic and environmental factors. A personalized approach using multiple treatments is likely required, but a robust trial strategy is elusive. The advance of personalized 3D culture models, combining induced stem cell (iPSC) and gene editing [93] with microfluidics ("lung-on-a-chip") technology [94] may allow us to proceed towards better treatment.

6. Epithelial EGFR/ADAM17 Axis: A Potential Therapeutic Target in CF and COPD Lung Disease

The design of anti-inflammatory agents aims mainly to decrease the neutrophil influx into the lung and concomitant inflammatory responses [8]. The importance of airway epithelial cells in inflammatory responses has been recognized for decades, but the signaling network is still under intense investigation [1, 95]. Airway epithelium serves as the first barrier and acts as defense against daily inhaled air pollutants and microbes by mucociliary clearance and secretion of a range of cytokines, cytokine receptors, growth factors, growth factor receptors, and antimicrobial peptides [1, 96]. Airway epithelial cells not only release inflammatory mediators to ASL but also signal to the underlying tissues (myocytes or fibroblasts). One of the mechanisms controlling paracrine and autocrine proinflammatory and profibrotic signaling in airway epithelium is the EGFR/ADAM17 axis.

A disintegrin and metalloproteinase 17 (ADAM17), also known as a tumor necrosis factor-α converting enzyme (TACE), is a transmembrane protein with proteolytic activity. It releases extracellular domains of its substrates, generally transmembrane proteins, to produce soluble bioactive signaling proteins, in a process called shedding. Mature ADAM17 consists of several functional domains (Figure 2), an extracellular metalloprotease domain (catalytic), a disintegrin domain, a membrane proximal domain (MPD) rich in cysteine residues, and a "conserved ADAM-seventeen dynamic interaction sequence" (CANDIS). Unlike other ADAM family members, it lacks an EGF-like domain [97–101]. These extracellular domains are connected to a transmembrane region and cytoplasmic tail with phosphorylation sites that likely are involved in ADAM17 activation or trafficking (Figure 2).

Ectodomain shedding mediated by the metalloprotease domain of ADAM17 provides a mechanism for initiation or inhibition of autocrine/paracrine signaling. So far, 76 proteins have been identified as substrates of ADAM17 [18]. They encompass membrane-bound cytokines (TNF-α), cytokine receptors (IL-6R, TNF-R), growth factors, in particular ligands of EGFR (TGF-α, AREG, EREG, HB-EGF, and epigen), adhesion proteins (L-selectin, ICAM-1), and transmembrane mucins (MUC-1). The shed soluble forms of these proteins are bioactive transducers of cell signaling via activation of cellular receptors on underlying neighboring cells (transactivation/paracrine activation), but they also are involved in activation of the shedding cells and neighboring cells (autocrine activation) (Figure 3).

Because most of the epidermal growth factor receptor (EGFR) ligands are cleaved by ADAM17, this sheddase has emerged as an important transducer of the airway epithelial autocrine and paracrine EGFR signaling (Figure 3). EGFR and ADAM17 are both involved in the broad spectrum of events that is characteristic of both CF and COPD lung disease, like excessive mucus expression [102–104], cytokine secretion [105], airway epithelial cell wound healing [106, 107], abnormal airway proliferation [108], maintenance of barrier integrity, and progressive lung tissue scarring [109]. They both are activated upon bacterial or viral infection and during inflammation [3, 5, 110]. While this is an effective and necessary response, it is suggested that exaggerated airway epithelial signaling in the chronic state may enhance inflammation and may lead to permanent damage to the lung structure.

EGFR functions as a sensor of airway epithelial integrity [111]. When cells have intact tight junctions, EGFR is not activated. But disruption of the epithelial cell integrity, either by mechanical injury or cytokine treatment (TNF-α/IFN-γ), leads to EGFR phosphorylation and concomitant inhibition of protein phosphatase 2A activity [112]. Cigarette smoke exposure of differentiated HBEC also leads to damage of the lung tissue observed as destruction of epithelial cell integrity, loss of E-cadherin/β-catenin complex, and disappearance of cilia [113]. This coincides with phosphorylation and perinuclear trafficking of EGFR [113] suggesting the importance of EGFR in maintenance of epithelial cell barrier integrity. The response of ADAM17 to loss of pulmonary epithelial cell integrity has been also shown by neuregulin-1 (NRG-1) shedding and concomitant activation of human epidermal growth factor receptor-2 (HER2) [114]. This raises the question whether and how EGFR and ADAM17 cooperate in sensing responses to airway injury.

6.1. EGFR and ADAM17 Are Important in Lung Development. Inactivation of ADAM17 by deletion of the zinc binding domain through homologous recombination in mice led to severely hypoplastic lungs at birth, reduced branching morphogenesis and alveolar development, impaired epithelial cell proliferation, and differentiation and delay in vasculogenesis [115]. Due to the early mortality of ADAM-KO mice, several alternative ADAM17 mouse models have been developed, with reduced activity (hypomorphic) or conditional mutants [116]. Conditional knockout ADAM17 mice

can serve as a model to investigate the role of ADAM17 in organogenesis, in specific cell types, and in inflammatory responses and tissue remodeling [20, 117]. Induced dysfunction of ADAM17 (ADAM17flox/flox SPC-rtTA TetO-Cre) in developing lung epithelial cells reduced saccular formation, cell proliferation, and lung epithelial cell differentiation, but the mice were born without severe respiratory distress [19]. Knocking out the gene in mesenchymal cells using a Dermo1-Cre transgene did not produce a detectable phenotype [19]. This suggests that epithelial, but not mesenchymal, ADAM17 plays a prominent role in lung development.

Other studies show that conditional knockout mice (ADAM17flox/flox with R26Cre-ER) treated with human neutrophil elastase have attenuated goblet cell metaplasia in comparison to the wild-type mice [118], suggesting that ADAM17 is involved in injury-induced airway metaplasia. Similar to ADAM17-KO mice, EGFR-KO mice survive for up to 8 days after birth and suffer from impaired epithelial development in several organs, including the lungs, underlining the need of a functional EGFR/ADAM17 axis for proper functioning and development of lung epithelium [119].

6.2. ADAM17 and EGFR Crosstalk. ADAM17 works in association with the tyrosine kinase receptor EGFR firstly by shedding most of its ligands, including AREG, HB-EGF, TNF-α, EPGN (epigen), and EREG (epiregulin) [18], resulting in activation of EGFR. In humans, only two EGFR ligands, EGF and betacellulin, are shed by ADAM10 [120, 121], a close relative of ADAM17 [18]. Crosstalk of ADAM17 and EGFR in inflammatory signaling transduction is further defined by the establishment of a positive ADAM17/EGFR feedback loop likely involving activation of ADAM17 via the EGFR/MAPK pathway [110, 122] (Figure 4). The exact molecular mechanism of this feedback signaling has not been firmly established. Moreover, ADAM17 regulates transcription of EGFR mRNA by cleavage of Notch1 and thus increases EGFR expression in a non-small lung carcinoma cell line [123], providing another positive feedback mechanism of the EGFR/ADAM17 axis (Figure 4).

In differentiated primary bronchial epithelial cells (HBEC-ALI), inhibitors of EGFR and of ADAM17 completely abolished ADAM17 substrate shedding as well as EGFR-dependent CXCL8 mRNA induction [87], illustrating the strong interrelationship between the key elements of the EGFR/ADAM17 axis (Figure 4).

6.3. The Role of ADAM17 and EGFR in Balanced Regulation of Lung Inflammation and Regeneration. ADAM17 is expressed in human bronchial epithelial cells in the large and small bronchi, in lung smooth muscle cells, lung muscular vessels, alveolar macrophages, perivascular leukocytes, and lung endothelial cells. It has been long recognized as an important regulator of lung tissue homeostasis [116, 124, 125], pro- and anti-inflammatory responses [18], and tissue regeneration [122, 126, 127].

The anti- and proinflammatory properties of the EGFR/ADAM17 signaling pathway are context and cell type dependent [18]. For instance, in airway epithelial cells, ADAM17

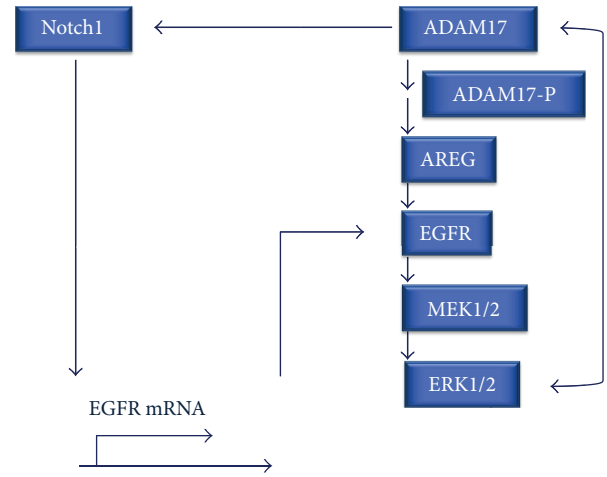

Figure 4: The ADAM17/EGFR axis. The EGFR/ADAM17 positive feedback loop likely involves activation of ADAM17 via the EGFR/MAPK pathway and direct interaction of ERK1/2 with ADAM17. Additionally, by cleavage of Notch1 from a non-small lung carcinoma cell line ADAM17 regulates transcription of EGFR mRNA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

together with EGFR induces mRNA expression and protein release of CXCL8, a neutrophil chemotactic factor that promotes inflammation [7, 87, 105]. However, by shedding TNF receptor type 2 (TNFR2) [128], which antagonizes TNF-α, ADAM17 exhibits also anti-inflammatory properties [129]. Thus, it is likely that the type of stimulus and substrate selection determine the effect of the EGFR and ADAM17 activity [130–132].

EGFR/ADAM17 signaling in tissue regeneration encompasses wound healing [122], proliferation [126, 127], differentiation [19, 133], and cell migration [134]. Due to the involvement of EGFR/ADAM17 paracrine and autocrine signaling in several lung disorders [109, 116], modulation of ADAM17 and EGFR activation in both COPD and CF is important, to keep the balance between anti-inflammatory processes and promotion of inflammation, and also between regeneration and excessive tissue remodeling. Indeed, we recently found that cigarette smoke induced shedding of the ADAM17 substrate amphiregulin (AREG), and IL-6R was enhanced in differentiated bronchial cells in culture obtained from COPD patients compared to non-COPD, suggesting that epigenetic factors controlling the activity of the ADAM17/EGFR axis are affected in COPD [87].

6.4. Release of Cytokines, Growth Factors, and Mucins Depends on ADAM17/EGFR Signaling. External stress factors like oxidative stress, viral and bacterial toxins, and CS exposure activate the ADAM17/EGFR signaling pathway. Pathogens inhaled into the airways, or exposure to other stimuli, activate Toll-like receptors (TLR) [135–137] and G-coupled receptors (GPCR) [138] that crosstalk with downstream ADAM17/EGFR signaling. As a result of cigarette smoke extract exposure, secretion of downstream proinflammatory cytokines including CXCL8 [7, 139], growth factors (TGFα,

AREG, and HB-EGF) [87, 105], cytokine receptor IL-6R [87], mucins (MUC5AC), and phosphorylated MUC1 is induced in an ADAM17/EGFR-dependent manner [103, 113, 140, 141]. This cascade of events involves mitogen-activated protein kinases (MAPK), MAPK1 (ERK1), MAPK2 (ERK2), and p38-MAPK [3, 142]. Upon activation of the ERK or p38 MAPK pathway, ADAM17 dissociates from an endogenous extracellular tissue inhibitor of metalloproteinase-3 (TIMP-3) [143-145], accumulates on the cell surface [146, 147], and induces release of TGF-α [146]. Acrolein, an active component of cigarette smoke, which induces MUC5AC mRNA in an ADAM17 and EGFR dependent manner, decreases TIMP-3 transcript levels, also suggesting a role of TIMP-3 in ADAM17 activation.

6.5. CFTR Deficiency Affects the EGFR/ADAM17 Axis. Several lines of evidence suggest that CFTR deficiency affects the activity of the EGFR/ADAM17 axis. F508del CFTR mutant mice respond differently from normal to airway injury by naphthalene [148]. A week after injury, we observed a significantly enhanced mRNA expression of amphiregulin (AREG) compared to normal [149]. AREG mRNA expression is dependent both on ADAM17 and EGFR activity in human bronchial epithelial cells [87], suggesting a link between CFTR deficiency and EGFR/ADAM17 activity during resolution of airway injury. Furthermore, inhibition of CFTR with a small molecule (inh-172) in NCI-H292 cells reportedly activates CXCL8 production in an EGFR/ADAM17-dependent way [61]. However, the relationship between ADAM17/EGFR signaling and CFTR deficiency is still poorly understood and may involve a variety of mechanisms [150].

Exaggerated responses mediated by the EGFR/ADAM17/MAPK pathway have also been reported in CFTR-deficient cells compared to CFTR expressing counterparts. The CFTR-deficient cell line IB3 produces more CXCL8 than an isogenic CFTR-expressing cell line. The CFTR-deficient cell line CuFi-1 produces more CXCL8 in response to heat-inactivated *P. aeruginosa* than a non-CFTR-deficient cell line (NuLi-1), and this involves EGFR phosphorylation and ERK1/2 activation [151]. Most of these studies use undifferentiated, submerged immortalized cell lines [152], compare genetically diverse cell lines (like CuFi and NuLi or IB3 and C38 cells) [61, 151, 153, 154], or use a CFTR inhibitor [61] with reported off-target effects [155]. Further studies in primary human bronchial epithelial cells in air-liquid interface culture, and models that allow comparison of genetically identical populations in parallel, including CFTR-deficient and corrected induced pluripotent stem cells (iPSC) and inducible CFTR-expressing CFBE cells, are currently performed to further support the notion that CFTR is involved in the activity of the EGFR/ADAM17 axis and may contribute to abnormal resolution of injury and inflammation in CF lung disease.

6.6. Redox Potential: A Possible Link between CFTR Deficiency and ADAM17/EGFR Signaling? The intra- and extracellular redox potential changes in response to physiological processes and in pathophysiological conditions [156]. Reactive oxygen species (ROS), produced during cellular stress [74], are an important regulator of redox state and they are also involved in activation of the EGFR/ADAM17 signaling pathway, affecting TGF-α and AREG release and mucin expression [103, 104, 135]. Some studies point towards the role of NADPH oxidases (NOX), in particular dual oxidase 1 (DUOX1) [104, 135, 157] or dual oxidase 2 (DUOX2) [136], which produce ROS at an extracellular or possibly intravesicular domain. ATP-mediated DUOX1 activation involves a TGF-α/ADAM17/EGFR/ERK signaling pathway [157]. Recent studies indicate a role of DUOX1 in allergen-dependent SRC/EGFR activation in airway cells [158]. However, the mechanism by which ROS affect EGFR/ADAM17 signaling in intact airway cells is likely highly complex and still remains not well understood.

ROS do not only affect receptors, phosphatases, and kinases in the ADAM17/EGFR pathway, but ADAM17 [159] and EGFR [160] themselves are redox-sensitive proteins. EGFR has intracellular cysteine residues in the active site that are targets of ROS and determine EGFR kinase activity, likely through association of EGFR with NADPH oxidase, NOX2 [160]. ADAM17 activity is regulated by thiol-disulfide isomerization in the extracellular MPD domain mediated by protein disulfide isomerase (PDI), an oxidoreductase sensitive to redox changes [161]. PDI, by direct interaction with the membrane proximal domain (MPD) [159], changes the disulfide bridge pattern and thus the conformation of the extracellular protease domain from open active to closed inactive state leading to the inhibition of ADAM17 activity (Figure 5) [101, 162]. Redox-dependent conformational changes likely make ADAM17 sensitive to the extracellular redox potential [159].

Since CFTR deficiency is thought to increase ROS levels [163], which would activate ADAM17 and EGFR [122] and inactivate protein phosphatases [164], we propose that redox signaling is a plausible link between EGFR/ADAM17 activity and CFTR deficiency. CFTR deficiency may change the extracellular redox potential to more oxidized in airway, because CFTR is involved in epithelial glutathione transport at the apical membrane [27, 165], which serves as a natural antioxidant [30]. This would in turn enhance the activity of ADAM17 through the mechanism illustrated in Figure 5. Polymorphisms in the glutathione pathway are modifiers of CF lung pathology, emphasizing the importance of this pathway [166]. Further studies are required to establish the relationship between glutathione transport, extracellular redox potential, and the activity of the EGFR/ADAM17 axis in CF airways. This can be achieved by application of fluorescent protein redox probes that can be expressed in different cellular compartments [156] in advanced airway culture models.

7. Role of Amphiregulin in EGFR/ADAM17-Dependent Lung Disease

One of the prominent ADAM17-dependent EGFR agonists produced ubiquitously by the human lung is amphiregulin (AREG) [167] by human airway epithelial cells [87, 105], smooth muscle cells [168], and fibroblasts [111]. AREG is also expressed by infiltrating and resident lung myeloid cells,

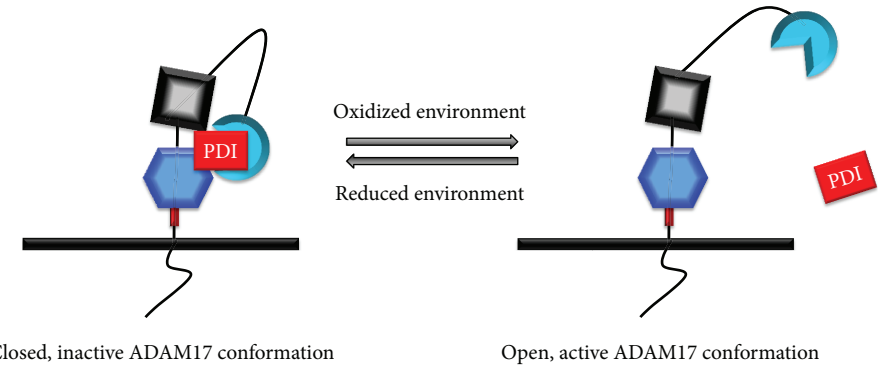

Figure 5: ADAM17 is a redox-sensitive protein. The ADAM17 membrane proximal domain (MPD), which is in close proximity to the active site, is sensitive to extracellular (or intravesicular) redox changes. This redox-sensitive ADAM17 activity is regulated by thiol-disulfide isomerization mediated by protein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

including activated macrophages [169], eosinophils [170], dendritic cells [171], neutrophils [172], and mast cells [173]. Human AREG is synthetized as an N-glycosylated transmembrane precursor (50 kDa)[174] with a basolateral sorting motif [175] and shed mainly by ADAM17 [110]. Proteolytic cleavage of the AREG extracellular domain releases several AREG-soluble forms, predominantly an α-N-glycosylated 43 kDa form [174], which is one of the EGFR ligands, with lower affinity to EGFR than EGF and TGF-α.

7.1. AREG Is Induced in Lung Disease. AREG is involved in inflammation and repair responses through autocrine and paracrine activation of EGFR, and generally induced in lung disease [176]. In CF sputum samples, elevated levels of AREG have been shown in airway blood neutrophils [172]. In lung biopsies from asthma patients, more AREG is expressed than in healthy controls [167]. Other studies showed that in sputum of asthma patients AREG is upregulated only during an acute attack [177], suggesting its role in quick cellular responses to the triggers. Increase of AREG in sputum samples from children with asthma negatively correlates with lung function [178] and positively correlates with the number of eosinophils [179, 180].

Epithelial secretion of AREG in situ has not been investigated in CF and COPD in comparison to controls. However, Zuo et al. reported enhanced AREG expression in smoking-induced airway lesions [181], and we observed a stronger AREG shedding response to cigarette smoke in cultured airway cells from COPD patients compared to normal [87] suggesting a possible involvement in the progression of lung disease. Current studies are aimed at elucidating the role of CFTR deficiency in AREG shedding.

7.2. Regulation of AREG Transcription and Shedding. In vitro, AREG mRNA expression and protein release are induced upon exposure to different stress factors like histamine [167], diesel exhaust particles [6], cigarette smoke extract exposure [182], cigarette smoke [87], and rhinoviruses [3]. Also, AREG protein secretion is dependent on the EGFR/MAPK pathway in an airway epithelial cell line treated with particulate matter [183]. In differentiated primary airway cells, CS induction of AREG mRNA levels is abolished by ADAM17 and EGFR inhibitors, consistent with a prominent role of the EGFR/ADAM17 axis in AREG signaling and mRNA synthesis [87].

7.3. AREG Affects Mucus and Cytokine Secretion in Asthmatic Patients. In asthmatic patients, AREG produced by mast cells enhances mucus production [184]. AREG-dependent MUC5AC mRNA level induction has been shown upon exposure to particulate matter in NCI-H292 cells [185] and differentiated primary airway cells NHBE-ALI [177]. AREG-dependent secretion of MUC5AC, TGF-β1, and CXCL8 was also observed in epithelial cell line culture supernatants [179]. All these findings suggest an important role of AREG in mucus secretion and cytokine release in airway epithelial cells.

7.4. AREG in Paracrine Signaling and Tissue Remodeling. In addition to its role in epithelial proliferation and differentiation (autocrine), AREG is also involved in fibrotic and inflammatory responses (Figure 3). Conditioned media from AREG-stimulated airway epithelial cells induced expression of CXCL8, VEGF, COX-2, and AREG in human airway smooth muscle cells, providing proof for paracrine crosstalk between epithelial cells and connective tissue in an AREG-dependent manner [168](Figure 6). Inhibition of AREG or EGFR in TGF-β1-stimulated lung fibroblasts diminished AREG-dependent fibroblast proliferation, expression of α-smooth muscle actin and collagen [186], strongly suggesting a role of EGFR/ADAM17/AREG signaling in pulmonary fibrosis in vivo. AREG stimulation also induces airway smooth muscle cell proliferation, which leads to airway remodeling in vivo [180].

These data together suggest that exaggerated and chronic AREG release may contribute to mucus plugging, excessive

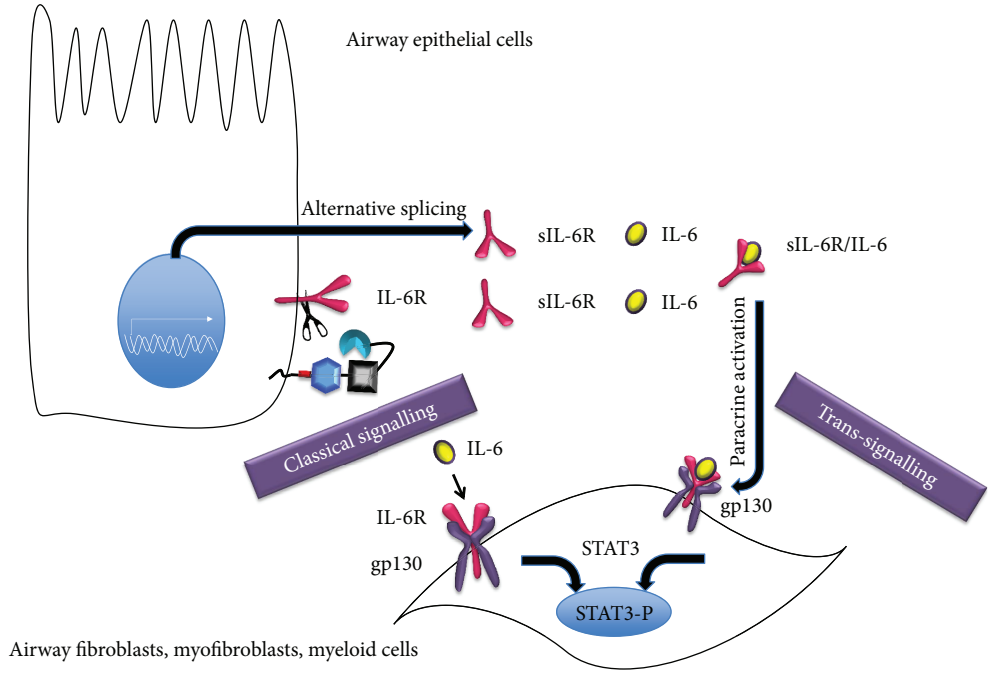

Figure 6: Classic and trans IL-6R signaling. In "classic" signaling membrane-bound IL-6R stimulated by secreted IL-6 associates with a homodimer of signal transducing glycoprotein (gp130) to activate downstream signaling molecules, including the ubiquitous transcription factor STAT-3. In trans-signaling,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

inflammation, and tissue remodeling in CF and COPD. Therefore, the EGFR/ADAM17/AREG signaling pathway is a potential therapeutic target to regulate both inflammation and lung cell proliferation in COPD and CF. However, AREG is only one of the many players involved in the autocrine and paracrine signaling leading to lung pathology. There is no available literature presenting intervention in AREG function in clinical trials.

8. Role of IL-6R in Human Lung Inflammation and Tissue Regeneration

Another prominent ADAM17 substrate involved in lung disease is the IL-6 coreceptor IL-6R. In "classic" signaling, this transmembrane receptor type I binds to IL-6, which evokes an association with a homodimer of signal transducing glycoprotein, gp130. This trimer dimerizes to form a hexameric complex composed of IL-6, IL-6R, and gp130 [187] (Figure 6). Alternatively, the extracellular domain of IL-6R can also evoke a trans-signaling cascade, when it is shed by ADAM17, producing a soluble form (sIL-6R) (Figure 6) [188] or ADAM10 [188–190]. sIL-6R can be also generated by alternative splicing of IL-6R mRNA [191–193]. However, data suggest that shedding rather than the alternative splicing takes part in trigger-induced generation of the sIL-6R [128]. Indeed, we observed that in primary bronchial epithelial cells in air liquid culture, cigarette smoke enhanced ADAM17-dependent sIL-6R shedding but not the production of the alternatively spliced mRNA [87]. Both the alternatively spliced form and the shed form of sIL-6R can create functional complexes with IL-6 (IL-6/sIL-6R) [194], mediating a transfer of stress responses from epithelial cells to underlying mesenchymal and myeloid cells, involved in tissue remodeling and inflammation (trans-signaling, Figure 6).

IL-6 activated membrane bound IL-6R has anti-inflammatory, antiapoptotic, and regenerative properties [128, 195–198]. In contrast, trans-signaling mediated by IL-6/sIL-6R complexes binding to gp130 is thought to maintain inflammation and promote inflammation-associated cancer [128, 195, 199, 200] (Figure 6).

Importantly, IL-6R expression is not ubiquitous, but restricted to specific cell types, such as lymphoid cells, hepatocytes, and airway epithelial cells [4, 128, 201, 202]. However, cells that do not express IL-6R but do express gp130 can still transduce IL-6 signals via binding of IL-6/sIL-6R complexes in trans. Thus, smooth muscle cells and endothelial cells are responsive to IL-6 through IL-6/sIL-6R trans-signaling provided by epithelial cells [203, 204] (Figure 6).

The role of IL-6R in CF and COPD has not been studied in detail. In COPD patients, elevated levels of IL-6R have been observed in peripheral blood leukocytes [205] and sputum samples [206]. Recently, genetic variants of IL-6R have been linked with lung function [207] and COPD severity [208]. In contrast to elevated IL-6R levels in sputum of COPD patients [207], the levels of sIL-6R in BALF from CF patients were not different in comparison to control, possibly due to enhanced degradation of sIL-6R by serine proteases

[209]. Furthermore, it is not evident what the impact of luminal IL-6R on the activity of the subepithelial tissue is, which is separated from the lumen by tight junctions. Shedding of IL-6R from epithelial cells occurs mainly towards the basal side where it can engage in paracrine and autocrine signaling (Figure 6) [4, 87]. Inhibition of sIL-6R by intraperitoneal injection of a recombinant decoy receptor (gp130Fc) attenuates pulmonary fibrosis, whereas activation of IL-6 trans-signaling in cell lines enhances fibroblast proliferation and extracellular matrix protein production [210], which are the hallmarks of the pulmonary fibrosis progression.

Together, these data show the importance of IL-6R trans-signaling in inflammation and lung remodeling and offer possibilities for therapeutic interventions [14].

8.1. Species Specificity of IL-6R Signaling. Murine models have been invaluable in the study of inflammatory disease, but should be analyzed with caution. Species differences in IL-6R mediated signaling have been observed. Human IL-6 stimulates human and murine cells, whereas murine IL-6 only stimulates murine IL-6R signaling [211]. Because of this species specificity, transgenic mice expressing human sIL-6R from a liver promoter did not bind the endogenous murine IL-6, and as a consequence the transgenic animals do not have a transgene specific phenotype [211, 212]. Garbers et al. reported that human IL-6R is a substrate of human ADAM17, but murine IL-6R is a substrate of murine ADAM10 [213]. However, Schumacher et al. subsequently revealed that trigger-induced shedding of both human and mouse IL-6R is mediated by murine ADAM17, but constitutive release of IL-6R is largely mediated by mADAM10 in mice [190]. Additionally, in humans, but not in mice, the sIL-6R can be generated by translation from an alternatively spliced mRNA [190, 214]. Of note, the soluble gp130 form, which circulates in human plasma, blocks IL-6R trans-signaling responses and does not show species specificity, meaning that it interacts with human and mouse sIL-6R complexes [215]. All of this has implications in the extrapolation of IL-6R data obtained in mouse models to human, but is consistent with a prominent role of EGFR/ADAM17 sIL-6R trans-signaling in human lung pathology.

8.2. Dual Control of the STAT3 Transcription Pathway by the EGFR/ADAM17 Axis. IL-6R-mediated signaling leads to activation of the transcription factor STAT3 [216], ERK [217], and PI3K [201], linking this signaling pathway with the EGFR/ADAM17 axis. STAT3 is also activated through a parallel pathway in trans-signaling, which involves shed EGFR ligands [218](Figure 7). The STAT3 pathway is involved in tissue repair [198], carcinogenesis [9, 219], and immune responses [220]. Notably, STAT3 was found to be a modifier gene of cystic fibrosis lung disease [221], and enhanced STAT3 phosphorylation was observed in lung tissue from smokers and COPD patients [222]. Consequently, a disturbance in the control of the complex EGFR/ADAM17/STAT3 pathway would likely play a role in Cystic Fibrosis and COPD chronic lung disease.

9. ADAM17 Phosphorylation and Trafficking

The serine- and threonine-rich ADAM17 cytoplasmic tail [112] has three phosphorylation sites that have been proposed to activate ADAM17: Thr735, Ser791, and Ser819 (Figure 2). Pro-ADAM17 and mature ADAM17 are phosphorylated at Thr735 under resting conditions, but phorbol ester (PMA) treatment further increases phosphorylation at this site [223]. ERK 1/2 and p38 MAP kinase phosphorylate ADAM17 at Thr735 [126: Diaz-Rodriguez, 2002 #237] [224], and activate ADAM17 proteolytic activity, leading to shedding of IL-6R and TGF-alpha [126]. Stimulation of ADAM17 increases its phosphorylation without changing the total protein level, suggesting that phosphorylation plays a role in the regulation of the proteolytic activity of ADAM17. However, mutation of the phosphorylation sites individually or in combination and even removal of the whole cytoplasmic tail has no significant effect on stimulated shedding in cell models, suggesting that phosphorylation may be a minor regulatory mechanism of ADAM17 proteolytic activity, or that it plays a context and cell type dependent role in intracellular transport, processing, and maturation [112, 121, 225–227].

9.1. The Role of ADAM17, AREG/IL-6R, and EGFR Trafficking in Cellular Signaling. The majority of EGFR signaling is believed to occur at the plasma membrane. However, many studies show that upon ligand-dependent activation, EGFR is rapidly internalized into endosomes, where hypothetically it may continue to signal [228, 229]. Up till now, the triggered endocytosis of EGFR has been interpreted as signal attenuation, due to lysosomal degradation [230]. However, recent reviews elaborate on the role of EGFR endocytosis and recycling in active signal transduction [10, 230–233]. How triggered intracellular trafficking of EGFR relates to the regulation of the EGFR/ADAM17 axis has not been explained in detail. So far, these studies largely focus on deregulated EGFR trafficking in the context of carcinogenesis. However, EGFR trafficking in CFTR deficiency and its effect on the signaling cascade has not been investigated.

Most reports assume that ADAM17 cleaves and sheds it substrates at the extracellular membrane. However, ADAMs and its substrates have been localized in various subcellular compartments like lysosomes [234], endosomes [175, 235], and exosomes [233]; thus, it is difficult to identify an exact site of ADAM17 mediated shedding and how it interacts with EGFR [236].

Doedens et al. proposed that endocytosis from the cell surface is a pre-requisite for ADAM17 catalytic activity [237]. Lorenzen et al. suggested that ADAM17 and its substrate meet in the ER/Golgi pathway and that the prodomain does not interfere with the enzyme-substrate interaction [100]. Xu et al. showed that ADAM17 is localized at the cell surface as a dimer and associates with tissue inhibitor of metalloproteinase-3 (TIMP-3) [126]. Upon activation with PMA or anisomycin, the amount of ADAM17 at the cell surface increases, followed by dissociation of ADAM17 from TIMP-3, and ADAM17 dimers convert to monomers.

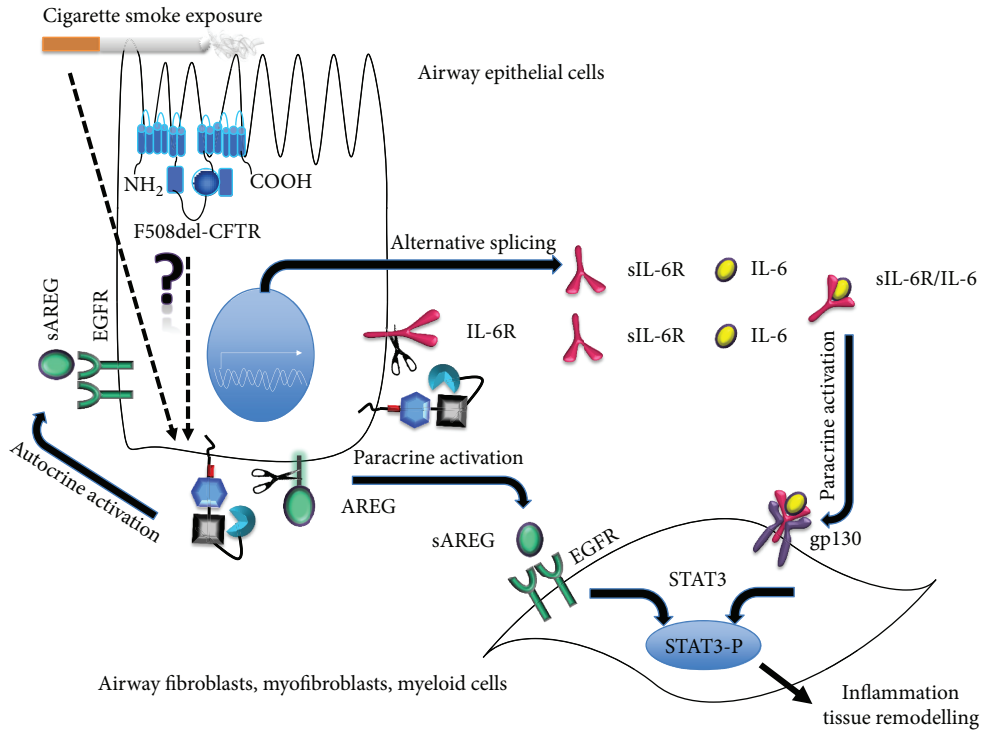

Figure 7: ADAM17/EGFR signaling an important player in CF and COPD lung disease. In airway epithelial cells in culture, both CFTR deficiency [61] and cigarette smoke exposure [87] cause enhanced activity of the EGFR/ADAM17 signaling cascade, and thus enhanced release of proinflammatory cytokines and growth factors that are ADAM17 substrates and signaling molecules that are downstream of the EGFR/MAPK pathway (Figure 1) including the proinflammatory CXCL8 (IL-8). In chronic lung disease, this could contribute to mucous metaplasia, inflammation, and tissue remodeling. A potential link between CFTR deficiency, COPD, and EGFR/ADAM17 activity (dashed arrows) is the extracellular redox potential, which is in part dependent on CFTR-related glutathione transport [30, 165, 166], and which in turn regulates ADAM17 activity (Figure 5). In COPD, CS induced long-term downregulation of CFTR expression [16, 47, 80] can have the same long-term effect, in addition to the acute oxidative stress caused by cigarette smoke. In this figure, we focus on two canonical ADAM17 substrates: proinflammatory IL-6 receptor (IL-6R) and growth factor amphiregulin (AREG). AREG is proteolytically cleaved by ADAM17 from airway epithelial cells and activates EGFR in the airway epithelial cells and on the underlying fibroblasts. IL-6R trans-signaling requires ADAM17-mediated shedding of the extracellular domain or alternative mRNA splicing. The soluble sIL-6R binds to IL-6 creating IL-6/sIL-6R complexes that trans-activate gp130 on the underlying myofibroblasts and myeloid cells. Signals transduced by AREG and IL-6R shed from airway epithelial cells converge in combined activation of the transcription factor STAT3 in myofibroblasts and smooth muscle cells. STAT3 is involved in fibrotic responses and inflammatory lung disease, and a modifier of CF lung disease [221]. Based on available literature cited in text, enhanced EGFR/ADAM17 signaling may contribute to hyperinflammation and epithelial metaplasia, fibroblast and smooth muscle cell activation, angiogenesis, and net deposition of extracellular matrix (tissue remodeling) observed in COPD and CF lung disease.

According to the authors, these monomers correspond to the active form of ADAM17 and are predominantly found in cytoplasm [147], suggesting that the monomers are internalized, and shedding may occur intracellularly. Also, recent reports suggest that ADAM-mediated cleavage occurs from an intracellular vesicular pool in several cell types. Triggered shedding of FasL by ADAM10 and ADAM17 by T-lymphocytes involves trafficking of proteases and substrates to an intracellular membrane raft compartment [234]. Moreover, two proteins that regulate ADAM17-dependent shedding of EGFR ligands, annexins, and a phosphofurin acidic cluster sorting protein 2 are close to ADAM17 in the intracellular vesicular compartment [235] as shown by proximity ligation assay (PLA). Consistent with this, we have shown by PLA that upon CS exposure ADAM17 and ADAM17-P appear in close proximity with its substrates AREG and IL-6R in the intracellular compartment in ALI-HBEC, whereas under basal condition ADAM17 or ADAM17-P substrate complexes were infrequent [87]. Together, reports from various cellular models suggest that the shedding process may occur in an intracellular compartment and that triggered activity involves active trafficking of ADAM17 and its substrates. It remains to be established whether ADAM17 sheds its substrate in intracellular vesicles followed by secretion or whether the ADAM17/substrate complexes in these vesicles need to be transported to the membrane first in order to deliver ADAM17 and its substrate for cleavage. In both cases, a vesicle trafficking and membrane fusion event is involved, which may be subject to regulation, adding another level of complexity.

A further aspect of the relationship between EGFR/ADAM17 signaling and membrane trafficking concerns the production and intercellular exchange of exosomes. Higginbotham et al. showed the importance of paracrine exosomal

AREG-mediated signaling in breast cancer cells [233]. Recipient LM2-4175 cells rapidly take up AREG-containing exosomes in an EGFR-dependent manner and enhanced invasion of LM2-4175 cells through matrigel [233]. Also, EGFR- and ADAM17-containing exosomes have been described [238, 239]. Such exosomes are considered important in the resolution of tissue injury and inflammation, presumably because they allow delivery of functional signaling complexes from triggered cells to neighboring cells. While most available studies address the role of exosomes in the progression of cancer, their role in chronic lung disease and possible implications for future treatment is under study [240].

10. Summary and Conclusions

Several lines of evidence, discussed above, suggest that the ADAM17/EGFR axis and downstream regulatory pathways are hyperactive in CF and COPD chronic lung disease, promoting inflammation and tissue remodeling by shedding EGFR binding growth factors and proinflammatory agonists from airway epithelial cells. This may contribute to inflammation, epithelial metaplasia, fibroblast and smooth muscle cell activation, and net deposition of extracellular matrix. Taken together, we propose that pathology-driven trans-signaling at least in part depends on airway epithelial AREG and IL-6R shedding. Importantly, signals transduced by shed AREG and IL-6R from airway epithelial cells may converge in activation of the transcription factor STAT3 in lung fibroblasts, myofibroblasts, and smooth muscle cells (Figure 7). Moreover, unbalanced ligand shedding towards the submucosa in CF and COPD and likely other chronic lung disease will affect the activity of resident and infiltrating myeloid cells, including dendritic cells, macrophages, and neutrophils.

While evidence in cellular and animals models is compelling, direct evidence for EGFR/ADAM17 hyperactivity from patient lung tissue *in situ* is scarce. This is in part due to a lack of appropriate airway material for analysis, especially from early disease and healthy controls. In a recent study, Zuo et al. show enhanced AREG expression in biopsies from smokers lungs, associated with remodeling lesions [181], consistent with enhanced EGFR activity as shown in primary cells in culture [87, 181]. However, successful pharmacological interventions in the EGFR/ADAM17 pathway have not been reported to our knowledge. Activation of CFTR activity may help to reduce EGFR/ADAM17 activity and resolve COPD pathology [85], but no clinical evidence for this is yet available. Targeting EGFR/ADAM17 in CF patients has not been attempted. Conversely, it would be important to study patients treated with CFTR-targeted medication for evidence of reduced activity of the EFGR/ADAM17 axis in biomarker and biopsy studies.

Genetic analysis of the COPD and CF populations, aiming at identification of genes that determine the pathology ("modifier genes") would also provide important evidence for the involvement of the EGFR/ADAM17 axis. So far, as already cited above, STAT3, controlled by the EGFR/ADAM17 axis (Figure 7), is a modifier of CF lung disease [221]. IL-6R, a prominent ADAM17 substrate linking the axis to STAT3 in trans-signaling, is a modifier of COPD as well as asthma [207, 208]. So far, polymorphisms associated with the ADAM17, AREG, or EGFR genes have not been directly linked to chronic lung disease, but that may be due to limitations of the studies.

A possible mechanistic link between EGFR/ADAM17 activity, CF, and COPD is suggested by the observation that CFTR activity is diminished in COPD airways. Reduced CFTR activity interferes with glutathione transport in CF airways, adding to oxidative stress, which would activate the EGFR/ADAM17 axis both on CF and COPD. Further studies in vivo and in vitro are required to establish this.

Interventions in the EGFR/ADAM17 pathway may reduce CF and COPD lung pathology. However, since long-term systemic delivery of available EGFR and ADAM17 inhibitors likely causes undesirable side effects, novel methods to control this pathways will likely be required. Studies of stress-induced dynamic trafficking of the membrane proteins involved in the EGFR/ADAM17 axis in advanced 3D airway culture models may allow the development of novel modulators that will allow a more targeted approach to suit the requirements for treatment of CF and COPD lung disease.

Disclosure

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Conflicts of Interest

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

Acknowledgements

Marta Stolarczyk was supported by the Dutch Lung Foundation (Longfonds, Grant no. 3.3.10.027). The authors thank Susanne Bogers MSc for her help in editing the final manuscript and literature list.

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Hindawi
Mediators of Inflammation
Volume 2017, Article ID 3028082, 14 pages
https://doi.org/10.1155/2017/3028082

Research Article

Cigarette Smoke Exposure Inhibits Bacterial Killing via TFEB-Mediated Autophagy Impairment and Resulting Phagocytosis Defect

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Received 12 August 2017; Accepted 3 October 2017; Published 28 December 2017

Academic Editor: Amit Gaggar

Introduction. Cigarette smoke (CS) exposure is the leading risk factor for COPD-emphysema pathogenesis. A common characteristic of COPD is impaired phagocytosis that causes frequent exacerbations in patients leading to increased morbidity. However, the underlying mechanism is unclear. Hence, we investigated if CS exposure causes autophagy impairment as a mechanism for diminished bacterial clearance via phagocytosis by utilizing murine macrophages (RAW264.7 cells) and *Pseudomonas aeruginosa* (PA01-GFP) as an experimental model. *Methods*. Briefly, RAW cells were treated with cigarette smoke extract (CSE), chloroquine (autophagy inhibitor), TFEB-shRNA, CFTR(inh)-172, and/or fisetin prior to bacterial infection for functional analysis. *Results*. Bacterial clearance of PA01-GFP was significantly impaired while its survival was promoted by CSE ($p < 0.01$), autophagy inhibition ($p < 0.05$; $p < 0.01$), TFEB knockdown ($p < 0.01$; $p < 0.001$), and inhibition of CFTR function ($p < 0.001$; $p < 0.01$) in comparison to the control group(s) that was significantly recovered by autophagy-inducing antioxidant drug, fisetin, treatment ($p < 0.05$; $p < 0.01$; and $p < 0.001$). Moreover, investigations into other pharmacological properties of fisetin show that it has significant mucolytic and bactericidal activities ($p < 0.01$; $p < 0.001$), which warrants further investigation. *Conclusions*. Our data suggests that CS-mediated autophagy impairment as a critical mechanism involved in the resulting phagocytic defect, as well as the therapeutic potential of autophagy-inducing drugs in restoring is CS-impaired phagocytosis.

1. Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by chronic inflammation, emphysema, and recurring chronic infections of the lower airways [1–4]. Currently, COPD is the fourth leading cause of death in the United States and is expected to become the third largest cause globally by 2020 [1–3, 5]. One plausible reason for this increase in mortality is an increased prevalence of cigarette smoking, which is one of the major risk factors for COPD pathogenesis [1, 4].

As mentioned above, chronic infection is a major contributor to the worsening and progression of the obstructive lung disease. Specifically, the lower respiratory tract of COPD patients is often faced with bacterial colonization and viral infections. The most common bacterial pathogens responsible for these infections are *Streptococcus pneumoniae*, nontypeable *Haemophilus influenzae*, and *Pseudomonas aeruginosa* [2, 3, 6–9]. The presence of these pathogens and the resulting infections result in exacerbations that increase inflammation and decrease lung function, leading to the progression of COPD and resulting increase in hospitalization and mortality [2, 3, 6, 7, 10, 11]. Although the exact cause of chronic infections in COPD is unknown, there is increasing concentration on designing novel therapeutics to improve the morbidity of COPD subjects because chronic antibiotic treatments over time develop resistance [2, 7, 12–15].

A critical aspect of host defense against bacterial infections are highly phagocytic cells called macrophages [3, 7, 10, 16]. The ability of these cells to remove pathogens through phagocytosis is necessary for controlling debilitating lung infections [3, 7, 11, 16]. Therefore, phagocytosis is a necessary cellular process that recognizes foreign pathogens/particles and removes them [3, 7]. Our preliminary studies suggested that the chronic infections of the lower airway in COPD might involve dysfunction of the phagocytic ability of alveolar macrophages [2, 3, 10]. One possible mechanism for the diminished phagocytosis in alveolar macrophages is cigarette smoke- (CS-) induced autophagy impairment [2, 12–14, 17–19]. Briefly, autophagy is a homeostatic cellular process that degrades misfolded proteins, damaged organelles, and pathogens, which can be impaired by chronic CS exposure, the leading cause of COPD-emphysema pathogenesis [1, 12, 14–16, 19–21]. Despite studies that have demonstrated CS exposure may impair phagocytosis in macrophages [2, 9, 11], the exact mechanism remains unknown. Thus, in this study, we aimed to investigate the specific mechanism by which CS impairs bacterial clearance. We first focused on evaluating the role of transcription factor EB (TFEB), the master autophagy regulator that induces the transcription of various autophagy/lysosomal biogenesis genes based on our recent data suggesting its critical role in COPD-emphysema pathogenesis [15, 19, 22].

Briefly, these preliminary studies revealed that CS exposure induces localization of TFEB to aggresome bodies, which was associated with a decrease in lung function and increased severity of emphysema in COPD subjects. [15]. This finding led us to evaluate possible therapeutic approaches to induce TFEB expression as a way of inducing autophagy [15]. One drug that we have investigated for TFEB induction is a flavonoid called fisetin. Fisetin is an over-the-counter dietary supplement that acts as an antioxidant for brain health [15, 22, 23]. In recent studies from our lab and others, fisetin has been shown to induce TFEB and consequentially autophagy [15, 18, 19].

Hence, this study investigated further the mechanism of CS-impaired bacterial phagocytosis in COPD in order to explain the mechanism and reason for recurring exacerbations. First, we verified the pathogenic role of CS-induced autophagy impairment as a mechanism for diminished phagocytosis that may account for the chronic exacerbations in COPD. We also found that fisetin was effective in restoring CS-impaired bacterial phagocytosis. Moreover, fisetin also demonstrated an added therapeutic potential as a possible mucolytic and bactericidal.

2. Materials and Methods

2.1. Reagents and Treatments. The murine macrophage cell line, RAW264.7 was used as an *in vitro* model to investigate cigarette smoke exposure and its impact on phagocytosis. Standard cell culture procedures as previously described were used [14]. Briefly, cells were maintained at 37°C in 5% CO_2 in DMEM/F12 media with 10% fetal growth serum (RMBIO) and 1% PS (penicillin and streptomycin; Invitrogen). For *in vitro* cigarette smoke exposure, cigarette smoke extract (CSE) was prepared by burning two to three 3R4F research-grade cigarettes (Tobacco Research Institute, University of Kentucky, Lexington, KY) and aerating into serum-free DMEM/F12 media (20 ml). An OD (320 nm) of 0.74 was considered to be 100% CSE, and working CSE concentrations were adjusted using cell culture media. As a model of CS-induced autophagy inhibition in RAW264.7 cells, chloroquine (30 μM) was used for 8 hrs of pretreatments. To investigate the role of TFEB in the regulation of autophagy- and xenophagy-mediated phagocytosis, TFEB expression was knocked down in RAW cells by transfecting them with TFEB-Mission™ shRNA (Sigma) for 24 hrs using the Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad CA) following the protocol provided by the manufacturer. Similarly, cystic fibrosis transmembrane conductance regulator (CFTR) was inhibited in the macrophages using CFTR(inh)-172 inhibitor (Sigma-Aldrich, St. Louis, MO) at a concentration of 10 μM for 8 hrs to examine the possible role of CFTR in CS-impaired phagocytosis.

2.1.1. Pseudomonas aeruginosa Infection Model. To investigate if the phagocytic defect could be ameliorated by TFEB-mediated autophagy induction, fisetin treatment was utilized. Autophagy inducer, cysteamine (250 μM; Sigma) was used as a positive control in the bactericidal and mucolytic experiments. For experiments involving infection, *Pseudomonas aeruginosa* (*P. aeruginosa*) strain *PA01*-GFP was cultured for 15–18 hrs in Luria Bertani (LB; Thermo Fisher Scientific, Waltham, MA) broth with carbenicillin (1%, Sigma) to select for PA01-GFP at 37°C and 250 rpm, in a shaking incubator. This culture of *PA01*-GFP was added directly to DMEM/F12 media in each well at a multiplicity of infection (MOI) of 10.

2.2. Immunoblotting. Our previously described immunoblotting method [20, 24] was used to quantify changes in the expression of TFEB (master autophagy regulator), CFTR, p62 (aggresome marker), and β-actin in the soluble protein fractions of RAW264.7 cells. TFEB was procured from Santa Cruz Biotechnology, CFTR-181 in-house [25] and β-actin from Sigma.

2.3. Fluorescence Microscopy for Quantification of Phagocytosis. RAW264.7 cells were plated onto 12 or 24 well plates and pretreated for 8 hrs with fisetin (20 μM), CSE (5%), chloroquine (10 μM), and CFTR(inh)-172 (inhibitor; 10 μM) or transfected for 24 hrs with TFEB-Mission™ shRNA. After treatment, these cells were infected with *PA01*-GFP at an MOI of 10 for 3 hrs prior to fluorescence microscopy. Fluorescence images were captured using the ZOE™ Florescent Cell Imager (Bio-Rad). *PA*01-GFP-infected (fluorescent) and total (bright-field) macrophages in the same field were counted in order to analyze the phagocytic ability of the cells. The percentage of macrophages infected with *PA01*-GFP-infected macrophages as compared to the total number of macrophages was quantified to calculate the bacterial clearance by macrophages.

2.4. Bacterial Survival Assay. RAW cells were seeded into 12 or 24 well plates and treated for 8 hrs with fisetin

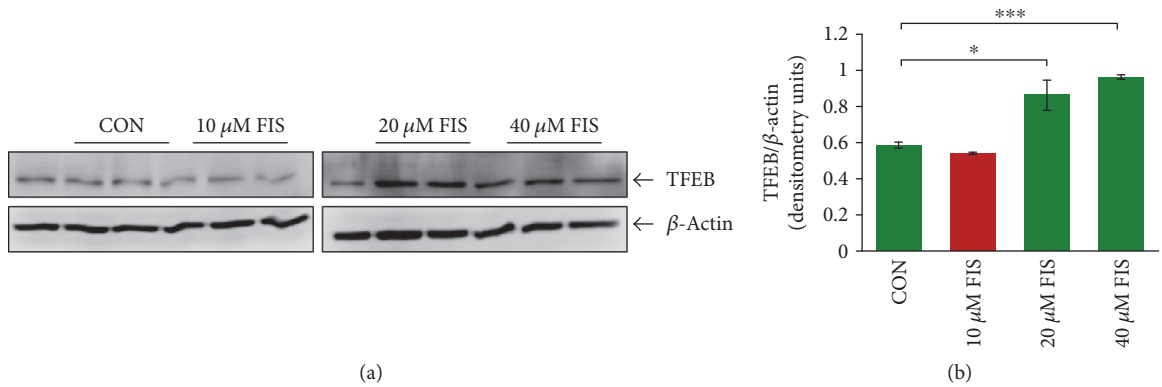


Figure 1: Fisetin induces TFEB expression in murine macrophages. (a) RAW264.7 cells were treated with increasing dosages of fisetin (0, 10, 20, and 40 μM) for 8 hrs. Following treatment, the cells were lysed, and the total protein lysate was isolated for immunoblotting to determine changes in TFEB expression. The Western blot analysis shows an increase in TFEB (autophagy regulator; soluble) expression in the macrophages treated with 20 μM and 40 μM fisetin. β-Actin was used as a loading control. (b) Densitometry analysis of TFEB expression was normalized to β-actin. Data represent $n = 4$ in each group, and error bars depict mean ± SEM, $^{*}p < 0.05$; $^{***}p < 0.001$. The 20 μM dose of fisetin induces a significant increase in TFEB expression, which was thus selected for further experimental investigation.

(20 μM), CSE (5%), chloroquine (30 μM), and CFTR(inh)-172 (10 μM) or transfected for 18 hrs with TFEB-Mission™ shRNA. Following this treatment, the cells were infected with *PA01*-GFP at an MOI of 10 for 3 hrs. Next, 50 or 100 μl of media was collected from these cultures and spread on 2% LB agar plates supplemented with 1% carbenicillin to select for *P. aeruginosa*. These plates were incubated at 37°C for 24 hrs and bacterial colony-forming units (CFUs) were counted to quantify bacterial survival.

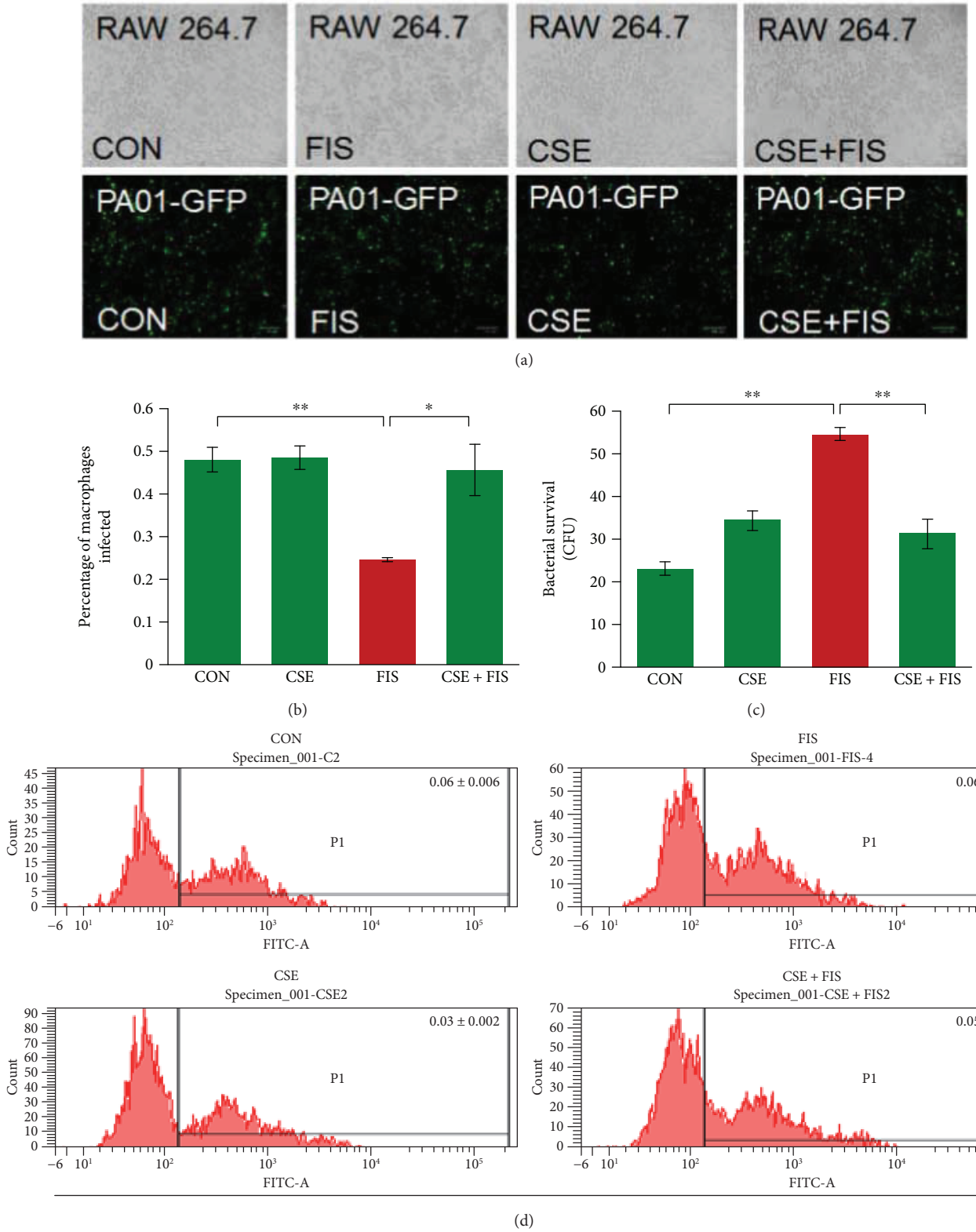
2.5. Bactericidal and Mucolytic Experiments. The 5% mucin (Sigma) solution in PBS was treated with fisetin (20 μM) or cysteamine (positive control; 250 μM) and stirred for 12 hrs until dissolved. The 300 μl of these mucin solutions was pipetted into the top of a 1 mL sterile pipette, and the travel time to reach the bottom of the pipette was recorded. This was used to calculate velocity by dividing the length of the pipette (28 cm) by the recorded time, which was used to quantify mucolytic activity of fisetin. Finally, to evaluate the bactericidal properties of fisetin, *PA01*-GFP was cultured and treated with fisetin (20 and 40 μM) or cysteamine (positive control; 250 μM). The 100 μl of the bacterial culture was plated in a 96-well plate and the OD (600 nm) was recorded as a measurement of bacterial growth every 3 hrs for 18 hrs.

2.6. Statistical Analysis. The data is presented as the mean ± standard error of the mean (SEM) and differences between the various groups were tested using standard *t*-test. The differences were considered significant if the p value was ≤0.05. Densitometry analysis of the results from immunoblotting and fluorescence microscopy was done utilizing ImageJ software (NIH, Bethesda, MD) as we previously described [2].

3. Results

3.1. TFEB Expression Is Induced by Fisetin in Murine Macrophages. Previous publications have shown that fisetin has the ability to induce autophagy [15, 18, 19, 22]; hence, we decided to investigate its effectiveness in recovering CS-impaired phagocytosis. However, it was first necessary to determine the dose of fisetin to be used for further experimentation; thus, by treating RAW264.7 cells with increasing doses of fisetin (0, 10, 20, and 40 μM) for 8 hrs, we first selected the appropriate dose. Following fisetin treatment, the total protein lysates were collected and the changes in TFEB expression were quantified. The data demonstrates that TFEB expression is significantly ($p < 0.05$ and $p < 0.001$) induced in RAW264.7 cells by both 20 and 40 μM fisetin treatments (Figures 1(a) and 1(b)). Thus, 20 μM fisetin dose was selected for further investigation because it was the lowest dose that induced TFEB expression.

3.2. CS Exposure Impairs Phagocytosis in RAW264.7 Cells. We next investigated if CS exposure impaired phagocytosis in murine macrophages and its underlying mechanism. In these experiments, RAW cells were pretreated with fisetin (20 μM) and/or CSE (5%) for 8 hrs followed by infection with PA01-GFP with an MOI of 10 for 3 hrs. Fluorescent microscopy images were captured using the Bio-Rad ZOE™ Florescent Cell Imager and analyzed using the ImageJ software. We found that CS exposure significantly ($p < 0.01$) decreased the number of intracellular bacteria as compared to controls, which was significantly ($p < 0.01$) recovered by fisetin treatment (Figures 2(a) and 2(b)). A similar trend was found with the treatment of another autophagy-inducing drug, cysteamine (see Supplementary Figure 1), which supported our mechanistic finding that CS exposure causes a phagocytic defect in murine macrophages via autophagy-impairment. To confirm this observation, the experiment was repeated and analyzed through flow cytometry. Analysis of the flow cytometry data showed that CSE-treated macrophages have a significantly ($p < 0.01$) lower number of PA01-GFP bacteria that was significantly ($p < 0.05$) increased by fisetin treatment (Figures 2(d) and 2(e)). As a functional read out, bacterial survival was quantified by plating 100 μl of the cell culture media from the



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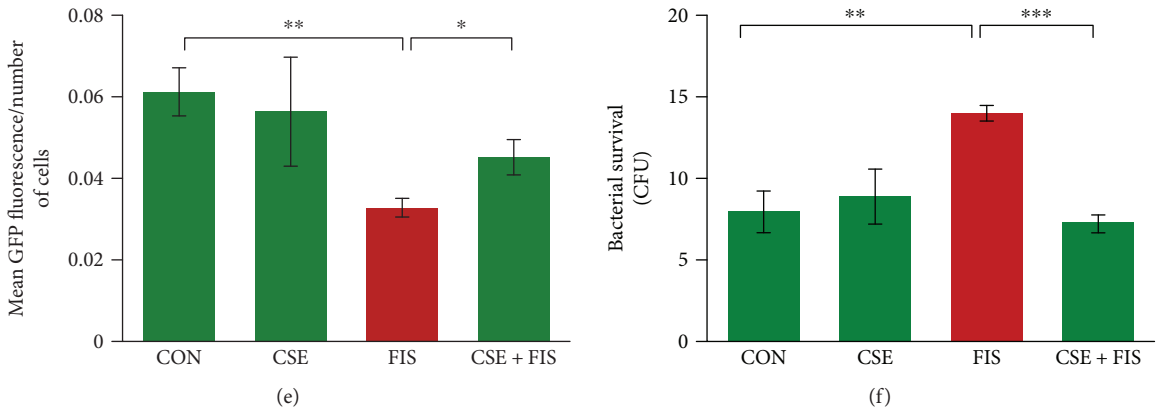

Figure 2: CS exposure impairs bacterial clearance by macrophages that promote bacterial survival. (a) RAW cells were pretreated with fisetin (20 μM) and or CSE (5%) for 8 hrs. Following treatment, the cells were infected with *PA01*-GFP for 3 hrs at a MOI of 10. After infection, the cells were washed twice with sterile PBS, followed by bright-field and fluorescence microscopy (scale bar, 100 μm). These florescent images were utilized to quantify the number of infected cells (intracellular bacteria) using the ImageJ software. The data shows that CSE treatment significantly impairs bacterial clearance, indicated by a decrease in the number of intracellular bacteria, which was significantly recovered by fisetin. (b) The data from images shown in (a) are represented here as mean ± SEM of percentage of macrophages infected, $n = 3$, $^{**}p < 0.01$; $^{*}p < 0.05$. (c) The cell culture media (100 μl) from the experimental groups shown in (a) were spread on 2% LB agar plates and incubated for 24 hrs at 37°C. The number of colony-forming units (CFU) was counted to quantify the number of extracellular bacteria as a representation of bacterial survival. CSE treatment resulted in significantly increased bacterial survival that was controlled by fisetin treatment. Data represents mean ± SEM of CFUs, $n = 3$, $^{**}p < 0.01$. (d) Flow cytometry results of RAW cells treated with the same groups as shown in (a) showed the CS-induced phagocytic defect as well as verify fisetin's ability to recover CSE-impaired macrophage phagocytic function. (e) The flow cytometry data expressed as mean of positive GFP florescent phagocytic cells; shown as mean ± SEM, $n = 3$, $^{**}p < 0.01$; $^{*}p < 0.05$. (f) Bacterial survival assay of cell culture media (100 μl) from flow cytometry experiment showed a significant increase in the number of CFUs after CSE treatment, which was significantly reduced by fisetin. The data is representative of mean ± SEM of CFUs, $n = 3$, $^{**}p < 0.01$; $^{***}p < 0.001$. This data suggests that CS exposure impairs phagocytosis in murine macrophages that can be restored by autophagy induction.

microscopy and flow cytometry experiments on 2% LB agar plates and incubating these plates for 24 hrs at 37°C, in order to count the colony-forming units (CFU). The plates from the CSE-treated group in both experiments had significantly ($p < 0.01$) higher bacterial survival as compared to the control, while the fisetin treatment significantly ($p < 0.01$, $p < 0.001$) diminished bacterial survival (Figures 2(c) and 2(f)). Our findings suggest that CS exposure impairs bacterial phagocytosis and increases its survival in murine macrophages that can be recovered by fisetin through autophagy induction.

3.3. Inhibition of Autophagy Impairs Clearance and Promotes Survival of PA01 Bacteria in Murine Macrophages. CS exposure has been found to inhibit autophagy and cause aggresome formation in chronic pulmonary diseases such as COPD [1, 14, 15, 20]. Therefore, we investigated CS-impaired autophagy as a possible mechanism for the dysfunctional phagocytosis in RAW cells (macrophages), by pretreating these cells with fisetin (20 μM) and/or chloroquine (60 μM) for 8 hrs. Chloroquine is an autophagy inhibitor that was utilized as a positive control for CS-impaired autophagy. Following treatment, these cells were infected with PA01-GFP at an MOI of 10 for 3 hrs as described above. Afterwards, fluorescent images were captured for analysis, which demonstrated that autophagy inhibition significantly ($p < 0.01$) impairs bacterial clearance in RAW cells that can be significantly ($p < 0.05$) recovered by fisetin treatment (Figures 3(a) and 3(b)). To further explore the effect on phagocytosis, bacterial survival was analyzed by plating the experimental media on 2% LB agar plates and performing a CFU count. The results from this bacterial survival assay showed that autophagy impairment by chloroquine significantly ($p < 0.05$) increased bacterial survival, which was significantly ($p < 0.05$) reduced by fisetin treatment (Figure 3(c)). These results verify that autophagy inhibition mediated by CS exposure results in phagocytosis dysfunction in murine macrophages.

3.4. TFEB Knockdown Causes a Phagocytic Dysfunction in Murine Macrophages. Previous research has shown that TFEB is the master autophagy regulator that initiates the transcription of various autophagy/lysosomal-related genes [15, 19, 26–28]. It has also been demonstrated that CS exposure results in TFEB localization in aggresome bodies causing decreased lung function [15]. Hence, we explored TFEB's role in autophagy- and xenophagy-impaired phagocytosis by knocking down TFEB expression in RAW cells followed by pretreatment with fisetin (20 μM) for 8 hrs and infection with PA01-GFP at an MOI of 10 for 3 hrs. Fluorescent images were captured and then analyzed using the ImageJ software showing that bacterial clearance was significantly impaired ($p < 0.001$) by TFEB knockdown. Moreover, fisetin treatment was able to significantly ($p < 0.001$) rescue bacterial clearance (Figures 4(a) and 4(b)). Next, bacterial survival was quantified by counting the CFUs using cell culture media

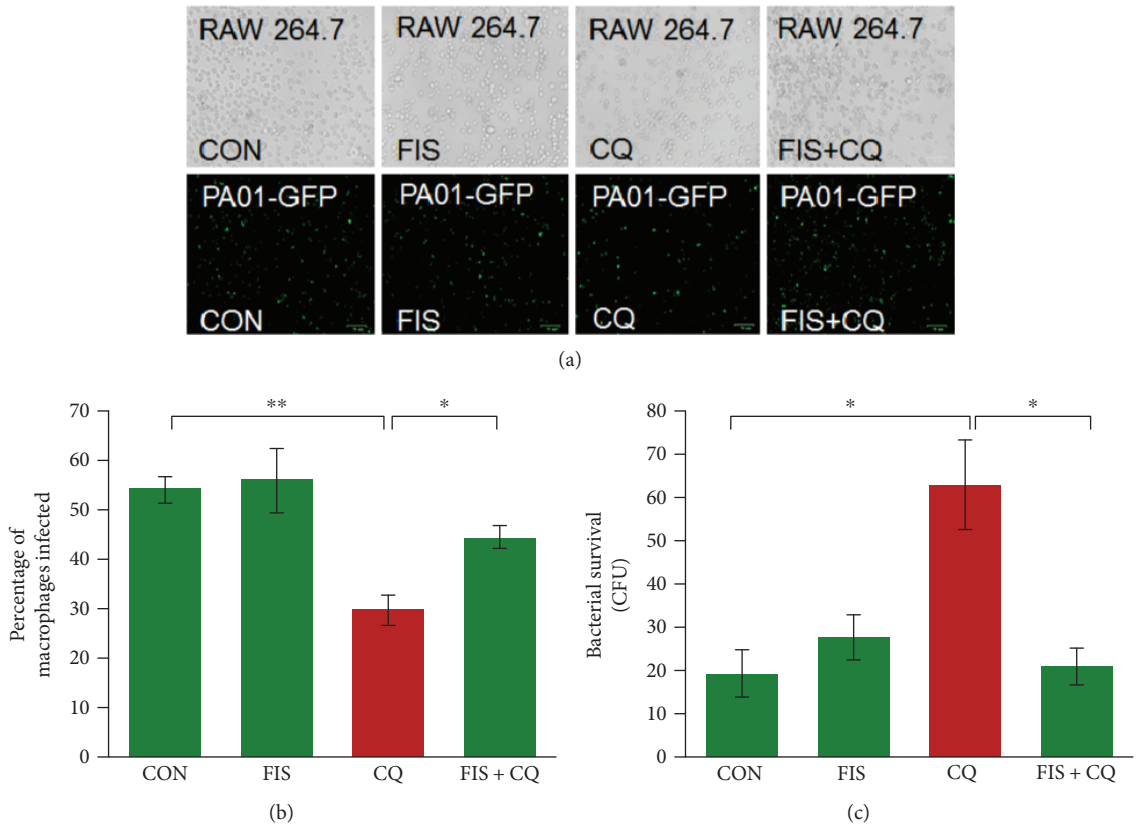

Figure 3: Autophagy inhibition impairs phagocytosis in murine macrophages. (a) RAW cells were pretreated with fisetin (20 μM) and chloroquine (60 μM) for 8 hrs. Then, the cells were infected with *PA*01-GFP for 3 hrs at a MOI of 10. After infection, the cells were washed twice with PBS followed by bright-field and fluorescence microscopy (scale bar, 70 μm). ImageJ software was utilized to count the number of infected cells (intracellular bacteria). Data shows that chloroquine treatment impairs phagocytosis as indicated by the observations of significantly lower numbers of intracellular bacteria in chloroquine-treated cells, while fisetin demonstrated the ability to recover phagocytosis shown by a significant increase in the number of intracellular bacteria. (b) The data shown in (a) demonstrated the mean ± SEM of percentage of macrophages infected, $n = 3$, $^{**}p < 0.01$; $^{*}p < 0.05$. (c) The media (100 μl) from the experimental groups in (a) were spread on 2% LB agar plates and incubated for 24 hrs at 37°C, and the number of CFUs was counted to quantify the number of extracellular bacteria as a representation of survival. Data suggests that autophagy inhibition leads to the impairment of PA01-GFP clearance, as extracellular bacterial survival was significantly higher in cells treated with chloroquine, an autophagy inhibitor. Moreover, fisetin significantly reduces the bacterial survival as anticipated. Data represents mean ± SEM of CFUs, $n = 3$, $^{**}p < 0.01$ and verifies that autophagy inhibition leads to the impairment of phagocytosis in RAW cells.

from this experiment, as described above. TFEB knockdown cells had significantly ($p < 0.01$) higher bacterial survival as compared to that of the control that was controlled significantly ($p < 0.001$) by treatment with fisetin (Figure 4(c)). Therefore, the data suggests that the regulation of autophagy by TFEB has a role in the CS-impaired phagocytosis.

3.5. CFTR Inhibition Impairs Phagocytosis in RAW264.7 Cells. CS exposure has been demonstrated to cause CFTR dysfunction through accumulation in aggresome bodies [2, 25, 29, 30]. This dysfunction has been associated with impaired autophagy and increased bacterial colonization [2, 31–34]. Thus, we investigated the role of CFTR dysfunction in impaired phagocytosis by pretreating RAW cells with the CFTR172 inhibitor (10 μM) and/or fisetin (20 μM) for 8 hrs followed by PA01-GFP infection with an MOI of 10 for 3 hrs. The analysis of fluorescent images showed that inhibition of CFTR significantly ($p < 0.001$) impaired bacterial clearance compared to the control, which was significantly ($p < 0.001$) recovered by fisetin treatment (Figures 5(a) and 5(b)). The phagocytic function of RAW cells was further evaluated by plating the cell culture media from this experiment on 2% LB agar plates for 24 hrs at 37°C and counting the CFUs in order to quantify bacterial survival. These results demonstrated that CFTR inhibition significantly ($p < 0.01$) increased bacterial survival, which was significantly ($p < 0.01$) decreased by fisetin treatment (Figure 5(c)). These results suggest that CFTR dysfunction also has a role in phagocytosis impairment. In order to investigate if fisetin can recover the CS-induced CFTR dysfunction, RAW cells were treated with CSE (5%) and/or fisetin (20 μM) for 8 hrs, and the total protein lysates were isolated. Immunoblotting was utilized to quantify changes in CFTR that demonstrates significant ($p < 0.05$) decrease in membrane CFTR expression (C-band) with CSE exposure, which was significantly ($p < 0.05$)

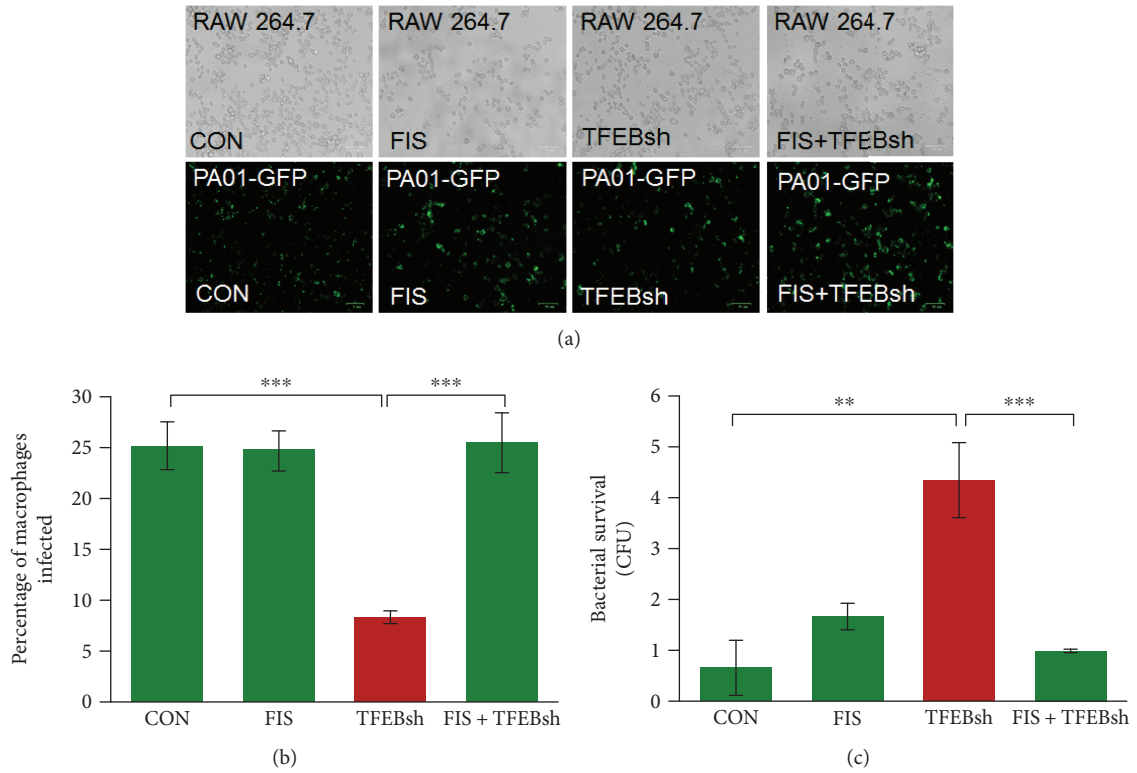

Figure 4: TFEB knockdown impairs phagocytosis in murine macrophages. (a) RAW cells were transfected with TFEB-Mission™ shRNA for 24 hrs. Following transfection, the cells were pretreated with fisetin (20 μM) for 8 hrs. Afterwards, cells were infected with *PA01*-GFP for 3 hrs at a MOI of 10. Following infection, the cells were washed twice with PBS followed by bright-field and fluorescence microscopy (scale bar, 70 μm). The florescent images were utilized to count the number of infected cells (intracellular bacteria) using ImageJ software. The data shows that the bacterial clearance is significantly inhibited as indicated by the significantly lower number of intracellular bacteria in TFEB knockdown cells. Meanwhile, fisetin significantly restores bacterial clearance, indicated by an increase in the number of intracellular bacteria. (b) Data from A, represented as percentage of macrophages infected, which are shown as mean ± SEM, $n = 4$, $^{***}p < 0.001$. (c) A bacterial survival assay was performed by spreading the cell culture media (100 μl) from the experimental groups in (a), on 2% LB agar plates that were incubated for 24 hrs at 37°C. CFUs were counted to quantify the extracellular bacteria survival. This data suggests that decreased TFEB expression significantly increases bacterial survival due to impaired phagocytosis. Moreover, fisetin was able to restore phagocytosis as determined by significantly fewer CFUs. The data represents mean ± SEM of CFUs, $n = 3$, $^{**}p < 0.01$; $^{***}p < 0.001$.

recovered by fisetin treatment (Figures 5(d) and 5(e)). Thus, the data suggests that CSE decreases membrane CFTR expression in murine macrophages, potentially due to CFTR misfolding, CS-impaired autophagy, and CFTR aggresome accumulation, further aggravating phagocytic response.

3.6. Fisetin Shows Promise as Bactericidal and Mucolytic for Treatment of COPD. The pathogenesis of many chronic pulmonary diseases involves chronic inflammation, recurrent exacerbations, and mucus buildup [2, 13, 17, 31, 35–39]. Thus, a therapeutic approach that controls these disease characteristics would be ideal for dealing with chronic exacerbations. Cysteamine is one such drug that has known antioxidant, anti-inflammatory, autophagy-inducing, mucolytic, and bactericidal properties [13, 14, 40, 41]. Similarly, fisetin (over-the-counter medication) also has antioxidant and anti-inflammatory properties, along with being a potent autophagy-inducing drug [15, 23, 42–44]. Furthermore, recent studies have shown that effective killing of various bacteria and viruses [45–48] can help with chronic or recurrent exacerbations. Hence, we investigated the bactericidal properties of fisetin against PA01-GFP. To do so, PA01 was grown for 15 hrs in LB broth followed by treatment with fisetin (20 or 40 μM). Upon treatment, the OD (600 nm) was recorded to quantify the number of bacteria in the culture. This was repeated every 3 hrs for 18 hrs. Analysis of the changes in OD showed that 40 μM fisetin significantly ($p < 0.01$) inhibited bacterial proliferation. Next, this experiment was repeated with fisetin (40 μM) or cysteamine (250 μM) treatment. This data verified that 40 μM fisetin treatment significantly ($p < 0.001$) impaired bacterial growth suggesting its bactericidal properties (Figures 6(a) and 6(b)). Next, we focused on the mucolytic potential of fisetin by stirring 5% mucin solutions overnight with or without fisetin (20 μM) or cysteamine (positive control; 250 μM). The mucin mix (300 μl) was then pipetted into the top of a 1 ml sterile pipette, and the velocity of mucin was recorded as a representation of changes in mucus viscosity. The data showed that both fisetin and cysteamine treatment significantly ($p < 0.001$) decreased the viscosity

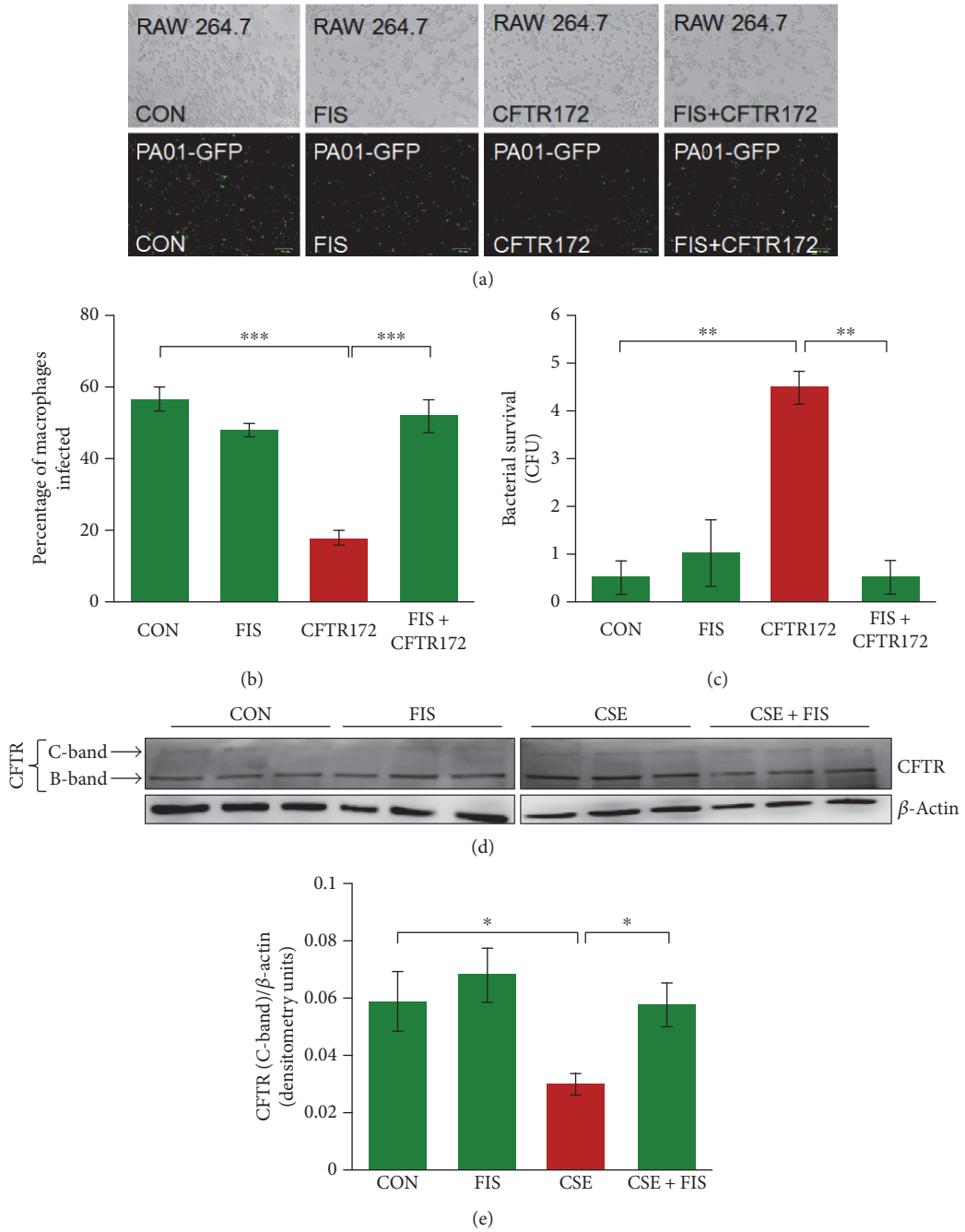

Figure 5: Fisetin recovers phagocytosis defect mediated by CFTR inhibition. (a) RAW cells were pretreated with CFTR172 inhibitor and/or fisetin (20 μM) for 8 hrs followed by infection with *PA01-GFP* for 3 hrs at a MOI of 10. After infection, the cells were washed twice with PBS and observed using bright-field and fluorescence microscopy (scale bar, 70 μm). The fluorescent images were used to count the number of macrophages infected (intracellular bacteria) using the ImageJ software. The data shows that the inhibition of CFTR impairs phagocytosis shown by a significantly lower number of intracellular bacteria, which was significantly recovered by fisetin treatment. (b) The data from (a) represented as the mean ± SEM of percentage of macrophages infected, $n = 3$, $^{***}p < 0.001$. (c) A bacterial survival assay was performed using the cellular media (100 μl) from experimental groups in A, which were plated on 2% LB agar plates and incubated for 24 hrs at 37°C. The colony-forming unit bacterial counts show that CFTR inhibition results in significantly higher numbers of CFUs representing impaired phagocytosis, which was significantly recovered by fisetin treatment. The data represents mean ± SEM of CFUs, $n = 2$, $^{**}p < 0.01$. (d) RAW cells were treated with CSE and/or fisetin (20 μM) for 8 hrs. After treatment, the total protein lysate was isolated and analyzed by immunoblotting for changes in the expression of CFTR and p62 expression. The Western blot analysis shows that membrane CFTR expression (C-band) was significantly decreased by CSE exposure, which was significantly recovered upon fisetin treatment. β-Actin was used as a loading control. (e) Densitometry analysis of CFTR and p62 expression was normalized to β-actin. Data represent $n = 3$ in each group, and error bars depict mean ± SEM, $^{**}p < 0.05$. Thus, data shows that CFTR inhibition affects the phagocytic ability of macrophages. Moreover, fisetin shows the ability to correct this CFTR-mediated phagocytic defect in RAW cells.

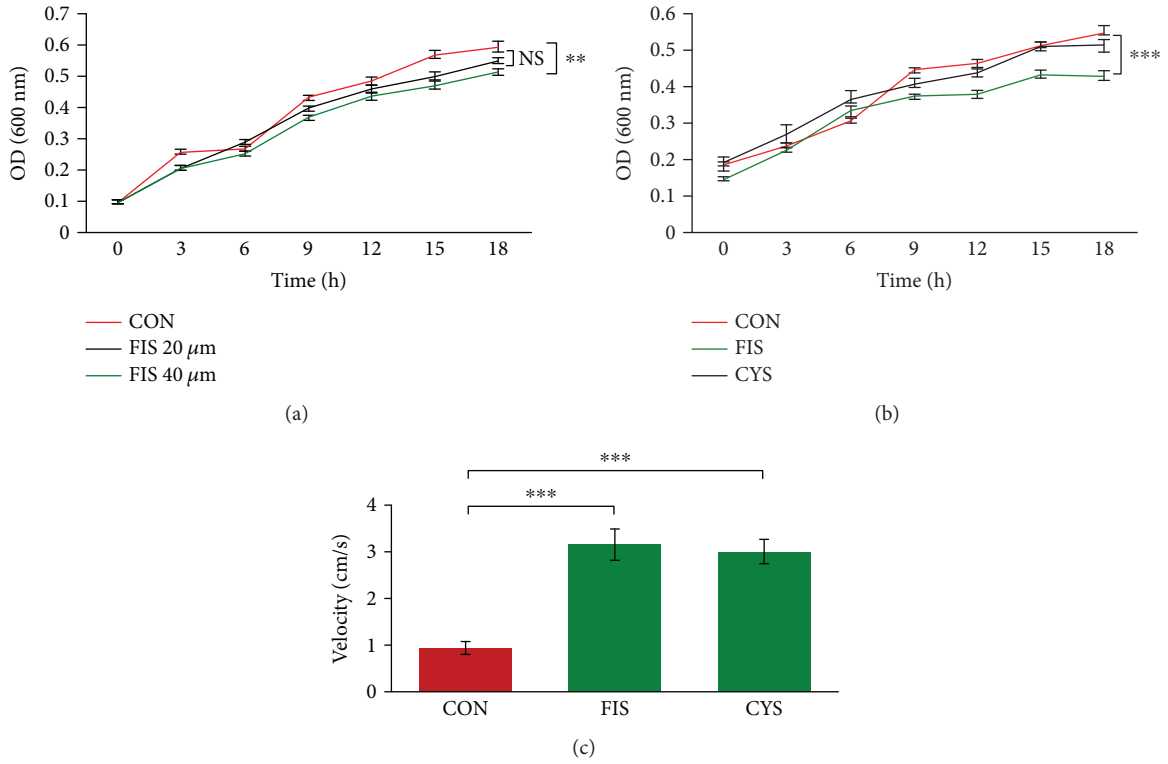

Figure 6: Fisetin demonstrates bactericidal and mucolytic properties. (a) *PA01* was grown for 15 hrs in LB broth then treated with fisetin (20 and 40 μM). Upon treatment, the OD (600 nm) was recorded to quantify the number of bacteria in the culture. Subsequently, the OD was taken every 3 hrs for 18 hrs to analyze changes in bacteria proliferation. The 40 μM fisetin demonstrates a significant inhibition of bacterial growth compared to the control. The data represents mean ± SEM, $n = 3$, $^{**}p < 0.01$. (b) The experimental procedure from (a) was repeated with fisetin (40 μM) or cysteamine (250 μM). The data shows that fisetin significantly reduces the number of bacteria compared to the control and cysteamine treatment. Data represents mean ± SEM, $n = 5$, $^{***}p < 0.001$. (c) The 5% mucin solution was stirred overnight with or without fisetin (20 μM) or cysteamine (250 μM). The 300 μl of this mucin mix was then pipetted into the top of a 1 mL sterile pipette, and the velocity of the mucin was recorded as a representation of changes in mucus viscosity or mucolytic activity. The data shows that both cysteamine (positive control) and fisetin significantly decreased the viscosity of mucin suggesting fisetin has a mucolytic potential. Data represents mean ± SEM, $n = 5$, $^{***}p < 0.001$.

of the mucin solution suggesting that fisetin has mucolytic potential (Figure 6(c)). These findings suggest that fisetin may be an effective treatment of recurrent or chronic exacerbations in COPD subjects.

4. Discussion

One aspect of CS-induced COPD-emphysema pathogenesis is constant bacterial colonization of the lower airways that provoke recurrent exacerbations in subjects causing high morbidity and mortality [2, 49–51]. Studies have suggested that CS exposure impairs phagocytosis, which may account for these exacerbations [2, 11, 52–54]; however, the mechanism was unknown. Our studies first verified that CS exposure significantly impaired bacterial (*P. aeruginosa* PA01-GFP) phagocytosis and improved its survival in murine macrophages. Next, to investigate a possible underlying mechanism for this dysfunction, we focused on autophagy and xenophagy, which is impaired by CS exposure. As a model of CS-induced autophagy and xenophagy impairment, we treated RAW cells with chloroquine, an autophagy inhibitor (as a positive control), and found that bacterial clearance was significantly hindered and its survival was significantly increased. Since, CS-impaired autophagy showed promise as an underlying mechanism for the phagocytic defect found in COPD-emphysema that can lead to recurrent exacerbations, we further investigated this process. We observed that the knockdown of master autophagy regulator, TFEB expression, in RAW cells significantly impaired the clearance of PA01-GFP and significantly enhanced its survival. This finding is important in understanding the mechanism because TFEB is the master autophagy regulator [15, 19]; thus, decreasing its expression leads to impairment of autophagy and xenophagy that we found to be a key regulator of phagocytosis in the murine macrophages. Hence, we concluded that CS-induced autophagy impairment might account for dysfunctional phagocytosis in COPD-emphysema subjects, the mechanism for which is described in Figure 7. Moreover, previous investigations have demonstrated the role of CFTR in the regulation of phagocytosis [2, 25, 33], as well as acquired CFTR dysfunction induced by CS exposure. Consequently, we found that inducing CFTR dysfunction with CFTR inhibitor 172 significantly impaired bacterial phagocytosis and increased its

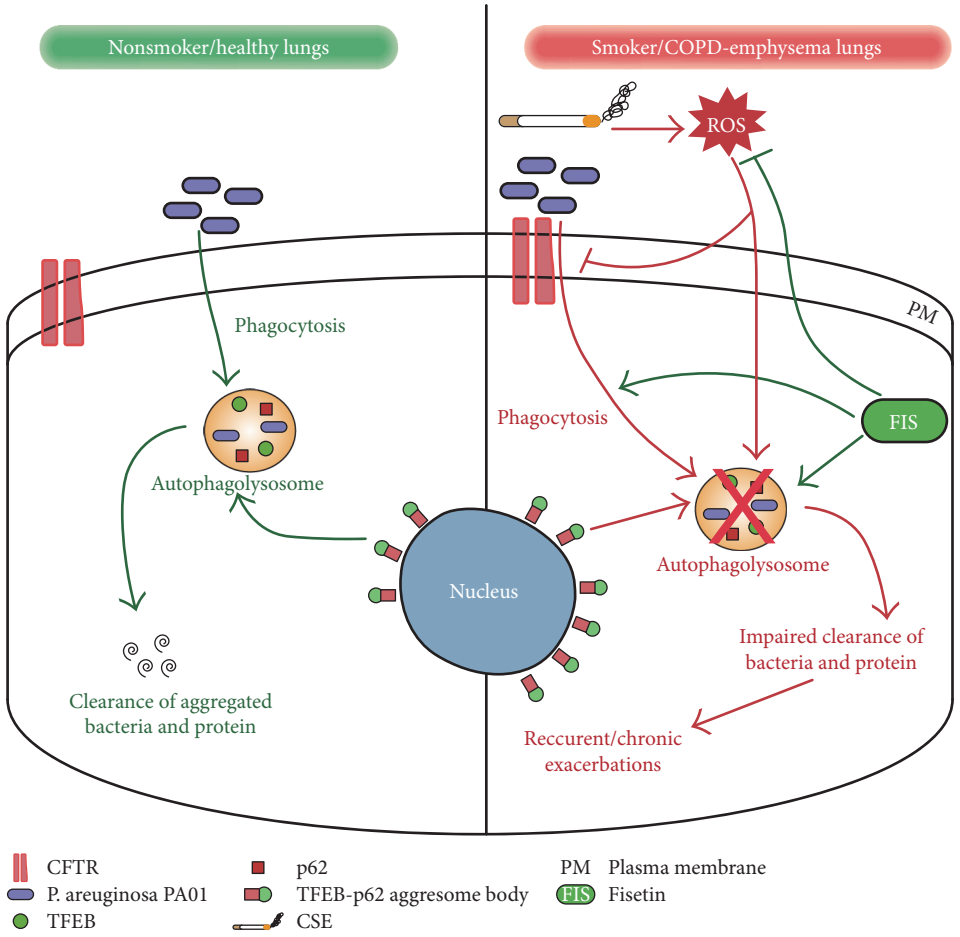

Figure 7: Schematic representation showing the mechanism of cigarette smoke-induced exacerbations in COPD via TFEB-mediated autophagy impairment. Our mechanistic analysis shows that CS/ROS exposure causes TFEB accumulation in aggresome bodies, thus impairing expression of autophagy-regulating proteins leading to chronic autophagy inhibition. Furthermore, CS exposure also causes a decrease in CFTR membrane expression, due to its similar accumulation in aggresome bodies further aggravating autophagy. This CS-mediated chronic autophagy impairment induces a phagocytic defect that results in an increase in bacterial infection as a mechanism for recurring or chronic exacerbations in COPD-emphysema subjects. Moreover, treatment with over-the-counter antioxidant medication, fisetin that induces TFEB expression to restore levels of autophagy proteins, corrects the CS-induced phagocytic defect. Thus, suggesting therapeutic potential of fisetin or autophagy-inducing drugs in controlling recurrent exacerbations in COPD-emphysema subjects.

survival in RAW cells, which suggest CFTR has a role in CS-induced phagocytosis that regulates TFEB-mediated autophagy. Therefore, our investigation shows for the first time that CS-impaired autophagy and xenophagy functions mediate an acquired CFTR defect as the underlying mechanism for the phagocytic defect found in COPD.

Moreover, it has been shown that CSE is high in reactive oxidant species (ROS) which has a deleterious effect on many cellular processes and respiratory function. Furthermore, ROS and CSE have been shown to inhibit autophagy [18, 20, 55], which supports our findings that CS-impaired autophagy has a role in the phagocytosis dysfunction observed in macrophages. Furthermore, ROS causes CFTR dysfunction [2, 25, 56], which we found to further exacerbate CS-induced autophagy impairment resulting in phagocytic defect. Thus, CS-induced autophagy and xenophagy impairment and CFTR dysfunction are synergistic mechanisms mediating phagocytic dysfunction seen in COPD subjects. Hence, we investigated if this phagocytosis dysfunction could be alleviated through the use of an autophagy mediating, antioxidant drug, fisetin. Fisetin is a flavonoid that has anti-inflammatory and antitumorigenic properties [15, 23, 44, 57]. Fisetin has also been shown to function as an autophagy inducer [15]. In this study, we found that induction of autophagy by an antioxidant, fisetin, significantly restored the CS-impaired phagocytic function and thus significantly reduced bacterial survival in RAW cells. Fisetin was also able to significantly restore phagocytosis when CFTR function was inhibited. These findings suggest that fisetin or other autophagy-inducing antioxidant drugs offer a novel therapeutic approach for the treatment of recurrent exacerbations in COPD-emphysema by targeting the mechanistic role of autophagy and xenophagy in phagocytosis dysfunction. This is significant because the constant treatment of these exacerbations with antibiotics leads to resistance in bacteria, resulting in increased

mortality [49, 58, 59]. Thus, by focusing on restoring the phagocytic function of macrophages in COPD subjects instead of targeting the bacteria directly, it is possible to prevent recurrent exacerbations and bacterial resistance in patients with COPD.

Furthermore, COPD and other chronic pulmonary diseases are characterized by inflammation, oxidative stress, increased mucus secretion, and bacterial infection [7, 12, 35, 38, 39, 60–62]. Hence, ideal treatments for these diseases would address all of these aspects instead of a treatment targeting only one specific characteristic of chronic obstructive pulmonary disease(s). Cysteamine is one such drug with properties that have been shown to influence these characteristics of chronic respiratory disease pathogenesis [13, 14, 41]; therefore, we investigated fisetin's therapeutic abilities in comparison to cysteamine (as a positive control). Fisetin has known antioxidant, anti-inflammatory, and bactericidal properties against some bacteria [23, 57]. Moreover, our investigation showed that fisetin could act as a mucolytic with comparable effectiveness to cysteamine, as well as inhibit the growth of PA01-GFP. These findings suggest that fisetin has promise in treating other pathogenic characteristics of obstructive lung diseases such as COPD-emphysema and cystic fibrosis. However, further investigation is necessary into the exact chemical properties of fisetin that account for its mucolytic and direct bactericidal activity.

Despite our findings of direct bactericidal activity in fisetin, this property only accounted for a 1.2-fold decrease in bacterial survival in our experiments. Meanwhile, in our phagocytosis experiments, the decrease in bacterial survival was fourfold; thus, it can be asserted that the significant decrease in bacterial survival seen in the fisetin-treated groups in these experiments was a result of autophagy induction. This assertion is possible because the effect of fisetin's direct bactericidal affect was minimal in comparison to our observations in phagocytosis-mediated bacterial clearance, which meant the restoration of phagocytosis-influenced bacterial killing in these results. This further supports that CS impairs TFEB-mediated autophagy as a mechanism for the phagocytic defect in COPD-emphysema subjects, as well as fisetin's therapeutic potential to restore phagocytosis through TFEB induction. Moreover, CS-acquired CFTR dysfunction is amplified by TFEB-mediated autophagy impairment as this results in aggresome arrest of CFTR as we recently described [25, 56]. Thus, as anticipated, autophagy-inducing antioxidants such as fisetin and cysteamine not only augment CS-impaired autophagy but also the resulting CFTR dysfunction and phagocytic defect.

5. Conclusion

In conclusion, we establish CS-impaired autophagy and xenophagy as a critical mechanism involved in the resulting phagocytic defect. Furthermore, autophagy-inducing drugs with anti-oxidant characteristics such as fisetin restore CS-impaired phagocytosis demonstrating its therapeutic potential in controlling recurrent exacerbations in COPD-emphysema and other chronic respiratory diseases.

Disclosure

The funders had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. The current affiliation of Neeraj Vij, MS, PhD, is President & CEO, VIJ BIOTECH LLC, Baltimore, MD USA.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this article.

Authors' Contributions

Neeraj Vij conceived and designed the study; Neeraj Vij, Garrett Pehote, Kathryn Brucia, and Manish Bodas analyzed and interpreted the study; Garrett Pehote, Manish Bodas, Kathryn Brucia, and Neeraj Vij contributed in the experiments; and Garrett Pehote, Neeraj Vij, Kathryn Brucia, and Manish Bodas drafted and edited the manuscript.

Acknowledgments

The study was supported by the Flight Attendant Medical Research Institute's (FAMRI) Young Clinical Scientist Award (YCSA_082131) to Neeraj Vij.

Supplementary Materials

Supplementary Figure 1. Cysteamine recovers CS induced phagocytic defect in murine macrophages. (A) The Raw cells were pre-treated with cysteamine (250 μM) and/or CSE (5%) for 8hrs. After treatment, the cells were infected with *PA01*-GFP for 3hrs at a MOI of 10. Then, the cells were washed twice with sterile PBS, followed by bright field and fluorescence microscopy (scale bar, 70 μm). These florescent images were utilized to quantify the number of infected cells (intracellular bacteria) using the ImageJ software. The data shows that CSE treatment significantly impairs bacterial clearance, indicated by a decrease in the number of intracellular bacteria, which was significantly recovered by cysteamine treatment. (B) The data from images shown in A are represented here as mean ± SEM of percentage of macrophages infected, $n = 3$, $^{***}p < 0.001$. (C) The cell culture media (100 μl) from the experimental groups shown in A were spread on 2% LB-agar plates and incubated for 24 hrs at 37°C. The number of colony forming units (CFU) was counted to quantify the number of extracellular bacteria as a representation of bacterial survival. CSE treatment resulted in significantly increased bacterial survival. Treatment with cysteamine showed a decrease in bacterial survival; however, it was insignificant. Data represents mean ± SEM of CFUs, $n = 3$, $^{*}p < 0.05$. *(Supplementary Materials)*

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Hindawi
Mediators of Inflammation
Volume 2017, Article ID 5047403, 10 pages
https://doi.org/10.1155/2017/5047403

Research Article

Comparison of Oropharyngeal Microbiota from Children with Asthma and Cystic Fibrosis

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Received 30 May 2017; Revised 24 August 2017; Accepted 22 October 2017; Published 27 December 2017

Academic Editor: Pierre-Régis Burgel

A genuine microbiota resides in the lungs which emanates from the colonization by the oropharyngeal microbiota. Changes in the oropharyngeal microbiota might be the source of dysbiosis observed in the lower airways in patients suffering from asthma or cystic fibrosis (CF). To examine this hypothesis, we compared the throat microbiota from healthy children ($n = 62$) and that from children with asthma ($n = 27$) and CF ($n = 57$) aged 6 to 12 years using 16S rRNA amplicon sequencing. Our results show high levels of similarities between healthy controls and children with asthma and CF revealing the existence of a core microbiome represented by *Prevotella*, *Streptococcus*, *Neisseria*, *Veillonella*, and *Haemophilus*. However, in CF, the global diversity, the bacterial load, and abundances of 53 OTUs were significantly reduced, whereas abundances of 6 OTUs representing opportunistic pathogens such as *Pseudomonas*, *Staphylococcus*, and *Streptococcus* were increased compared to those in healthy controls controls and asthmatics. Our data reveal a core microbiome in the throat of healthy children that persists in asthma and CF indicating shared host regulation favoring growth of commensals. Furthermore, we provide evidence for dysbiosis with a decrease in diversity and biomass associated with the presence of known pathogens consistent with impaired host defense in children with CF.

1. Introduction

Since their emergence, prokaryotes colonize all niches from the extremophilic ones to eukaryotic hosts. One of those niches, long time considered sterile, is the lower airways and the lungs of humans [1]. Of note, even a century ago, it was acknowledged that the lungs are under constant exposure to microorganisms contained in inhaled air and the upper respiratory tract [2]. The conclusion of sterility of the lower airways was based on negative results from standard microbiology that however favors the growth of pathogenic bacteria and was not designed to capture the full spectrum of bacterial species (especially anaerobes) [1].

Airway microbiology is still at the beginning of being deciphered, yet with the advances of research and next-generation sequencing, it is now established that the lower airways in healthy subjects are colonized by bacteria from the oropharyngeal microbiota dominated by members of the Firmicutes, Bacteroidetes, and Proteobacteria phyla [1]. Those findings were the keystone of a theory on the

Table 1: Demographic description of the cohort.

	Control	Asthma	CF
n	62	27	57
Male/female	28/34	21/6	46/11
Age in years (min–max)	10.10 (8–12)	10.00 (8–12)	10.61 (6–12)
FEV1 in L (min–max)	1.99 (1.15–3.18)	1.83 (1.26–2.35)	1.92 (0.88–3.34)
FEV1 z-score ± SD	−0.44 ± 1.08	−0.43 ± 0.79	−1.78 ± 1.37
FVC in L (min–max)	2.36 (1.44–3.87)	2.25 (1.53–3.34)	2.40 (1.04–3.86)
FVC z-score ± SD	−0.17 ± 0.79	0.12 ± 0.93	−1.42 ± 1.32
% antibiotic use within 4 weeks prior to sampling	1.61	3.70	45.61

FEV1: forced expiratory volume in 1 second; FVC: forced vital capacity. z-scores were calculated following the Global Lung Function Initiative (GLI) equation from 2012 [44].

acquisition of the airways' microbiome which is based on the island model: the lower airways' ("islands") microbiota is the result of the colonization from the upper airways ("mainland") regulated by the elimination from the host and conditions of regional growth [3, 4]. In a healthy subject, the balance between colonization and elimination leads to a neutral equilibrium where the most abundant microbes from the upper airways are the most frequently found bacteria in the lower airways [5].

With the advances of high-throughput sequencing, the view of lung microbiology shifted from a pathogen-centered view to a more global view of the whole microbiome [6, 7]. One of the most promising fields in lung research is to understand the crosstalk between the lung microbiota and respiratory epithelial surfaces especially in inflammatory airway diseases. Changes in the microbiome are found in several lung diseases associated with chronic airway inflammation including COPD, asthma, and CF [8–13]. In CF, by the age of 6 years, most children have experienced a bacterial infection with *H. influenza*, *S. aureus*, or *P. aeruginosa* [14–16]. In younger children with CF, the lower airways' microbiota is quite similar to the one observed in healthy subjects and is a subpopulation of the upper airways' microbiota [8, 17]. In asthma, it is currently under debate how bacterial and viral infections contribute to the increase in airway inflammation. Furthermore, it has been shown that environmental microbial exposures prevent the onset of disease [9, 10, 18]. Yet there is also evidence that microbiota in asthma patients shows signs of dysbiosis compared to that in healthy subjects with an increase in Proteobacteria, particularly *Moraxella* sp., and a decrease in Bacteroidetes, particularly *Prevotella* spp. [9, 10].

In children, it is difficult to get samples from the lower airways because they do not expectorate sputum and because sampling the lower airways by bronchial alveolar lavage is relatively invasive which makes it ethically not permissible except in extreme cases. Therefore, studies in children use oropharyngeal samples as a proxy sampling for lung microbiota. It was shown in healthy subjects and young children with CF that this sampling procedure reflects the lower airways' microbiome; based on the island model, the throat microbiota is even the source of microbial colonization [3, 5, 8, 17]. Therefore, the hypothesis of our study was to decipher if children with asthma and CF differ from healthy children regarding throat microbiota and if they differ from each other. To test that hypothesis, we compared the throat microbiota of healthy school-age children (6–12 y) with that of age-matched children with asthma and CF.

2. Materials and Methods

2.1. Subjects. This study was performed with throat swab samples from healthy school-age children and age-matched children with asthma and CF (Table 1). Children with CF were examined and sampled in the CF center in Heidelberg as approved by the Ethics Committee of the University of Heidelberg, and informed written consent was obtained from the patients, their parents, or legal guardians. The diagnosis of CF was based on the established diagnostic criteria [19]. Healthy controls and children with asthma were part of the cross-sectional GABRIELA study. The GABRIELA study was approved by the ethics committees of the participating universities and the regional data protection authorities. Asthma was defined as either (i) parent-reported wheeze during the last 12 months at two different time points of the study, (ii) when a positive answer a positive answer to the question "Did your child ever use an asthma spray?" was given, or (iii) when a doctor diagnosed asthma at least once or wheezy bronchitis more than once. Healthy controls were defined by the absence of all those indications (i.e., no doctor's diagnosis, no use of inhaler spray, and no wheeze at the two time points of the study).

2.2. Sample Collection and Storage. Airway samples from CF patients were obtained during routine visits at the CF center with oropharyngeal Eswabs (BD ESwab Collection Kit, Becton Dickinson, Heidelberg, Germany). Samples were treated in the first 24 h with PMA™ dye (Biotium Inc., Hayward, USA) to remove DNA from dead bacteria as previously described [8] and stored until DNA extraction at −20°C. A supplementary analysis was performed to evaluate the effect due to PMA treatment on a subset of the CF cohort (with and without PMA), and no significant differences in biomass and beta and alpha diversity were observed (Suppl. Figures 1 and 2). Throat swabs from asthmatic and control children were collected using sterile dry cotton-headed swabs (MASTASWAB MD 559, MAST Diagnostica GmbH, Germany). After sampling, the swab was immediately placed

back in the collection tube and stored within 24 h at −20°C. All DNA extractions were performed using the QIAamp Mini Kit (Qiagen, Hilden, Germany). Protease solution (7.2 mAU) and 200 μL of Buffer AL were added to the sample followed by a 15 sec vortex. Samples were incubated at 56°C for 10 min and then purified according to the manufacturer's protocol. DNA was eluted by adding 100 μL of buffer AE to the column, incubated for 1 min at room temperature, and centrifuged at 6000 ×g for 1 min. Negative controls were performed by doing the extraction without clinical samples.

2.3. Microbiome Analysis. DNA was amplified using universal bacterial primers flanking the V4 region (515F and 806R [20]). PCR was performed with numerous controls (positive control with a mock community (HM-782D, BEI Resources, Manassas, USA) and negative control of the PCR and extraction methods) to exclude contaminations [8]. Negative controls for both PCR and extraction did not yield any quantifiable amplicons. PCR products were ligated to the sequencing adapters and paired-end sequenced on an Illumina MiSeq system (250 cycles). Raw sequences were processed to remove low-quality reads and chimera. Sequences were subsampled to obtain the same number of reads per sample and then clustered as operational taxonomic units (OTU) (using the threshold of 3% of divergence). OTUs were classified at the taxonomic levels by comparison with sequences from the SILVA database using Mothur [21]. 5,312,076 of nonchimeric good-quality reads were obtained and subsampled to 7163 reads per sample to normalize the effort of sampling for every sample (Good's coverage of 99.1% (95.2–99.8)). The sequencing of a mock community with known species allowed us to calculate the overall error rate of the PCR and sequencing methodology. This error rate was 1.56×10^{-5}. Furthermore, we found 19 OTUs with abundance higher than 1% in the mock community when we were expecting 20. The taxonomical assignment of those OTUs showed a matching identity to the 20 expected species. Only two *Staphylococcus* species were clustered together in the same OTU due to identical 16S rRNA V4 sequences between *S. aureus* and *S. epidermidis*. The mean distance based on the Morisita-Horn index between the different mock communities used in different sequencing runs was 0.032.

Quantitative PCR (qPCR) was used to evaluate the number of 16S copies as a proxy measure of biomass. qPCR was performed using the Unibac primer (forward: 5′-TGG AGC ATG TGG TTT AAT TCG A-3′; reverse: 5′-TGC GGG ACT TAA CCC AAC A-3′) as previously described [8].

2.4. Statistical Analyses. The OTU table with all OTUs (normalized by subsampling 7163 reads per sample) obtained was used to calculate descriptive indices for alpha diversity (nonparametric Shannon index), richness (Chao1 richness estimate), and evenness (Shannon index-based measure of evenness). Variation in the alpha diversity and clinical parameters was tested with a pairwise Wilcoxon sum rank test. Beta diversity variations were evaluated at the OTU level via Principal Coordinate Analysis (PCoA) and PERMANOVA based on the Morisita-Horn similarity index [22]. R^2 indicating the strength of the explanatory variable on the distance between the microbiota is reported. Associations with lung function were analyzed by linear regression for continuous variables (R^2 indicating the strength of the correlation) or the pairwise Wilcoxon sum rank test for categorical variables.

We also performed an analysis with a model based on a negative binomial distribution (DESeq) [23] to detect differentially abundant OTUs between the groups (asthma versus CF, asthma versus healthy, and CF versus healthy). Adjustment for multiple testing was done using the Bonferroni-Hochberg method. All statistical analyses were performed with Mothur 1.37.4 and R 3.3.0 (mainly packages Phyloseq [24] to handle microbiome data, Vegan [25], and DESeq2 [23] for analysis). Scripts for Mothur, R, and the sequence data are deposited in figshare (https://figshare.com/s/1e69261612f3dfcacf42).

3. Results

3.1. A Decrease in Lung Function and Higher Antibiotic Usage Are Characteristic of the CF Cohort. Children with CF showed a decreased lung function with a FEV1 z-score of -1.78 ± 1.37 (versus -0.43 ± 0.79 for asthma (p value < 0.001) and -0.44 ± 1.08 for control (p value < 0.001)), a FVC z-score of -1.42 ± 1.32 (versus 0.12 ± 0.93 for asthma (p value < 0.001) and -0.17 ± 0.79 for control (p value < 0.001)), and a higher frequency of antibiotic usage (CF versus control: odds ratio = 47.81 (p value < 0.001) and CF versus asthma: odds ratio = 21.81 (p value < 0.001)) (Table 1).

3.2. Comparison of Oropharyngeal Microbiota Reveals Only Minor Shifts between Healthy Children and Children with Asthma or CF. The structure of the whole microbial community was compared using the Morisita-Horn similarity index and PERMANOVA analysis. Although significant differences between the three groups (CF versus control: $R^2 = 0.05$ (p value < 0.01), CF versus asthma: $R^2 = 0.09$ (p value < 0.01), and control versus asthma: $R^2 = 0.05$ (p value < 0.01)) were found, the low values of the R^2 (indicating the strength of the impact of the disease status on the differences between the microbial structure) and the major overlap of the three cohorts on the PCoA (Figure 1(a)) argue for only minor changes within the structure of the microbiome. This similarity is confirmed by the shared presence of the most common OTUs in each cohort (84 OTUs shared) (Figure 1(b)). Only 2 abundant OTUs were only present in CF patients; those belonged to the genus *Pseudomonas* and *Phyllobacterium*. One OTU belonging to the genus *Moraxella* was absent from the CF group, and one belonging to the genus *Aggregatibacter* was absent from the asthma groups. The difference between the three cohorts was mostly due to the modulation of the abundance of the most dominant genera and variation in the less abundant genera rather than to extinction of specific genera (Figure 1(c)). The CF cohort compared to controls and asthmatics showed an increasing trend in four dominant genera: *Prevotella*, *Neisseria*, *Veillonella*, and *Streptococcus*, while asthmatic children tended to have higher *Haemophilus* abundance compared to CF and controls.

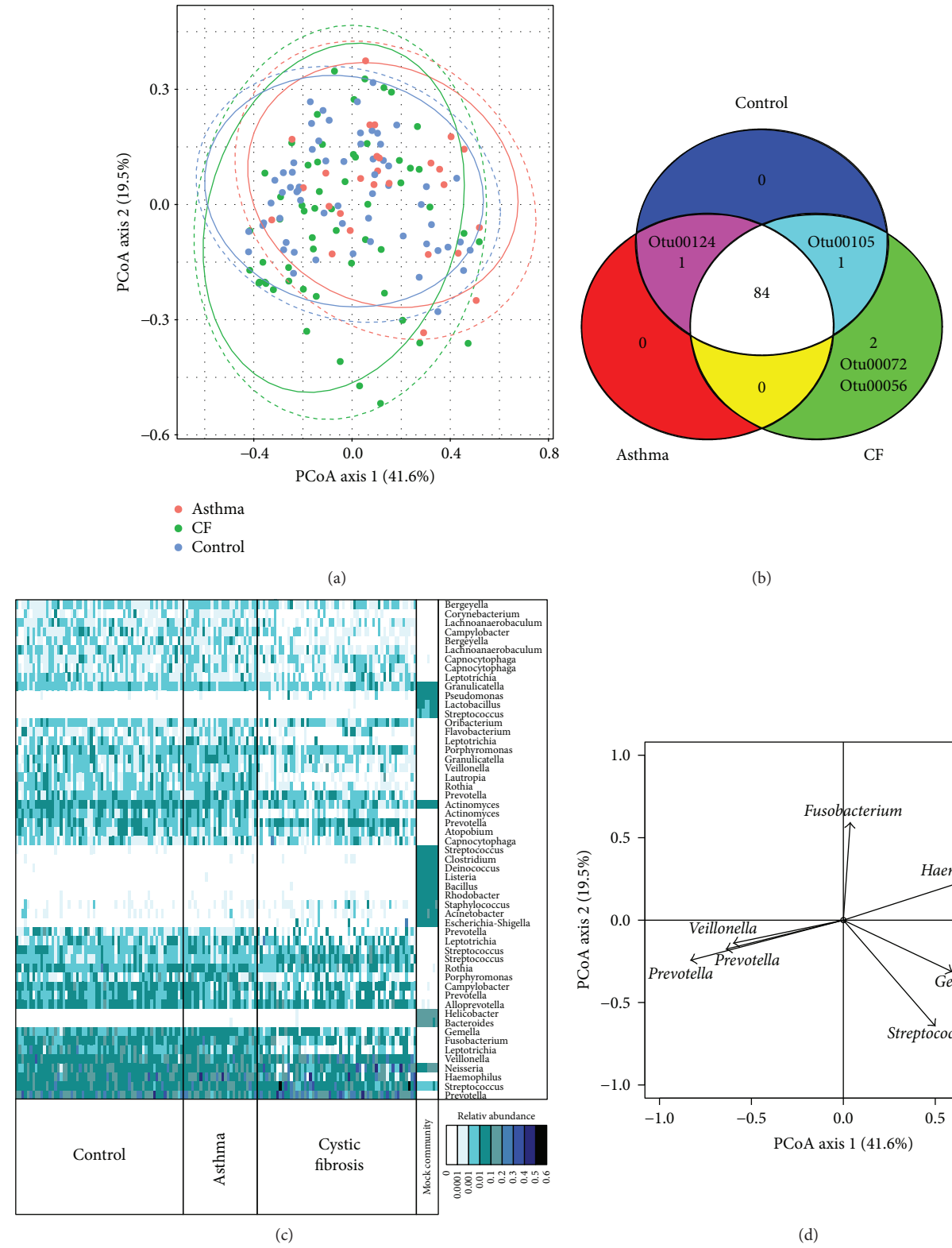

Figure 1: Structure of the throat microbiome of healthy children, children with asthma, and children with CF. (a) Microbial compositions of the whole microbiota were visualized by PCoA. The lines represent the 95% confidence interval assuming a multivariate *t*-distribution (full lines) or a multivariate normal distribution (dashed lines). (b) The presence/absence of the 88 most abundant bacteria (>0.1% relative abundance) was compared between the three groups and is displayed as a Venn diagram. (c) Relative abundance of the 55 most abundant OTUs in each sample clustered according to the cohorts. The mock community was an assembly of DNA from 20 known species with equimolar ribosomal RNA operon counts (100,000 copies per organism per μL). (d) Correlation plots of the major OTUs with the two first axes of the PCoA.

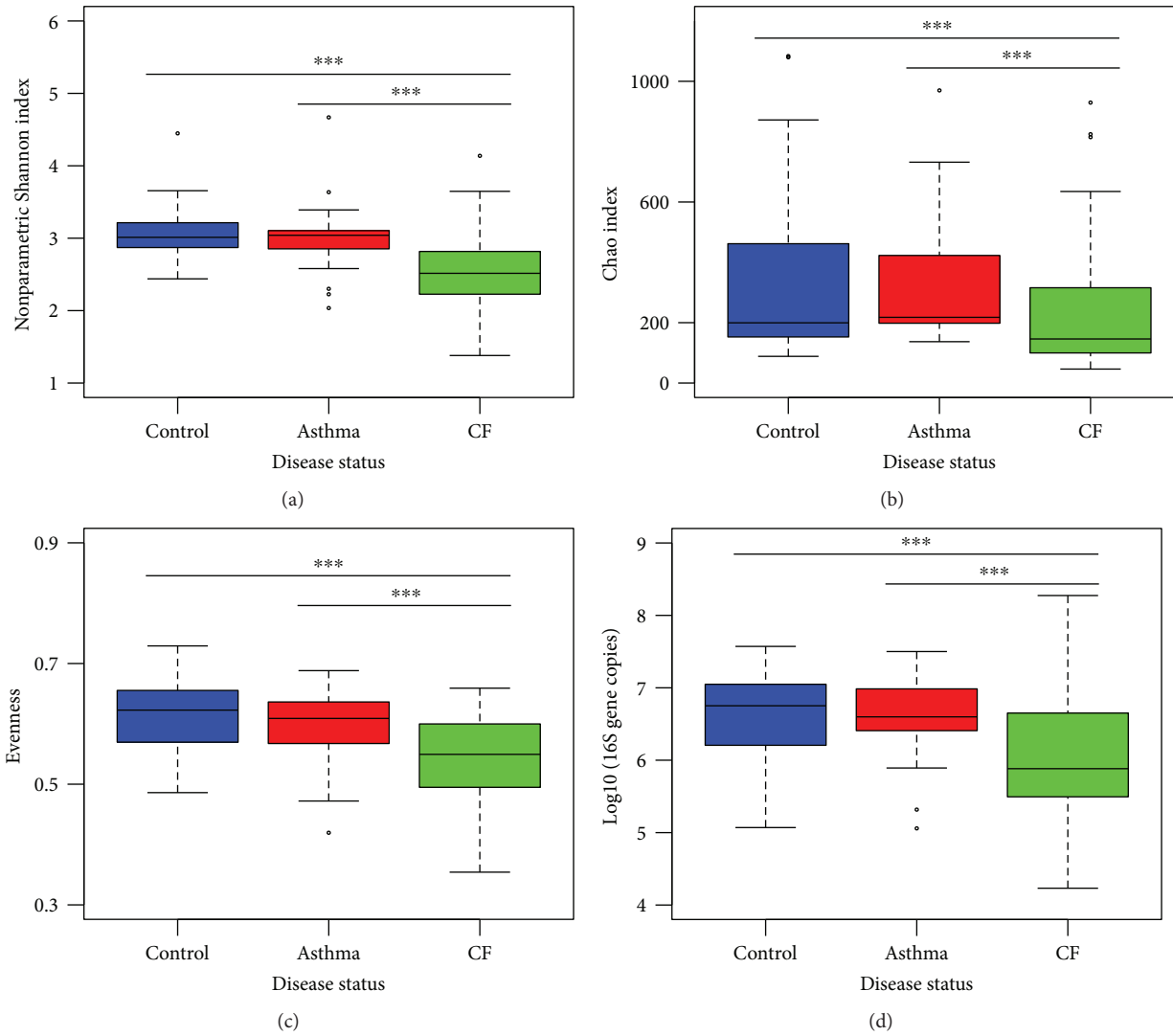

Figure 2: Diversity and biomass are decreased in the oropharyngeal microbiome of children with CF. (a) α-Diversity was assessed by the nonparametric Shannon index. (b) Richness was estimated by the Chao index. (c) Evenness was derived from a Shannon index-based measure of evenness. (d) Global biomass measured as the number of 16S rRNA gene copies. Statistical significance was calculated by the Wilcoxon test. $^{***}p$ value < 0.001.

The distribution over the two axes of the PCoA relied on differences in the following major components of the microbiota in the throat: OTUs belonging to the following genera *Prevotella*, *Veillonella*, *Streptococcus*, *Gemella*, *Haemophilus*, and *Fusobacterium* (Figure 1(d)). Samples from asthmatic patients were showing a significantly higher value on axis 1 of the PCoA compared to those from CF patients (p value < 0.01) and healthy people (p value < 0.05) indicating a higher abundance of the OTUs (mainly *Gemella* and *Haemophilus*) correlating positively with this axis of the PCoA and a lower abundance of OTUs belonging to *Prevotella* and *Veillonella* correlating negatively with axis 1. Gender and antibiotic usage within 4 weeks prior to sampling were also significantly affecting the microbiota structure of the full population (independent of the cohort) but with a small R^2 (gender: $R^2 = 0.03$ (p value < 0.01) and antibiotic usage: $R^2 = 0.01$ (p value < 0.01)).

When the cohort was analyzed separately, only the gender was affecting the CF cohort ($R^2 = 0.07$ (p value = 0.02)) and no antibiotic usage effect was observed. For the control and asthma cohort, no effects were observed for gender and antibiotic usage but the low prevalence of antibiotic usage did not allow strong statistical evaluation.

3.3. Oropharyngeal Microbiota from Children with CF Shows a Decrease in Alpha Diversity and Biomass. Microbiota of the throat from children with asthma versus control children did not differ in alpha diversity (p value = 0.56). However, throat microbiota from children with CF showed strong evidence of a decrease in the global alpha diversity compared to that from asthmatic and control children (p value < 0.001) (Figure 2(a)). The change in diversity was due to a difference in the total amount of species present (Figure 2(b)), as well as due to the distribution of the abundance of the species

(Figure 2(c)). Microbiota from children with CF was less diverse and less even indicating the overgrowth by one or few species. This observation correlated with the increase in the abundance of *Prevotella* and *Streptococcus* in the CF group.

No differences in the numbers of bacteria were observed between asthmatic and control children. However, children with CF harbored less bacteria in their throat (Figure 2(d)). The reduced α-diversity is also seen for children with lower lung function, even if only a trend is seen (FEV1 z-score: $R^2 = 0.17$ (p value = 0.06) and FVC z-score: $R^2 = 0.17$ (p value = 0.08)) probably reflecting the impact of CF.

Antibiotic usage within 4 weeks prior to sampling did not influence the alpha diversity within the CF cohort (p value = 1). Furthermore, also the nontreated CF samples showed a significantly lower alpha diversity than samples from patients with asthma (p value < 0.001) or control samples (p value < 0.001) indicating the effect of the disease status even without antibiotic therapy. However, biomass was slightly influenced by antibiotic usage as nontreated CF samples did not show a significant decrease in bacterial load compared to control and asthmatic samples, but treated CF samples showed a significant decrease in biomass. No significant difference in the biomass was observed between treated and nontreated CF samples (Suppl. Figure 3).

3.4. Differences in Oropharyngeal Microbiome Composition between Children with CF, Children with Asthma, and Healthy Controls. Between children with CF and healthy controls, 53 OTUs were differentially abundant (Figure 3). Most of them were decreased in the CF group except a small group composed of 6 OTUs belonging to *Streptococcus*, *Catonella*, *Enterobacteriaceae*, *Staphylococcus*, and *Pseudomonas*. Interestingly, *Staphylococcus* and *Pseudomonas* are well-known CF pathogens, and the OTUs belonging to *Pseudomonas* were the same as the ones in the mock community indicating that the species is *P. aeruginosa*. The same pattern was observed for the comparison between children with CF and children with asthma with a general decrease in the 20 differentially abundant OTUs in CF except for two OTUs belonging to the *Streptococcus* genus. Despite the visible change in the abundance in the heatmap, no significant differences were observed for the 5 other OTUs that appear more abundant in CF than in controls. This lack of statistical significance might be due to the low number of samples and the higher interindividual variability of those OTUs in the CF cohort.

4. Discussion

The aim of this study was to analyze whether differences occur in the throat microbiota relating to inflammatory airway diseases like asthma and CF in comparison to that in healthy children. Our results indicate that the microbiota of CF, asthmatic, and healthy children shows high levels of similarities with a strong core microbiota composed mostly of *Prevotella*, *Streptococcus*, *Neisseria*, *Veillonella*, and *Haemophilus*. The prevalence of those bacteria in the throat microbiota was demonstrated previously in healthy, CF, and asthmatic children indicating a close relationship, but our study is the first to compare the three groups with the same DNA extraction method, primer usage, and sequencing method thus controlling for potential technical bias [8, 9, 17]. Free of those biases, we were able to demonstrate that the CF group showed a decrease in both diversity and total bacterial load in the throat in comparison to asthmatic and control children. The decrease in diversity was due to an increase in the abundance of dominant genera, especially *Prevotella* and *Streptococcus*. The increase in the dominance influences the evenness of the bacterial community and the overall richness as it was observed before in CF and COPD [8, 13]. The decrease in the biomass would also indicate a less abundant microbiome in the throat of CF patients. The CF group also showed a significant increase in typical pathogens in the throat like *Pseudomonas*, *Staphylococcus*, and the atypical pathogen *Phyllobacterium* [26–28]. It could be hypothesized that the lower biomass in CF children compared to asthmatic and healthy children facilitates the colonization in the throat by additional pathogens as it decreases the competition pressure and colonization resistance by protective commensals represented by *Prevotella*, *Neisseria*, *Streptococcus*, and *Veillonella*. This theory is supported by the fact that in CF patients, the latter specific genera are decreasing in the lower airways after colonization by typical pathogens like *Pseudomonas aeruginosa* [8, 29]. The commensal and protective role of core throat bacteria found here and also in healthy subjects is still not elucidated; especially, the specific role of anaerobes in the lower airways remains controversial [30]. However, it was demonstrated that *Prevotella* and other anaerobes exhibit antimicrobial activities and a specific strain of *Prevotella* possessing an altered LPS had less immunestimulatory activities [31, 32]. Our data cannot exclude the hypothesis that CFTR mutation is modifying the niches and the regional growth for specific CF pathogens in the lungs and that those modifications are the driving force of the establishment of pathogens in the lungs.

The strong similarities between the throat microbiota from healthy children and that from asthmatic children were already demonstrated within a larger cohort in which no differences were detectable between asthma and control children [9]. Our data with a subsample of this study and using a different analysis (DESeq) confirm these findings, indicating the absence of effects of asthma in the throat. Yet, those results are in contradiction with those of other studies with smaller cohorts [33, 34], in which a significant increase in *Haemophilus* and a decrease in *Prevotella* were seen in asthmatic children. On closer examination, our data show a similar trend, yet overall no severe or significant dysbiosis in the examined age group was observed. In our setting, statistical tests did not indicate significance. The analysis with a model based on a negative binomial distribution (DESeq) is less sensitive to false positives than Metastat or a generalized linear model analysis when applied to a small-size cohort. As shown by Depner et al., we did not observe a difference in the total bacterial load between asthma and control children [9].

The strong overlap and shared core microbiome between the three groups are an evidence of the importance of the

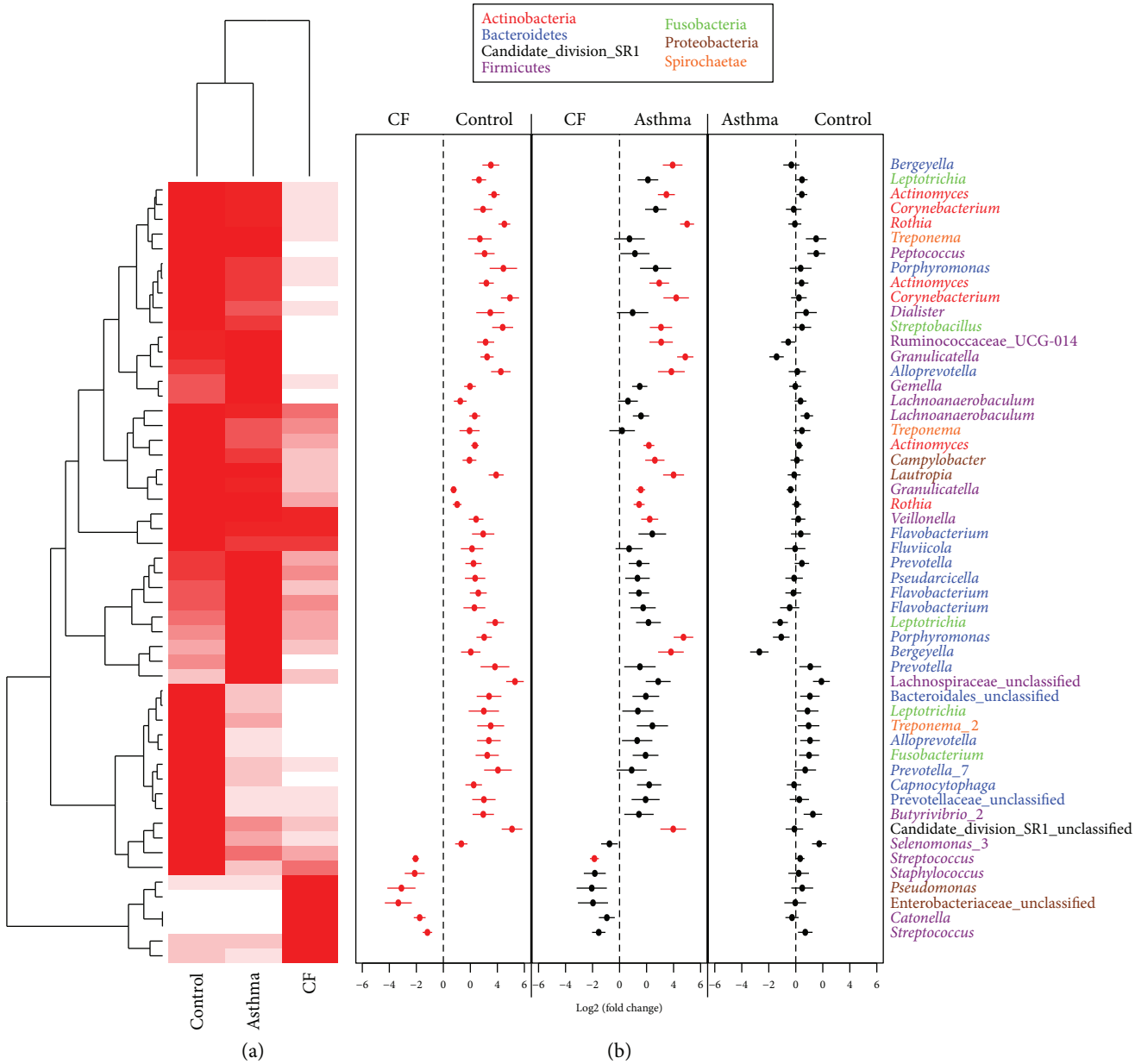

FIGURE 3: Differences in OTU abundance between healthy children, children with asthma, and children with CF. Differential abundance of each OTU was tested via a method based on the negative binomial distribution and is represented in a heatmap for the significantly differentially abundant OTU. Mean abundance was normalized within each OTU to the maximal values (normalized value$_{OTU1}$ = (relative abundance$_{OTU1}$)/maximum (relative abundance$_{OTU1}$)). The color code is from white (minimal abundance: 0) to red (maximal abundance: 1). Fold changes between the groups and standard errors are displayed in (b). Red dots indicate significant differences after correction for multiple testing. OTUs are named following their genus classification and colored following their phylum classification.

regulation of the upper airways' microbiota by the host. As patients with asthma and CF come from different environments and disease status and showed the highly similar microbiota, this indicates a positive selection to conserve the structure of the microbiota. It seems that there is a tight immune regulation of the microbiota in the throat that allows only the growth of commensals or at least nonpathogenic bacteria [35]. However, this regulation seems to be slightly unbalanced in CF patients leading to a decrease in diversity and total amount of bacteria correlating with the increased chance to harbor a typical CF pathogen (*Staphylococcus* or *Pseudomonas*). As we did not observe changes in asthma, the imbalance observed in CF could be due to impaired host defense mechanisms associated with CFTR dysfunction in the airways, changes in regional growth condition in the lungs, and/or CF-specific antibiotic regimen [36–38].

Our study focused on oropharyngeal swabs. Yet in the light of recent results on healthy, asthmatic, and CF children and the current theory on acquisition and establishment of the airway microbiome, the throat microbiota will have a direct influence on the lower airways and

therefore on the colonization probability [4, 8, 10, 17]. Therefore, our findings might indicate that the core microbiome found between the three cohorts can be the source of commensals that will colonize the lower airways. The colonization by those commensals is regulated by the host in a complex balance between migration and elimination [4]. In this context, a lower migration of protective commensals may increase the chance of pathogenic colonization in the lungs for CF [39]. This correlates with the finding that a decrease in diversity in the lower airways of CF patients was linked to a higher chance of further infection and a more severe inflammatory response [16, 40]. However, as our study did not analyze lower airways' samples, we can only speculate that the core microbiome found in the three cohorts and the slight differences specific to CF also occur in the lower airways.

The limitation of our study is that despite the common DNA extraction method, primer choice, and sequencing technology used, there are still some differences in the methodology applied to the CF cohort and the two other cohorts. One methodological discrepancy was the use of different swabs and PMA treatment that might affect both diversity and biomass. However, we showed by comparing 10 samples with and without PMA that, in our study cohort, PMA did not significantly affect both biomass and diversity (Suppl. Figures 1 and 2). Finally, the two cohort sampling methods used different types of swabs that might influence the sampling of microbial community; therefore, this bias still has to be taken in consideration. However, the most important technical bias in microbiota studies are the DNA extraction method, primer choice, and sequencing technology, and therefore, in this study using the same primers and DNA extraction and sequencing technology, we abrogated most of the major bias [41–43]. Furthermore, the sampling size of the asthmatic cohort was smaller compared to that of the CF and control cohort; thus, differences between this cohort and the two others have to be interpreted carefully.

In conclusion, this study analyzed for the first time the throat microbiota of healthy school-age children and children with asthma and CF with comparable methodology. Our results show that the three patient groups showed a high level of similarity indicating a core microbiome and host regulation that favors the growth of commensals. However, the CF group showed a decreased diversity and biomass of the microbiota associated with the presence of known CF pathogens consistent with impaired host defenses associated with CFTR malfunction in the airways.

Conflicts of Interest

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

Authors' Contributions

Alexander H. Dalpke, Erika von Mutius, and Marcus Mall contributed equally as senior authors.

Acknowledgments

During the work on this project, Dr. Antje Legatzki has passed away.

Supplementary Materials

Supplementary Figure 1: PMA treatment of the DNA did influence neither diversity nor biomass of the CF microbiome in the analyzed cohort. A subset of samples ($n = 10$) from the CF cohort was analyzed for changes in diversity and global biomass with and without PMA treatment. No significant differences were observed. Supplementary Figure 2: PMA treatment of the DNA did not influence the structure of the CF microbiome in the analyzed cohort. A subset of samples ($n = 10$) from the CF cohort was analyzed for changes in the microbial structure with and without PMA treatment. No significant differences in the structure of the microbiome were observed. Paired samples are in the same color. The lines represent the 95% confidence interval assuming a multivariate t-distribution (full lines) or a multivariate normal distribution (dashed lines). Supplementary Figure 3: impact of antibiotic therapy prior to sampling on the diversity and biomass of the microbiome in the analyzed cohort. Samples were classified in two groups "treated" and "nontreated" based on the usage of antibiotic within 4 weeks prior to sampling. Statistical significance was calculated by the pairwise Wilcoxon test. $^{***}p$ value < 0.001. *(Supplementary Materials)*

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Hindawi
Mediators of Inflammation
Volume 2017, Article ID 6506953, 20 pages
https://doi.org/10.1155/2017/6506953

Research Article

Effect of IRAK-M on Airway Inflammation Induced by Cigarette Smoking

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Received 12 February 2017; Revised 16 May 2017; Accepted 29 May 2017; Published 29 August 2017

Academic Editor: Catherine Greene

Background. IRAK-M, negatively regulating Toll-like receptor, is shown the dual properties in the varied disease contexts. We studied the effect of IRAK-M deficiency on cigarette smoking- (CS-) induced airway inflammation under acute or subacute conditions in a mouse model. *Methods*. A number of cellular and molecular techniques were used to detect the differences between IRAK-M knockout (KO) and wild type (WT) mice exposed to 3-day or 7-week CS. *Results*. Airway inflammation was comparable between IRAK-M KO and WT mice under 3-day CS exposure. Upon short-term CS exposure and lipopolysaccharide (LPS) inhalation, IRAK-M KO mice demonstrated worse airway inflammation, significantly higher percentage of Th17 cells and concentrations of proinflammatory cytokines in the lungs, and significantly elevated expression of costimulatory molecules CD40 and CD86 by lung dendritic cells (DCs) or macrophages. Conversely, 7-week CS exposed IRAK-M KO mice demonstrated significantly attenuated airway inflammation, significantly lower concentrations of proinflammatory cytokines in the lungs, significantly increased percentage of Tregs, and lower expression of CD11b and CD86 by lung DCs or macrophages. *Conclusions*. IRAK-M plays distinctive effect on CS-induced airway inflammation, and influences Treg/Th17 balance and expression of costimulatory molecules by DCs and macrophages, depending on duration and intensity of stimulus.

1. Introduction

Cigarette smoking (CS) is a recognized risk factor for several airway inflammatory diseases, particularly chronic obstructive pulmonary disease (COPD) [1]. CS impairs the physical integrity and immunodefense functions of airway epithelium, leading to increased susceptibility to bacterial infection of the airways [1, 2]. CS is also a profound proinflammatory stimulus that triggers airway inflammation by activating innate and adaptive immune cells, such as dendritic cells (DCs), macrophages, and T cells. T cells are one of the predominant cell types in the pathogenesis of COPD, with $CD4^+$ and $CD8^+$ T cells being seen in both the airways and parenchyma of COPD patients [3, 4]. COPD-associated Th1 cells, known as Th17 cells, have been found in the lungs of COPD patients. Evidence from animal model showing the imbalance of T helper 17 cells (Th17)/T-regulatory cells (Treg) induced by CS supported the role of Treg and Th17 cells in the immunopathogenesis of CS-induced lung pathology [5].

Toll-like receptors (TLRs), expressed on airway epithelium, recognize lipopolysaccharide (LPS) which activates intracellular molecules, such as myeloid differentiation factor 88 (MyD88) and IL-1 receptor-associated kinases (IRAKs)

Table 1: Sequences for real-time quantitative RT-PCR primers.

qRT-PCR genes	Accession number	Primer sequences (5′—3′)
T-bet	NM_019507.2	F: GTTCAACCAGCACCAGACAGAG
		R: TGGTCCACCAAGACCACATC
GATA3	NM_008091.3	F: GGATGTAAGTCGAGGCCCAAG
		R: ATTGCAAAGGTAGTGCCCGGTA
RORC	NM_001293734.1	F: GCTCCATATTTGACTTTTCCCACT
		R: GATGTTCCACTCTCCTCTTCTCTTG
FOXP3	NM_001199348.1	F: AGTGCCTGTGTCCTCAATGGTC
		R: AGGGCCAGCATAGGTGCAAG
GAPDH	NM_001289726.1	F:TTGTCTCCTGCGACTTCAACA
		R:TGGTCCAGGGTTTCTTACTCC

that lead to overproduction of proinflammatory cytokines [6]. IRAK-M, known as IRAK-3, is one of IRAK family members and functions as a negative regulator of TLR-mediated NFκB activation. Expression of IRAK-M is located in both airway epithelial cells and monocytes/macrophages of the healthy lungs [7, 8].

Previous studies reported the different effects of IL-6 and IL-22 on lung pathologies during inflammatory and repairing processes [9–11]. IRAK-M has also shown dual properties in various disease contexts. Induction of IRAK-M expression in some certain settings may be helpful in attenuating pathologies by limiting overproduction of proinflammatory cytokines and overactivation of innate immune response [6]. Chen et al. have reported that upregulation of IRAK-M in cardiac macrophages alleviates myocardial inflammation and prevents adverse cardiac remodeling in mouse models of myocardial infarction [12]. However, IRAK-M overexpression in some circumstances can increase host susceptibility to infectious- or noninfectious injury. In vitro studies showed that overexpression of airway epithelial IRAK-M inhibited innate immunity of airway epithelium against bacterial infection and increased epithelial infection to human rhinovirus- (HRV-) 16 [13, 14]. IRAK-M knockout (KO) mice exhibit accentuated inflammatory responses after bacterial and viral infections [7, 15, 16]. Interestingly, recent evidence showed that IRAK-M KO mice had attenuated lung fibrosis induced by bleomycin with a significant reduction of proinflammatory IL-13 in the airways [17].

Given these inconsistent effects of IRAK-M in regulating body immune responses to the environmental insults, its role in cigarette smoking- (CS-) induced airway inflammation remains open. In this study, we reported the distinctive effects of IRAK-M on airway inflammation in the various phases of CS exposure and type of stimulus possibly through influencing the surface expression of costimulatory molecules by DCs and macrophages of the lungs by using IRAK-M KO mice and differentiation of naïve T cells.

2. Materials and Methods

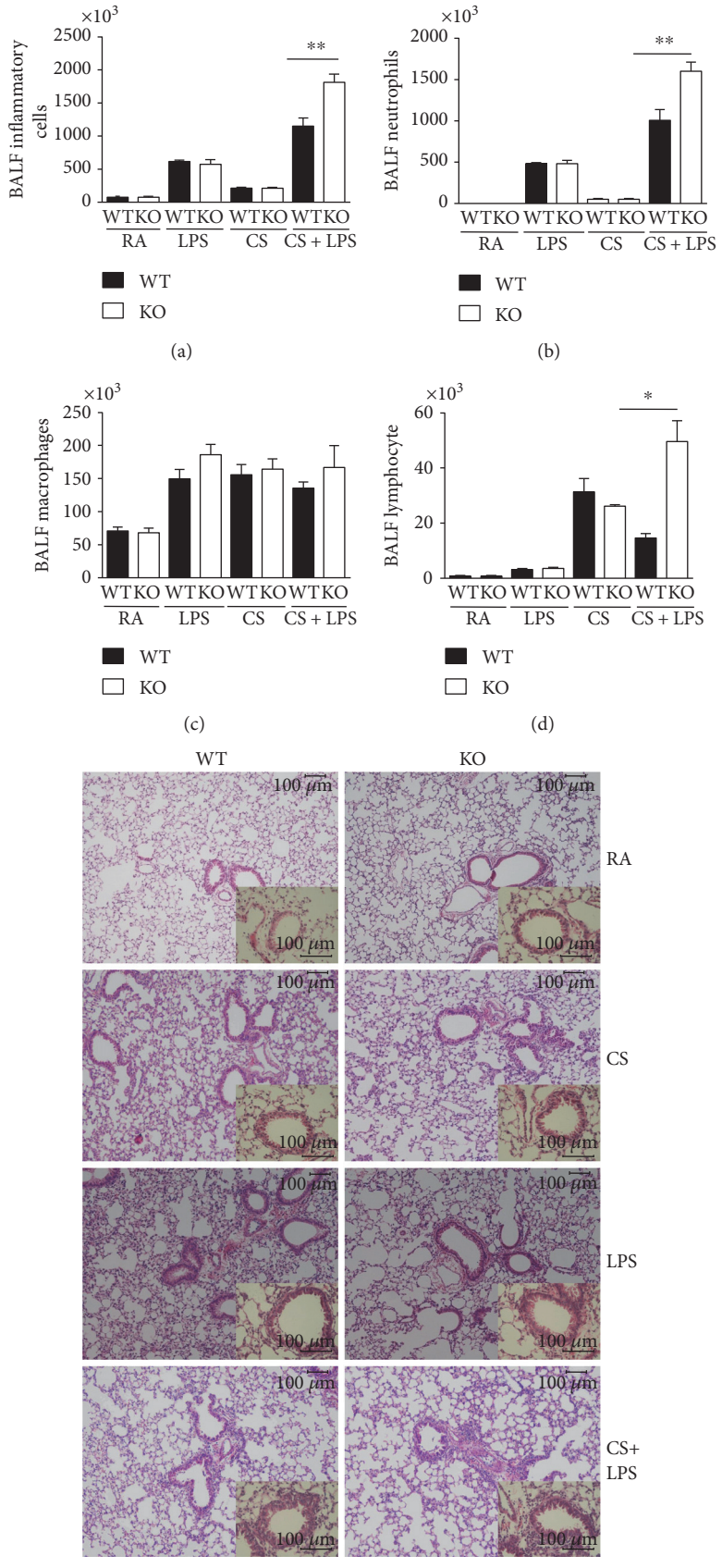
2.1. Mice. Wild-type (WT) mice with C57BL/6 background were purchased at age of 6 weeks from the Experimental Animal Research Center (Beijing, China). Mice deficient in IRAK-M (B6.129S1-Irak3^{tm1Flv}/J), originally from Jackson Laboratory (Bar Harbor, ME), were gifted by Dr. Nikolaos G. Frangogiannis and bred on B6 background for 10 backcrosses [7]. All animals were maintained in mouse facility at Peking Union Medical College Hospital (PUMCH). 8- to 10-week-old mice (~20 grams of weight) were used for all experiments. Mice were age-matched in acute CS exposure group and subacute CS exposure groups. The ages of mice were 12–15 weeks at the end of CS exposure.

All experiments were carried out according to international and institutional guidelines for animal care and approved by Peking Union Medical College Hospital Ethics Committee for animal experimentation.

2.2. Cigarette Smoke (CS) Exposure and LPS Inhalation. Mice were exposed to CS in a whole-body exposure system according to our previously described [18]. Briefly, mice were placed in a closed plastic box connected to a smoke generator. To establish acute or subacute animal model of airway inflammation induced by CS or LPS [18, 19], the mice were exposed to tobacco smoke of five cigarettes (reference cigarette 3R4F, University of Kentucky, USA) four times a day with 30 min smoke-free intervals between each smoke exposure for 3 days or for 7 weeks. Control mice were exposed to room air (RA). On the fourth day, some mice were challenged with PBS or 100 μg/ml of LPS (*Escherichia coli* serotype 0111:B4, Sigma) by inhalation for 30 mins according to previously described with minor modification [20–22]. LPS has been applied to replicate animal model of COPD [19]. Mice were sacrificed 24 hours after the last challenge with LPS or CS for further analysis.

2.3. Airway Resistance Test. Airway resistance (Rn) was determined as previously described for the invasive analysis of lung function using a computer-controlled small animal ventilator, the Flexivent system (Scireq, Montreal, PQ, Canada) [23].

2.4. Bronchoalveolar Lavage (BAL). Mice were euthanized, and BAL was performed as previously described method. Briefly, cells were obtained from BAL fluids, and cytospins (Thermo Electron, Waltham, MA) were prepared to determine the numbers of total inflammatory cells and differential



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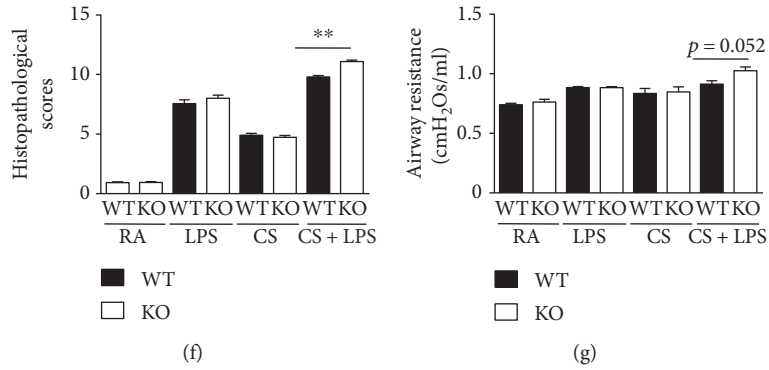


Figure 1: Increased airway inflammation in IRAK-M KO mice after 3-day CS exposure followed by LPS challenge. (a)–(d) The total inflammatory cells and differential populations recovered from BAL fluid. (e) Representative photomicrographs of hematoxylin & eosin-stained lung tissues showing that LPS/short-term CS-insulted IRAK-M$^{-/-}$ mice exhibited the typical pathological characteristics of airway inflammation evidenced by thickened airway epithelium and more inflammatory cells in the peribronchial area and around vessels compared with the similarly treated WT mice. (f) Semiquantative scorings of histopathologic changes. (g) IRAK-M KO mice showed marginally increased airway resistance compared with WT mice after combined exposure to short-term CS and LPS. Results are expressed as means ± SEM, $n = 5$–8 animals per group, $^{*}p < 0.05$, $^{**}p < 0.01$.

cells using a modified Wright-Giemsa staining. At least 400 cells were counted for one sample [18, 23].

2.5. Lung Histology and Semiquantitative Scorings of Airway Inflammation. For histology samples, lungs were perfused with saline and inflated with 4% paraformaldehyde at 25 cm H2O overnight after the last challenge. Hematoxylin & eosin (H&E) staining was performed at the Department of Pathology, Peking Union Medical College Hospital.

An index of pathological changes of airway inflammation in H&E slides was assessed in a blind manner by scoring the inflammatory cell infiltrated around airways and vessels according to the previously published methods [24, 25]. Briefly, a score of 0–3 on each section was used to reflect overall extent of airway inflammation (0: normal; 1: <25% of each section; 2: 25–75%; and 3: >75%).

2.6. Flow Cytometry Analysis. To prepare single cell suspensions, lungs were perfused with 20 ml cold PBS through the right ventricle, carefully minced, digested with collagenase type 1A and type IV bovine pancreatic DNase, and passed through a cell strainer. For detection of surface expression of costimualtory molecules, cells were stained with fluorochrome (FITC, PE, PerCP-Cyanine5.5, APC)-conjugated Abs (anti-mouse CD3, CD4, CD8, F480, CD11b, CD11c, CD40, CD80, CD83, and CD86) for 30 mins at 4°C according to previously published [26]. For measurement of expression of intracellular cytokines, cells were incubated with 50 ng/ml of PMA, 500 ng/ml of ionomycin, and GolgiStop (BD Biosciences) for 5 h at 37°C. Then, cells were stained with anti-CD3 and anti-CD8 for 30 min and next stained with mAbs (anti-IL-17A, anti-IL-4, anti-IFNγ, and anti-FOXP3) for 1 h. After wash by PBS containing 0.1% sodium azide, cells were subjected on FACSCalibur (BD Biosciences). All fluorochrome-conjugated Abs were purchased from eBioscience or Biolegend, and the corresponding isotype control Abs were added to "isotype samples."

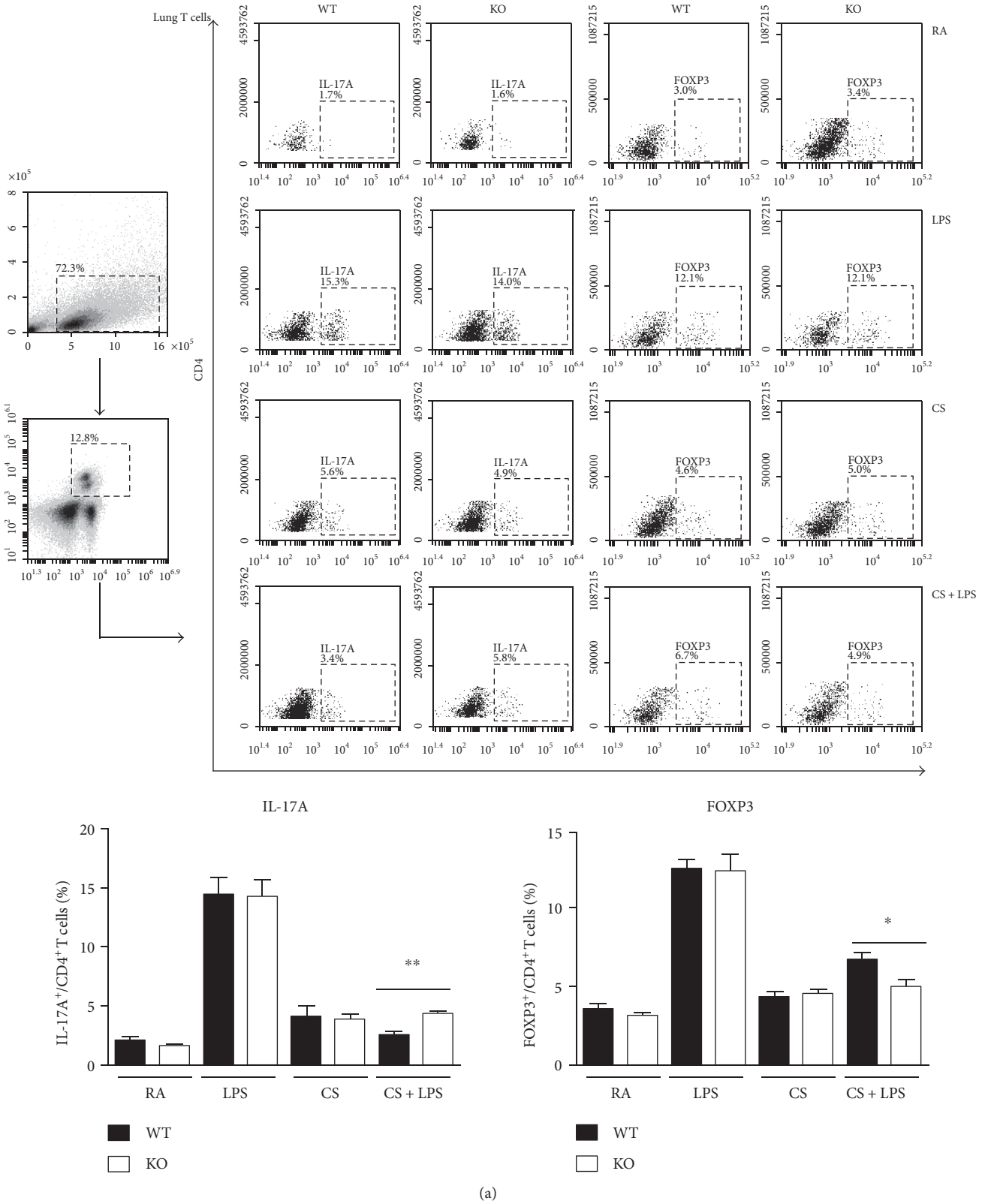
2.7. Quantification of Cytokines. The left lungs were harvested and homogenized in 1 ml PBS containing protease inhibitor cocktail (Sigma, St. Louis, MO) and centrifuged. Supernatants were filtered and kept at −80°C until analysis. Concentrations of cytokines were quantified by multiplex analysis kits as per the manufacturer's instruction (Biolegend, San Diego, CA). The detection limits of IL-1α, IL-1β, IL-6, IL-10, IL-12p70, IL-17α, IL-23, IL-27, IFNβ, IFNγ, MCP-1, TNFα, and GM-CSF were as follows: 1.78 pg/ml, 1.55 pg/ml, 1.27 pg/ml, 1.89 pg/ml, 1.68 pg/ml, 1.73 pg/ml, 8.19 pg/ml, 10.57 pg/ml, 10.95 pg/ml, 1.76 pg/ml, 1.8 pg/ml, 2.89 pg/ml, and 10.69 pg/ml, respectively.

2.8. RNA Extraction and Quantitative RT-PCR Analysis (qRT-PCR). Total RNA of the lung was extracted using TRIzol reagent according to the manufacturer's instructions. Then, cDNA was synthesized using a commercial cDNA kit (Takara Corporation, Japan). qRT-PCR was used to measure mRNA expression of RORC, FOXP3, GATA, and T-bet. The primers for qRT-PCR were listed in Table 1.

2.9. Statistical Analysis. Data are expressed as the mean ± SEM. Comparisons between multiple groups were carried out using analysis of variance (ANOVA), while comparisons between two groups were performed by Student's *t*-test. All data analyses were adopted by GraphPad PRISM software (version 6.0 for Windows; GraphPad, San Diego, CA, USA). $p < 0.05$ was considered significant.

3. Results

3.1. Effect of IRAK-M Loss on Airway Inflammation in Mice Challenged with LPS after Acute Exposure to CS. We evaluated the role of IRAK-M in airway inflammation in mice exposed to 3-day CS or inhaled LPS. Comparable airway inflammation (BAL inflammatory cells, aggregation of inflammatory cells around the airways and blood vessels, concentrations of cytokines, and airway resistance) was seen



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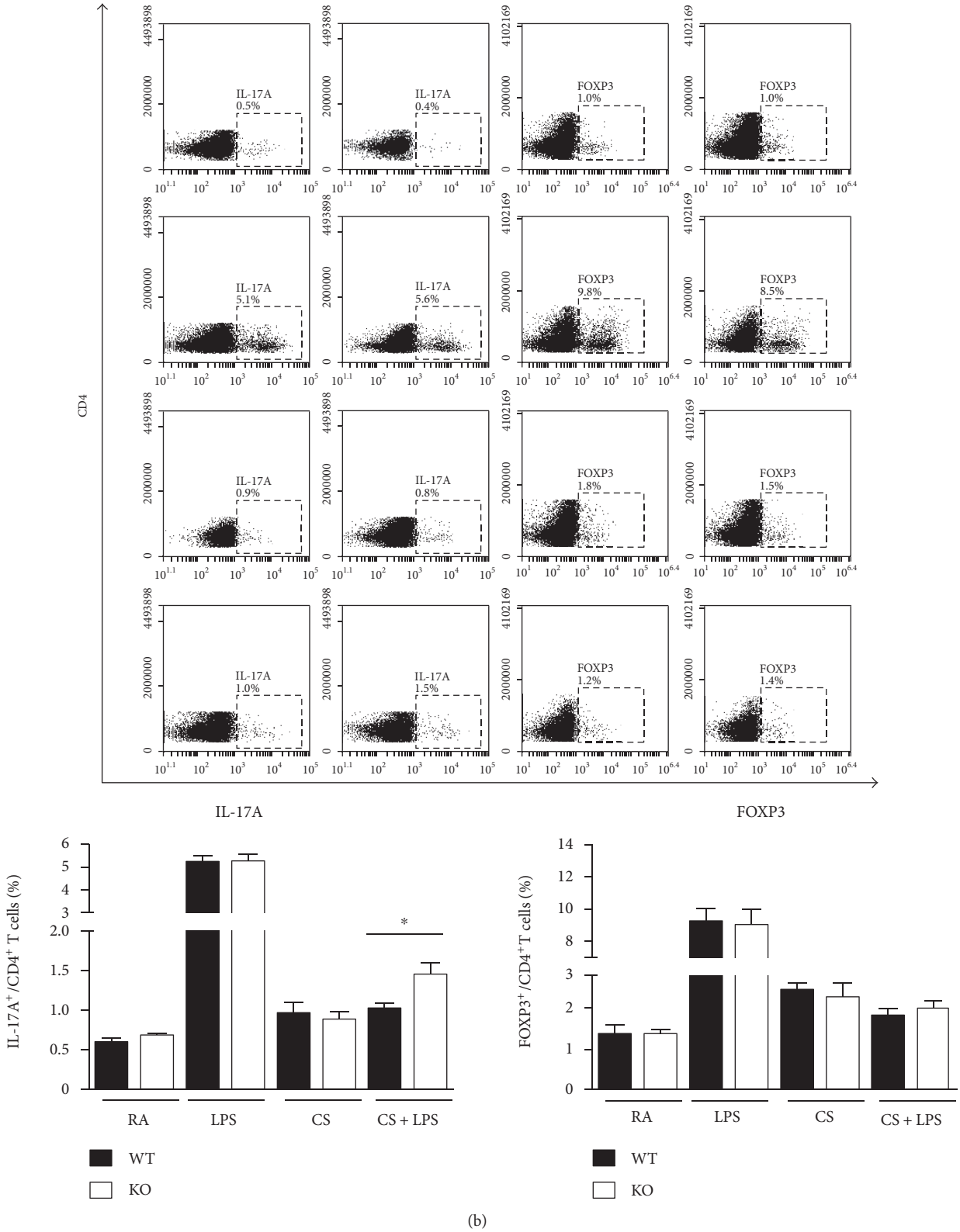
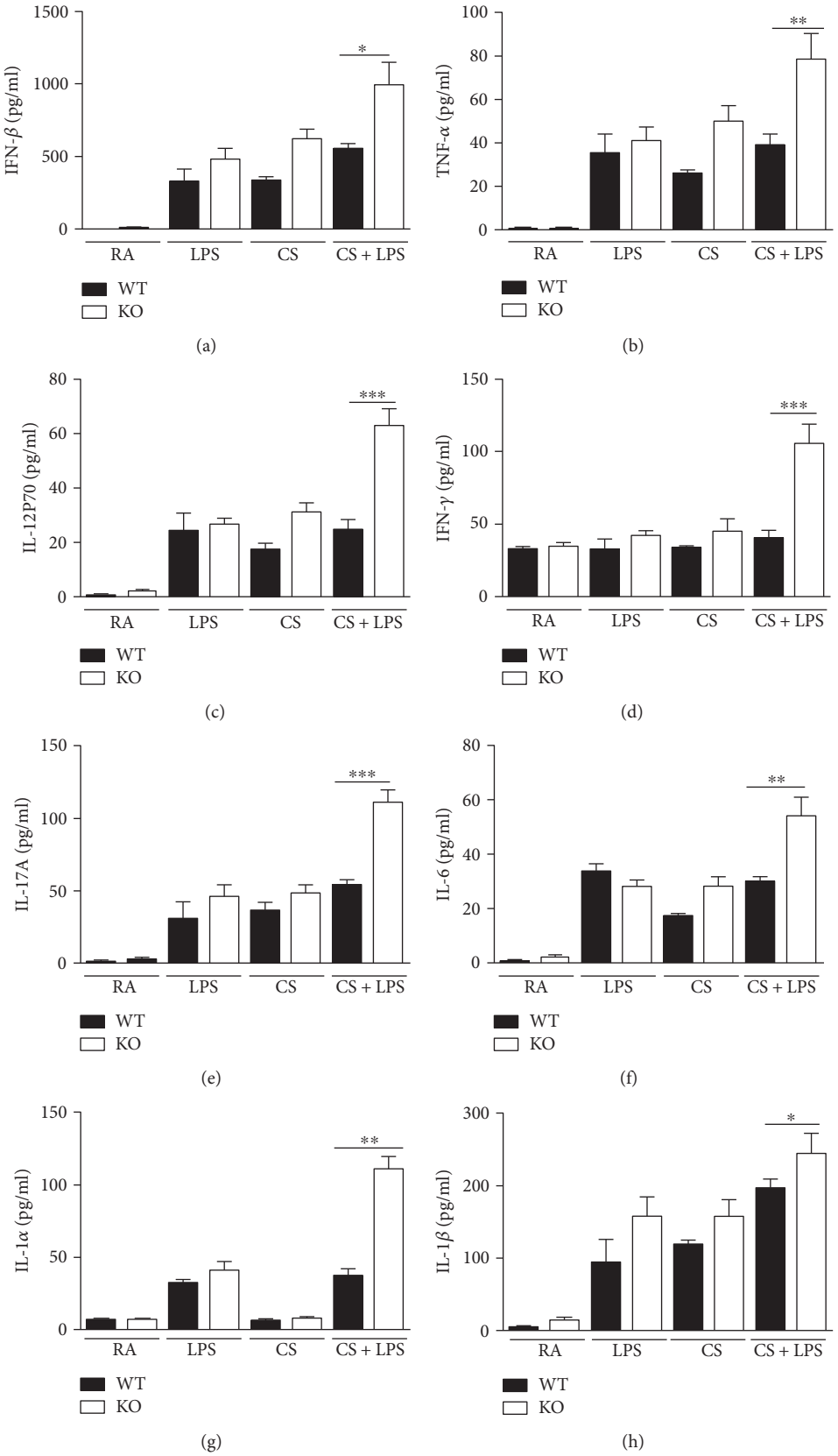


Figure 2: Effect of IRAK-M loss on T subsets after 3-day CS exposure followed by LPS challenge. FACS analysis of Th17 and Treg cells using specific Abs against Th17 and Treg markers in the lung (a) and spleen (b), the percentages of Th17 and Treg were presented as plotted. Results are expressed as means ± SEM, $n = 7–8$ animals per group, $^{*}p < 0.05$, $^{**}p < 0.01$.



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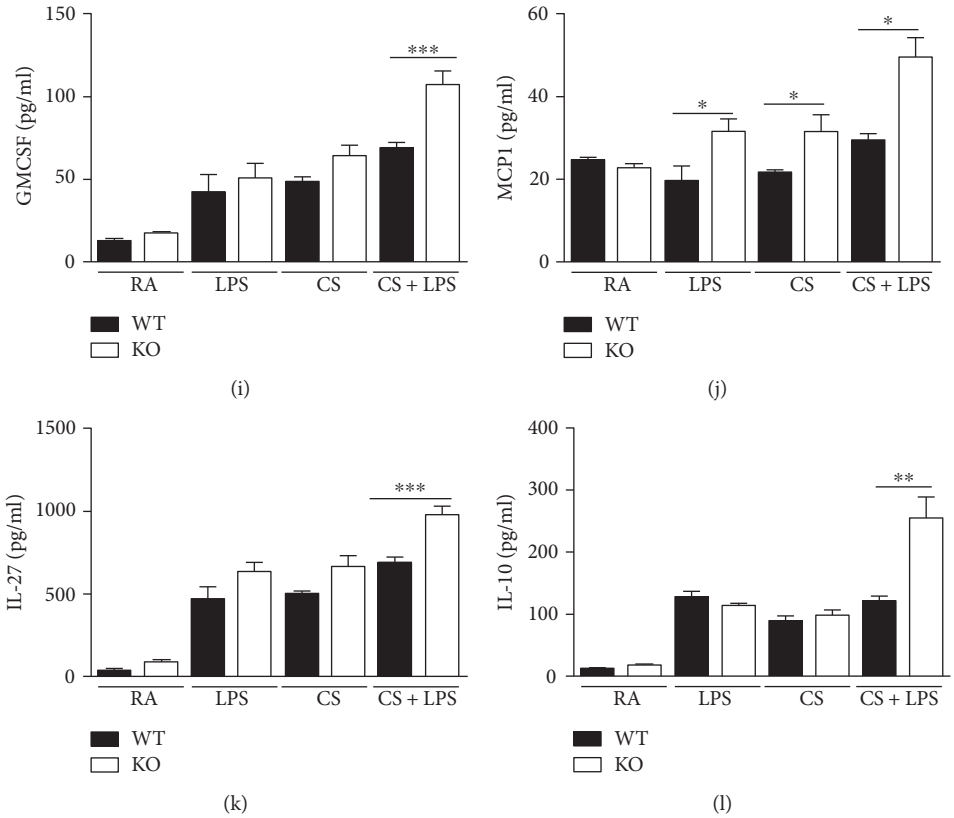


Figure 3: Elevated levels of inflammatory cytokines in lung homogenates in IRAK-M KO mice after 3-day CS exposure followed by LPS challenge. LEGENDplex analysis for levels of cytokines in lung homogenates. (a) Th1-associated cytokines, (b) Th17-related cytokines, (c) proinflammatory cytokines and mediators, and (d) anti-inflammatory cytokines. Results are expressed as means ± SEM, $n = 7–10$ animals per group, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

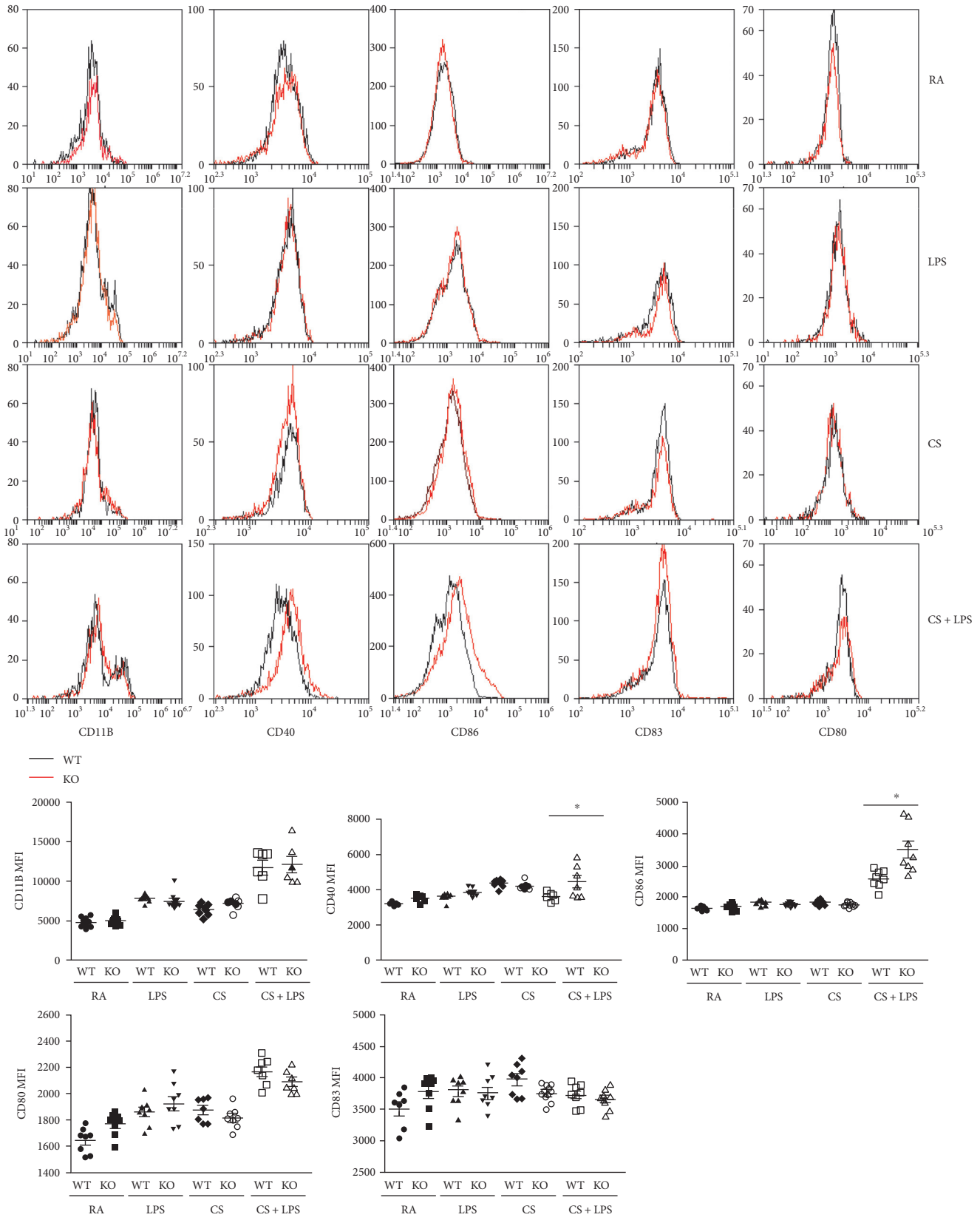
in both IRAK-M$^{-/-}$ and WT mice challenged with either short-term CS or a single dosage of LPS (Figures 1(a), 1(b), 1(c), 1(d), 1(e), 1(f), and 1(g)).

In order to test the differences in airway inflammation between IRAK-M KO mice and their WT counterparts, we next challenged mice with LPS inhalation after 3-day CS exposure [22]. Compared with the WT mice, IRAK-M$^{-/-}$ mice demonstrated more obvious lung inflammation evidenced by more inflammatory cells infiltrated in the airways (BAL total inflammatory cells, neutrophils, and lymphocytes) (Figures 1(a), 1(b), 1(c), and 1(d)) and deposited around peribronchus and vascular (Figure 1(e)). There was a significant increase in inflammation scores in IRAK-M KO mice compared with WT mice (Figure 1(f)). Airway resistance was marginally elevated in IRAK-M$^{-/-}$ mice relative to WT mice (Figure 1(g)).

3.2. Effect of IRAK-M Deficiency on T Cell Differentiation in Mice Challenged with LPS plus Short-Term CS Exposure. We next examined whether IRAK-M loss influenced Treg/Th17 imbalance in mice challenged with LPS after 3-day CS exposure. The percentage of Tregs (represented by CD3^{+}CD4^{+}FOXP3^{+}T) was significantly lower in lungs from IRAK-M$^{-/-}$ mice than that from WT mice; conversely, the percentage of Th17 (CD3^{+}CD4^{+}Th17A^{+}T) cells was significantly higher in lungs from IRAK-M KO mice than that from WT mice (Figure 2(a)). Under the stimulation with LPS following short CS exposure, we also observed a significant decrease in the percentage of Tregs in the spleen of IRAK-M KO mice compared to WT mice. A mild elevation of the percentage of Th17 in the spleen of IRAK-M KO mice challenged with LPS and acute CS was observed (Figure 2(b)).

3.3. Effect of IRAK-M Loss on Cytokine Production in Mice Challenged with LPS after Acute Exposure to CS. Previous study reported that cells deficient in IRAK-M had elevated secretion of Th1 skewing cytokines by DCs after TLR stimulation [27]. We challenged mice with LPS inhalation after 3-day CS exposure and observed that IRAK-M KO mice showed significantly higher levels of Th1-related cytokines (including TNFα, IL-12p70, IFNβ, and IFNγ) in lung homogenates than their WT littermates (Figure 3(a)). Consistently, significantly higher concentrations of Th17-associated cytokines (including IL-17A and IL-6) were seen in lung homogenates from IRAK-M KO mice after smoke-induced exacerbations of LPS infection (Figure 3(b)).

Because IRAK-M is also expressed by airway resident cells [8], we then measured the proinflammatory mediators released by airway epithelium. As shown in Figure 3(c), significantly elevated concentrations of epithelium-derived proinflammatory cytokines and chemokines (including GM-CSF, IL-1α, IL-1β, and MCP-1) were found in lung



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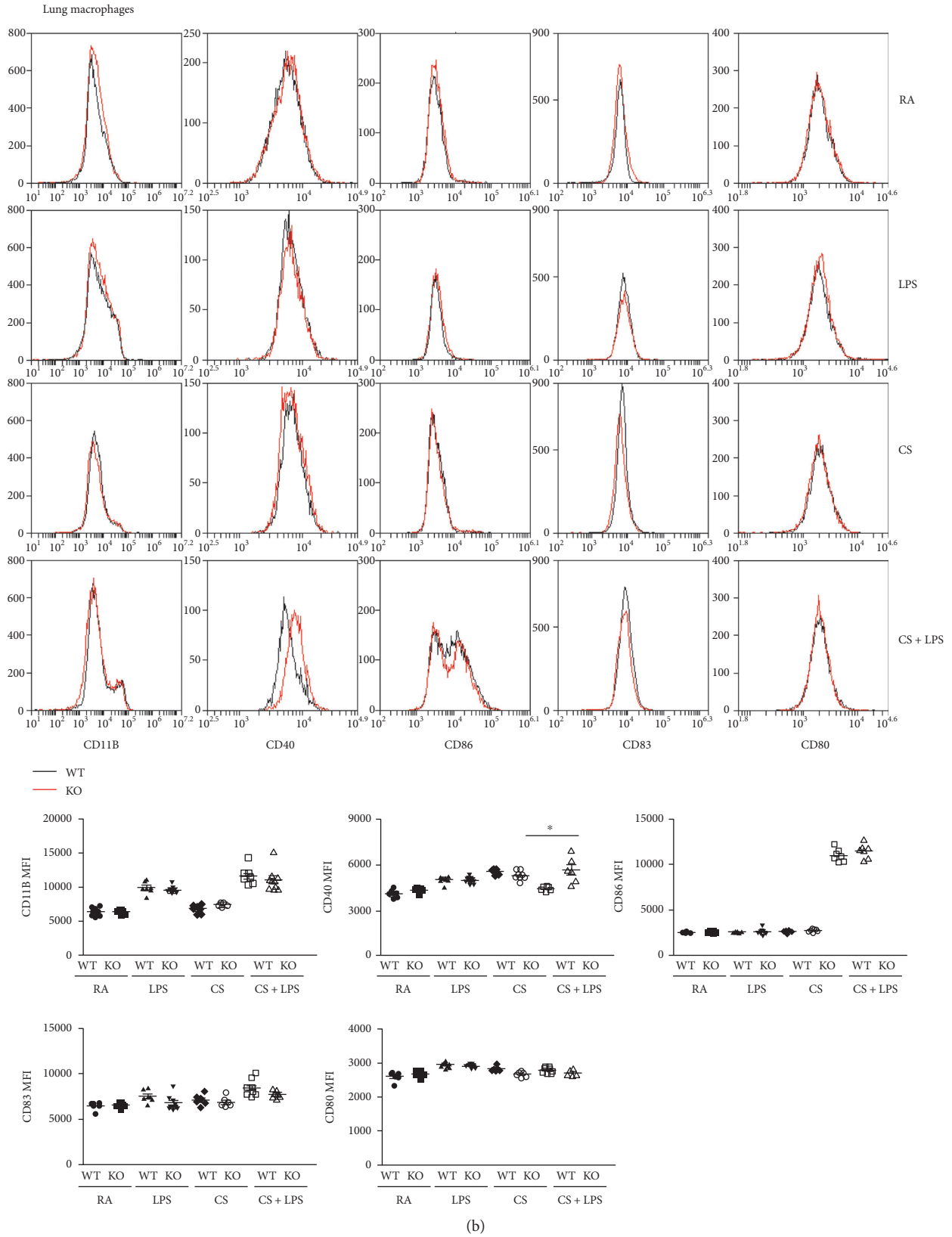


Figure 4: Effect of IRAK-M loss on surface expression of costimulatrory molecules on lung DCs and macrophages after 3-day CS exposure followed by LPS challenge. FACS analysis of surface expression of costimulatory molecules on (a) lung DCs and (b) macrophages using specific Abs against the indicated costimulatory molecule. Mean fluorescence intensity of indicated costimulatory molecules was plotted. Results are expressed as means ± SEM, $^{*}p < 0.05$.

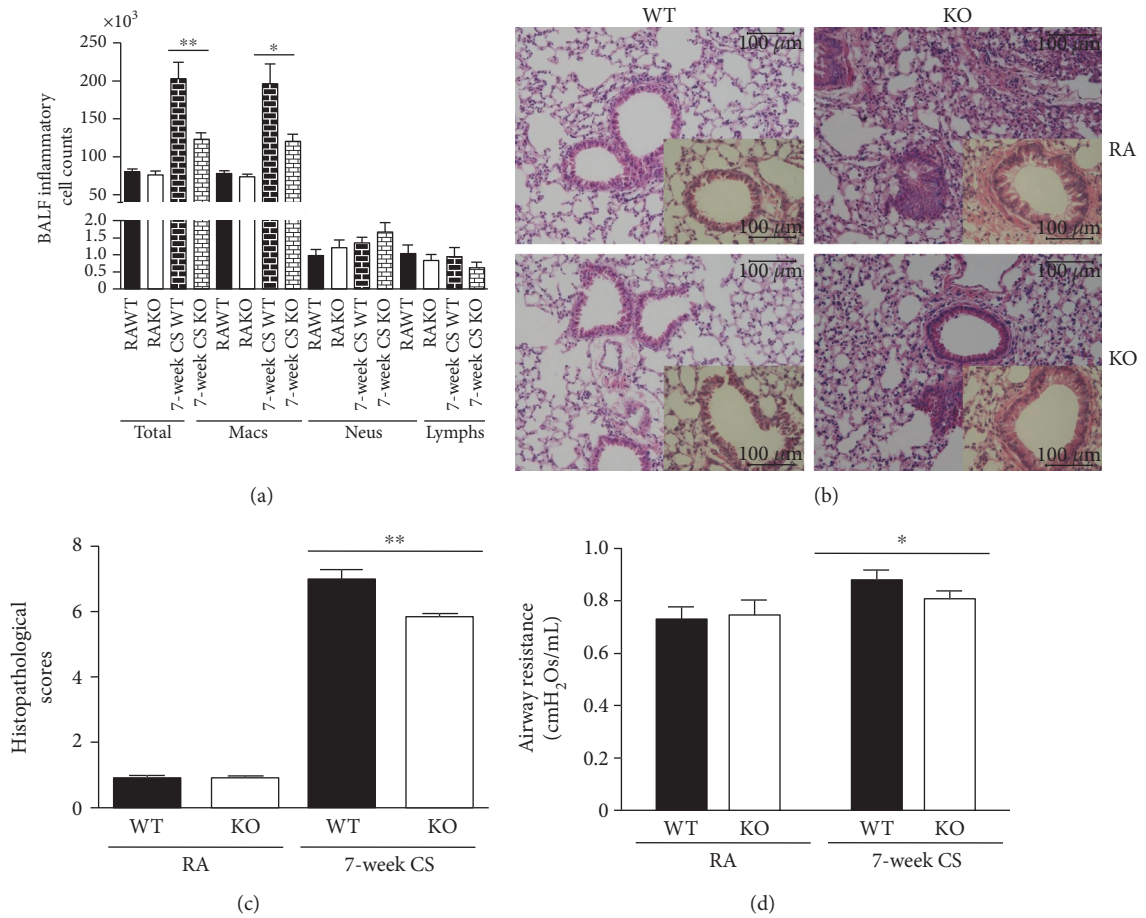


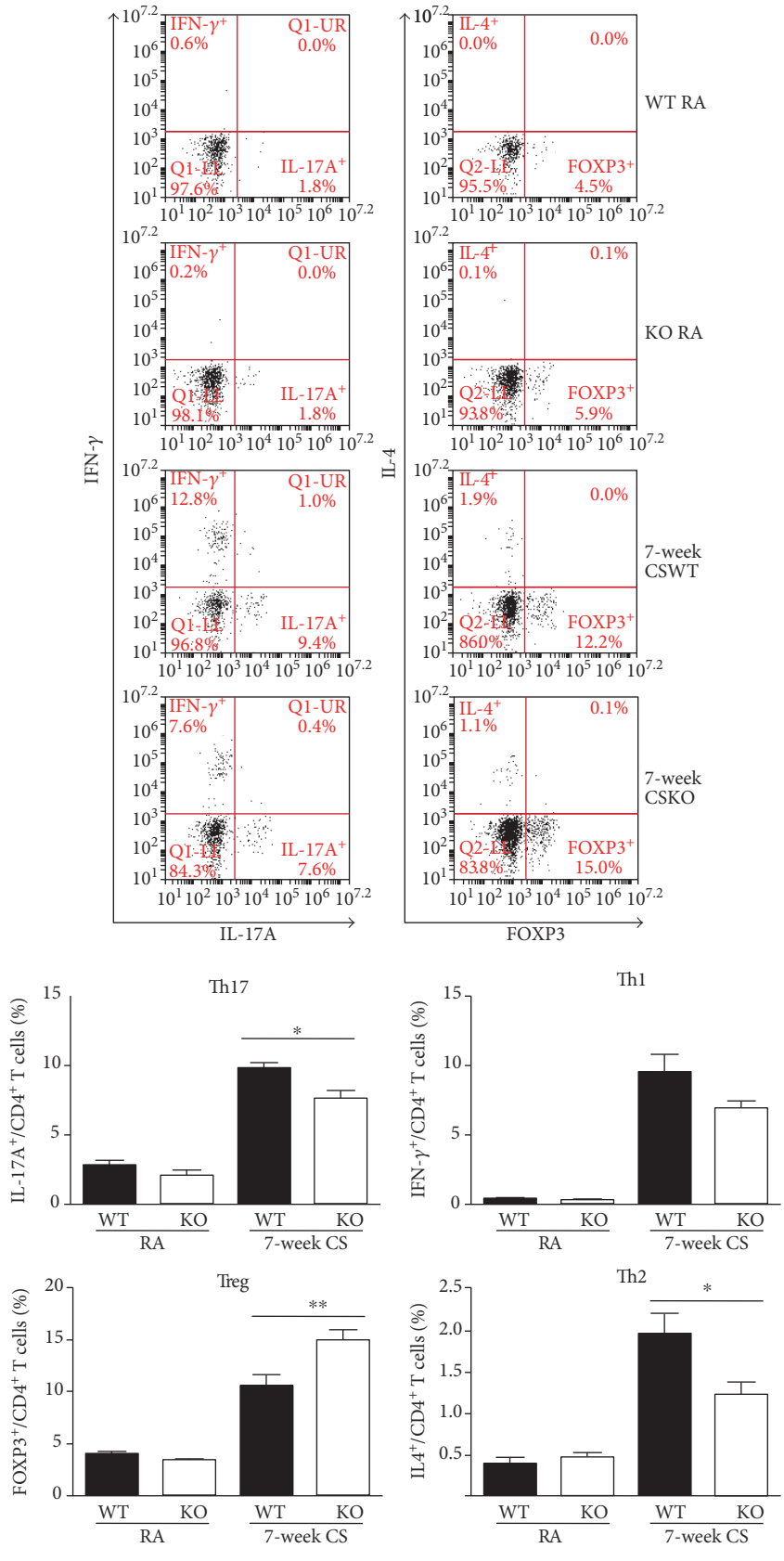
Figure 5: Attenuated airway inflammation and airway resistance, and lower concentration of cytokines in IRAK-M$^{-/-}$ mice after 7-week CS exposure. (a) The total inflammatory cells and differential populations recovered from BAL fluid. (b) Representative photomicrographs of hematoxylin & eosin-stained lung tissues showing that after 7-week CS exposure, IRAK-M KO mice exhibited attenuated pathological characteristics of airway inflammation evidenced by less thickened airway epithelium and less inflammatory cells in the peribronchial area and around vessels compared with the similarly treated WT mice. (c) IRAK-M KO mice showed significantly lower airway resistance compared with WT mice. Results are expressed as means ± SEM, $n = 5$–8 animals per group, $^{*}p < 0.05$, $^{**}p < 0.01$.

homogenates from IRAK-M$^{-/-}$ mice compared with WT mice. Additionally, we also observed the significant elevation of anti-inflammatory cytokines IL-10 and IL-27 in lung homogenates from IRAK-M KO mice, reflecting the nonspecific activation of inflammatory response to LPS and acute exposure to CS (Figure 3(d)).

3.4. Effect of IRAK-M Loss on Surface Expression of Costimulatory Molecules by DCs and Macrophages in Mice Challenged with LPS Inhalation following 3-Day CS Exposure. Dendritic cells (DCs), which function as not only innate lung sentinels but also orchestrators of adaptive immunity, are capable of initiating and maintaining the pathogenesis of COPD [28]. Lung DCs express IRAK-M that has been shown to bind CD80 and further inhibits activation of intracellular NF-κB/AP-1 [29]; we tested the effect of IRAK-M absence on surface expression of costimulatory molecules on lung DCs and macrophages in mice exposed to 3-day CS followed by LPS challenge using FACS analysis. There was significantly increased upregulation of costimulatory molecules CD40 and CD86 on lung DCs from IRAK-M KO mice compared to WT mice (Figure 4(a)).

In addition, significantly increased expression of CD40 by lung macrophages was seen in IRAK-M KO mice following LPS inhalation after 3-day CS insult compared with WT littermates treated under the same exposure condition (Figure 4(b)).

3.5. Effect of IRAK-M Ablation on Airway Inflammation in Mice under Subacute Exposure to CS. To reflect the effect of IRAK-M deficiency in airway inflammation under subacute CS exposure, mice were wholly exposed to 7-week CS. There was significantly less infiltration of total inflammatory cells and macrophages in BAL fluids from IRAK-M KO mice compared to WT mice (Figure 5(a)). Compared with IRAK-M KO mice, there was a greater aggregation of leukocytes seen in WT mice (Figure 5(b)). There was a significant decrease in inflammation scores in IRAK-M KO mice compared with WT mice (Figure 5(c)). Consistently, there was a significant inhibition of increased airway resistance induced by subacute CS exposure in IRAK-M KO mice compared to WT mice (Figure 5(d)).



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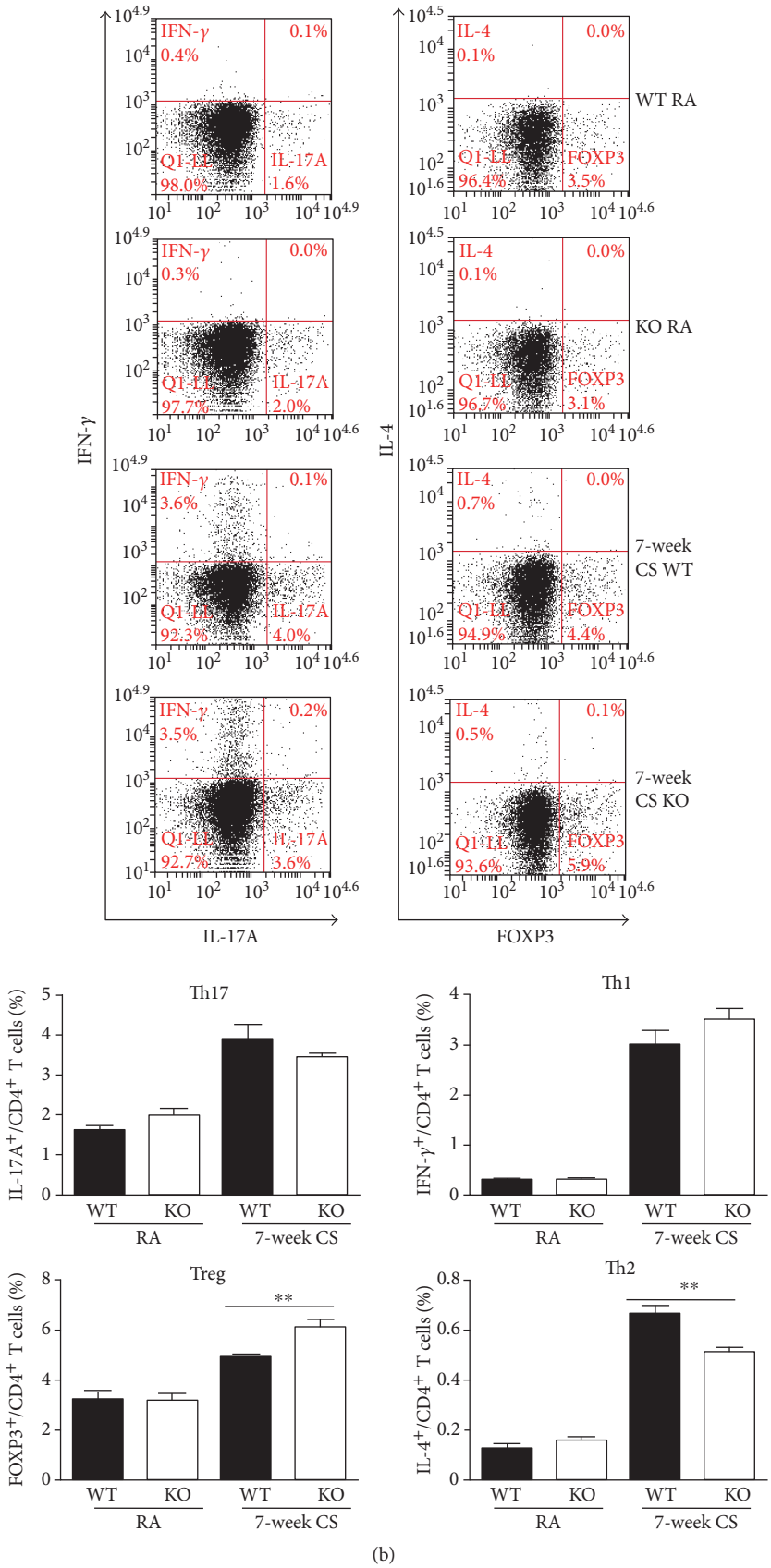

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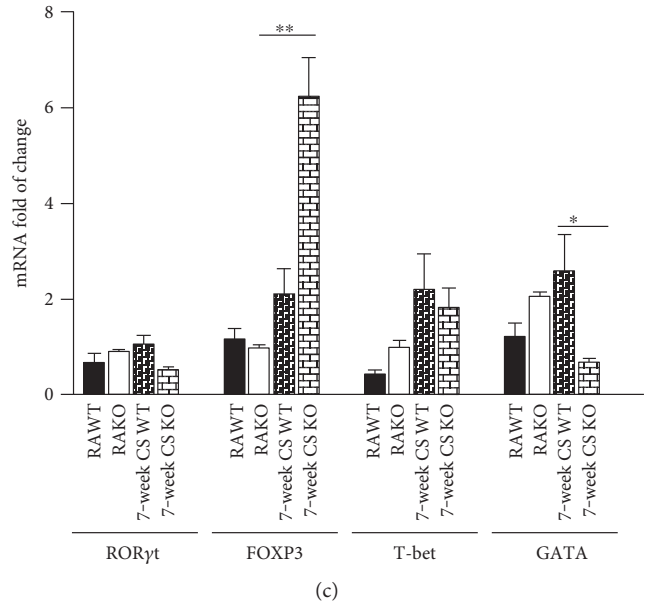


(c)

Figure 6: Effect of IRAK-M loss on T subsets after 7-week CS exposure. FACS analysis of Th17 and Treg cells using specific Abs against Th17 and Treg markers in the lung (a) and spleen (b), the percentages of Th17 and Treg were plotted. (c) qRT-PCR analysis of mRNA expression of specific nuclear transcriptional markers for Th1, Th2, Th17, and Treg. Results are expressed as means ± SEM, $n = 7$–8 animals per group, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{**}p < 0.001$.

3.6. Effect of IRAK-M Ablation on Treg/Th17 in Lungs of Mice under Subacute Exposure to CS. FACS analysis showed that there were significantly increased percentages of Tregs in lungs from subacute CS-exposed IRAK-M$^{-/-}$ mice compared with those from similarly treated WT mice; however, there were significantly less percentages of Th17 cells in IRAK-M KO mice than those in WT mice (Figure 6(a)). Compared to WT mice, IRAK-M KO mice showed a significant elevation of Tregs, not Th17, in the spleen after subacute CS exposure (Figure 6(b)). Significantly lower percentages of Th2 cells, not Th1 cells, were seen in mice deficient in IRAK-M compared to that in WT mice after 7-week CS challenge, indicating a role of IKAK-M in inducing more T cell differentiation into Th2 cells, not into Th1 cells, under long-term CS exposure (Figures 6(a) and 6(b)).

Retinoic acid-related orphan receptor γt (RORγt), a member of the nuclear hormone receptor superfamily, has been identified as a key transcription factor driving the Th17 differentiation program [30]. In contrast, the Treg cells are defined by the expression of the forkhead family transcription factor, FOXP3. qRT-PCR analysis on lung tissues also revealed a significantly higher mRNA expression of FOXP3 in IRAK-M KO mice than that in WT mice following subacute CS exposure (Figure 6(c)). mRNA expression of GATA was significantly induced by subacute CS exposure in WT mice, not in IRAK-M$^{-/-}$ mice. In addition, induction of mRNA expression of RORC was slightly inhibited in IRAK-M KO mice ($p = 0.09$) compared to WT mice (Figure 6(c)).

3.7. Effect of IRAK-M Loss on Cytokine Production in Mice Exposed to 7-Week CS. We next determined the concentrations of cytokines in lung homogenates after 7-week CS exposure. As shown in Figure 7, there was significantly lower levels of Th1-associated cytokines (including TNFα, IFNβ, IFNγ, and IL-12p70), Th17-related cytokines (including IL-17A, IL-6, and IL-23), and proinflammatory cytokines (including IL-1α, IL-1β, MCP-1, and GM-CSF) in lung homogenates of IRAK-M KO mice than those in WT mice. There was a significant decrease in the level of anti-inflammatory cytokine IL-27, not IL-10, in lung homogenates from IRAK-M KO mice relative to their WT counterparts in response to subacute CS injury, reflecting the nonspecific inhibition of immune activation under subacute CS stimulation in absence of IRAK-M.

3.8. Effect of IRAK-M Deficiency on Surface Expression of Costimulatory Molecules Induced by Subacute CS Exposure in Mice. Compared to air-exposed mice, expression of costimulatory molecule CD11b was lower on lung DCs from both IRAK-M KO and WT mice after 7-week CS exposure. There were no significant differences in expression of other costimulatory molecules by DCs between two types of mice (Figure 8(a)).

Chronic exposure to CS constitutively activates alveolar macrophages [31]. However, our FACS analysis showed that expression of CD11b and CD86 by lung macrophages was significantly lower in IRAK-M KO mice than that in WT mice after 7-week CS exposure (Figure 8(b)).

4. Discussion

IRAK-M is a MyD88-dependent inhibitor of TLR signaling and reported to display different functions depending on the milieu where it is expressed [6, 32]. We firstly to our knowledge examined the effects of IRAK-M on airway inflammation induced by acute CS exposure (3 days) followed by LPS inhalation and subacute CS exposure

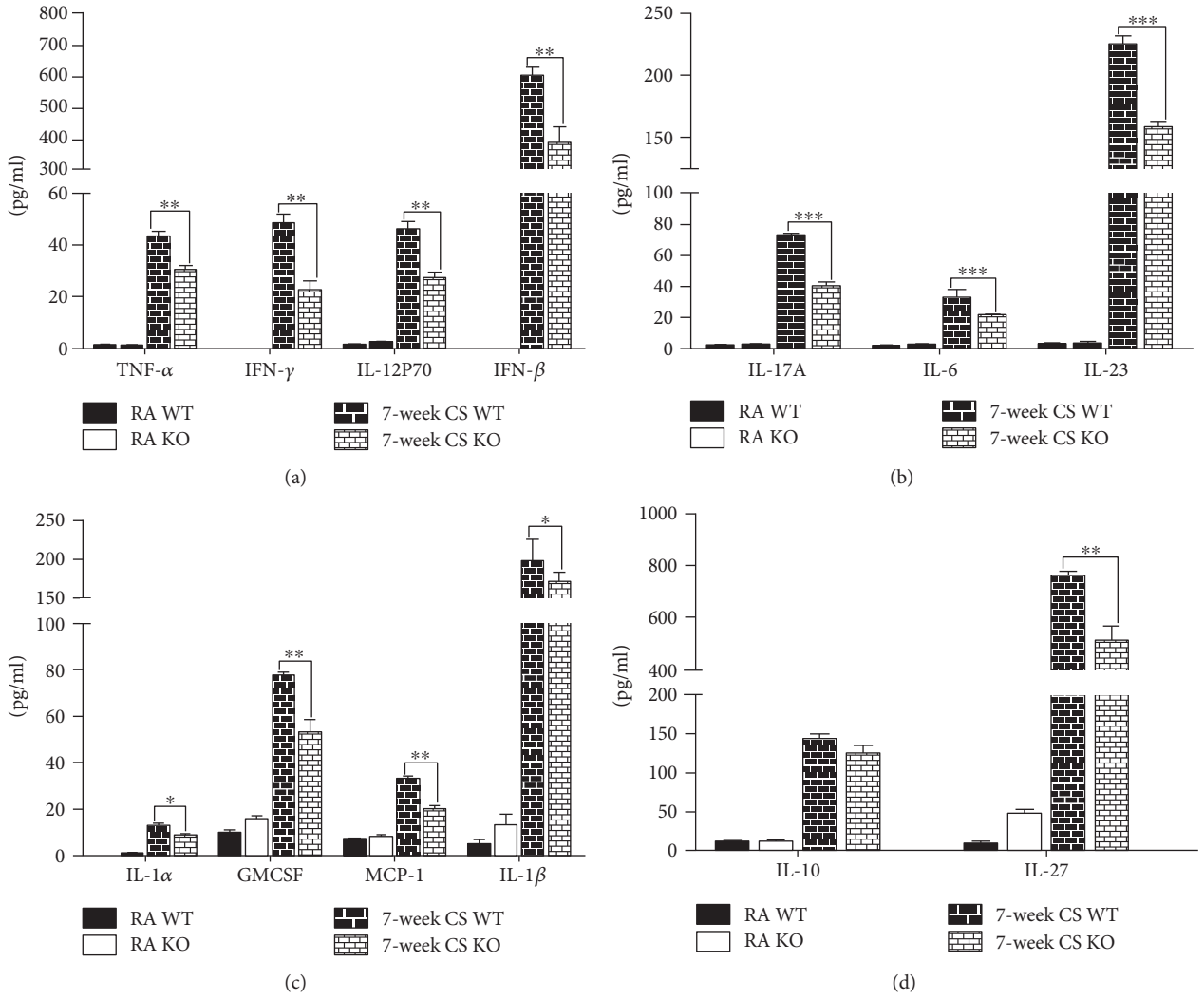
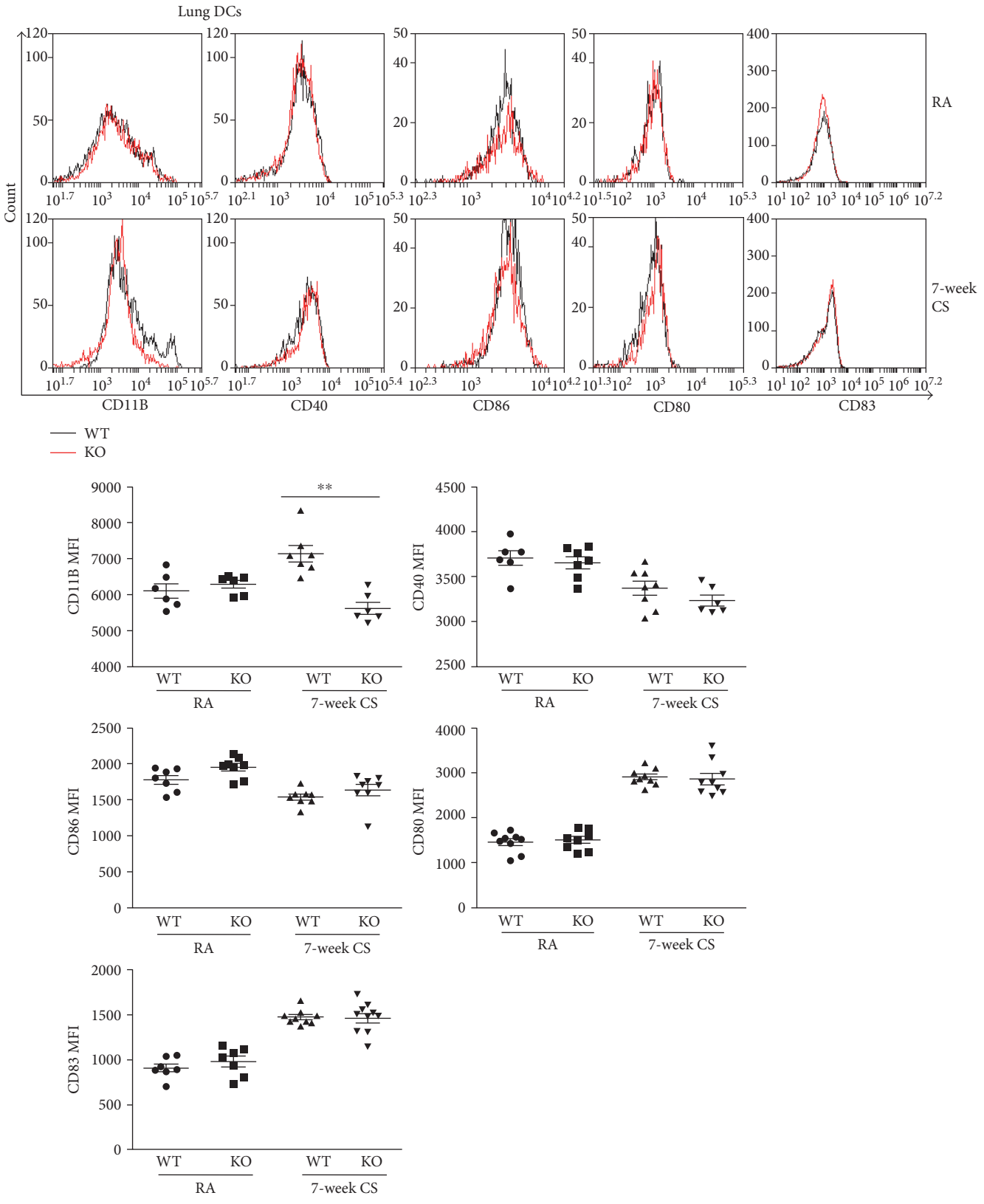


Figure 7: Decreased levels of inflammatory cytokines in lung homogenates in IRAK-M KO mice after 7-week CS exposure. LEGENDplex analysis for levels of cytokines in lung homogenates. (a) Th1-associated cytokines, (b) Th17-related cytokines, (c) proinflammatory cytokines and mediators, (d) anti-inflammatory cytokines. Results are expressed as means ± SEM, $n = 7$ animals per group, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

(7 weeks) using IRAK-M KO mice and WT littermates. In this present investigation, we showed IRAK-M expression temporally and spatially affected the pathological outcomes in the CS-induced airway inflammation with or without LPS. Compared with WT counterparts, mice deficient in IRAK-M showed significantly aggravated airway inflammation and more number of Th17 cells and less number of Treg in lungs after acute CS exposure followed by LPS challenge. Conversely, subacutely CS-exposed IRAK-M$^{-/-}$ mice manifested significantly attenuated infiltration of inflammatory cells into the airways and less number of Th17 cells and more Tregs in lungs compared to similarly administrated WT mice.

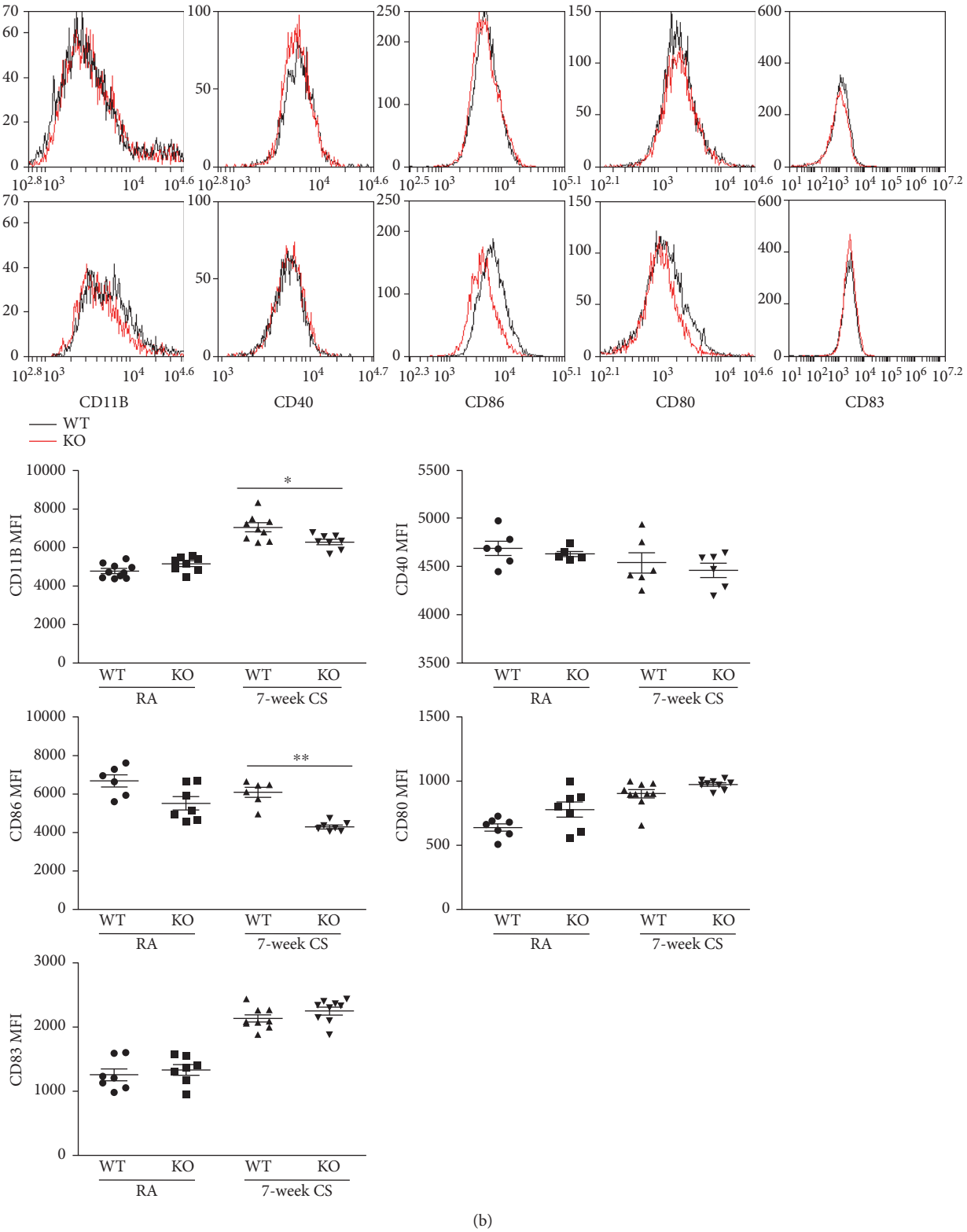
Exposure to CS is a risk factor for lung infection, and bacterial infections substantially contribute to exacerbations of COPD [33]. The airway inflammatory response in lung pathology induced by CS or bacteria is associated with infiltration of leukocytes, particularly, neutrophils and lymphocytes [34, 35]. We investigated the effect of IRAK-M loss on host defense against LPS in mice exposed to 3-day CS. IRAK-M KO mice displayed more severe lung inflammation and significantly increased accumulation of neutrophils and lymphocytes in the airways compared with WT mice. Consistently, concentrations of Th1-associated cytokines (IFNβ, IFNγ, and IL-12p70), proinflammatory cytokine TNFα, and Th17-related cytokines (IL-17A and IL-6) in BAL fluids were significantly higher in IRAK-M KO mice than those in the WT mice. Because IRAK-M expression is found in airway epithelium and macrophages [7, 8], we cautiously think that significant infiltration of neutrophils and lymphocytes was a downstream effect after administrated with LPS and acute CS exposure possibly mediated by IRAK-M.

Th17/Treg imbalance has been implicated in the immunopathology of COPD [1]. Th17 cells, a distinct lineage of activated CD4^{+} T cells, modulate tissue inflammation by producing IL-17A and IL-17F [36]. They also mediate immunity against extracellular pathogens [1].



(a)

Figure 8: Continued.



(b)

Figure 8: Effect of IRAK-M loss on surface expression of costimulatrory molecules on lung DCs and macrophages after 7-week CS exposure. FACS analysis of surface expression of costimulatory molecules on (a) lung DCs and (b) macrophages using specific Abs against the indicated costimulatory molecule. Mean fluorescence intensity of indicated costimulatory molecules was plotted. Results are expressed as means ± SEM, $^{*}p < 0.05$, $^{**}p < 0.01$.

Regulatory T cells (Treg) are subsets of $CD4^+$ T cells with immunoregulatory functions, which suppress inflammation by producing anti-inflammatory cytokines such as IL-10 [37].

Smokers with COPD had significantly fewer Treg cells in the lungs, less mRNA for FOXP3, and less IL-10 secretion from the whole lung than controls [38]. Consistent with the previous reports, we found an increase of the proportion of Th17 cells and a decrease of Tregs in both lungs and spleens from IRAK-M KO mice than WT mice after CS exposure plus LPS challenge.

Interestingly, our data showed that IRAK-M promoted airway inflammation induced by subacute exposure to CS in a mouse model. This is evident from the significant alleviation in infiltration of inflammatory cells around the airways and in pulmonary parenchyma, airway inflammatory response (for total BAL inflammatory cells and macrophages), and airway resistance seen in IRAK-M KO mice compared to those in WT mice. IRAK-M KO mice exposed to subacute CS showed decreased levels of Th17-related cytokines (including IL-6, IL-17A, and IL-23) and Th1-associated cytokines (e.g., TNFα, IFNβ, IFNγ, and IL-12p70), which may associate with reduced airway inflammation in these knockout mice. Consistent with the previous report demonstrating expression of Th1-type proinflammatory cytokines regulated by IRAK-M [39], our current data also showed that IRAK-M deficiency resulted in lower levels of Th1-type cytokines (TNFα, IFNβ, IFNγ, IL-12p70, and IL-12) and Th17-related cytokines (IL-17A, IL-6, and IL-23) in BAL fluid after 7-week CS exposure. Airway epithelial cells can be abnormally activated by CS and play an important role in the pathology of airway injury [40]. We also observed decreased expression of airway epithelium-related proinflammatory cytokines and chemokines secreted (including IL-1α, IL-1β, MCP-1, and GM-CSF) in the lungs of IRAK-M KO mice after subacute CS exposure, suggesting the inhibition of overactivation of airway epithelial cells in absence of IRAK-M. Taken together, IRAK-M deficiency alleviated lung inflammation possibly through regulating T cell subpopulations infiltrated in the lungs after subacute CS challenge.

By presenting antigen/MHC and costimulatory molecules to T cells, airway DCs play an important role in inducing activation and differentiation of naïve and effector $CD4^+$ and $CD8^+$ T cells, which both of these two cell types are involved in COPD pathogenesis [28]. Furthermore, disruptive airway epithelial barrier caused by CS makes lung DCs exposed to environmental challenges.

CS was reported to promote accumulation and survival of matured DCs in lung tissues from COPD patients [41]. Significant increases in costimulatory molecule expression by mature DCs contribute to an inappropriate immune response in the pathogenesis of COPD [42]. Pulmonary DCs of smoke-exposed mice were shown to be activated with surface expression of MHC II and costimulatory molecules CD40 and CD86 being significantly upregulated under 24-week CS exposure [43]. Quite interestingly, recent evidence has shown that long-term cigarette smoking extract (CSE) exposure downregulated CD11c/MHCII, CD83, CD86, and CD40 expression by DCs; however, short-term CSE stimulation upregulated surface expression of MHCII, CD83, CD86, and CD40 by lung DCs [44]. IRAK-M expression has been reported to inhibit DC activation and production of proinflammatory cytokines in response to *Helicobacter pylori* [45]. Airway macrophages function as the innate defense of the airways and drive the pulmonary immune response to CS and infection; thus, airway macrophages play important role in the pathogenesis of CS-induced airway inflammatory diseases [35]. In addition to its effects on DCs, IRAK-M modulates phenotype and function of mononuclear cells [17]. We used FACS analysis to reflect the effect of IRAK-M on expression of costimulatory molecules by DCs and macrophages under acute/subacute CS exposure. Under the acute CS exposure followed by LPS challenge, IRAK-M KO mice showed significant upregulation of costimulatory molecules CD40 and CD86 in lung DCs or macrophages compared with similarly treated WT mice. However, after 7-week CS exposure, significantly higher expression of CD11b by DCs and lower expression of CD86 by lung macrophages were seen in IRAK-M KO mice than those in WT mice. These observations might be attributed to the regulatory effect of IRAK-M on DCs and macrophages in the lungs, depending on the duration of stimulation and type of stimulus.

5. Conclusions

In the present study, we demonstrated that the role of IRAK-M signaling could differ between LPS-induced lung injury after short CS exposure and subchronic CS-mediated lung damage in a mouse model. IRAK-M provided airway protection against combined exposure to LPS and acute CS by influencing Treg/Th17 imbalance skewing to Th17 and upregulating expression of costimulatory molecules CD40 and CD86 by DCs and macrophages. In contrast, IRAK-M played a proinflammatory role in airway pathology under 7-week CS exposure by influencing Treg/Th17 imbalance skewing to Treg and downregulating expression of costimulatory molecules CD11b and CD86 by macrophages. An appropriate modulation of IRAK-M in airway inflammation induced by the duration of CS insult or type of stimulus might be helpful in searching for new targets to CS-associated airway pathology.

Conflicts of Interest

The authors declare that they have no competing interests.

Acknowledgments

This work was supported by grants from the National Natural Science Foundation of China (no. 81170040, no. 81470229).

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